# HER2 Specific Tumor Targeting with Dendrimer Conjugated Anti-HER2 mAb

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In the present study, we report the synthesis and human growth factor receptor-2 (HER2) specific tumor targeting properties of a dendrimer conjugated to anti-HER2 mAb (monoclonal antibody) conjugate. The polyamidoamine (PAMAM) dendrimer generation five (G5) was labeled with alexaFluor 488 and conjugated to anti-HER2 mAb. The binding and internalization of the antibody-conjugated dendrimer to HER2-expressing cells was evaluated by flow cytometry and confocal microscopy. Uniquely, the conjugate demonstrated cellular uptake and internalization in HER2-expressing cells as compared to free antibody. The time course of internalization and blocking experiments with free antibody suggest that the rapid and efficient cellular internalization of the dendrimer−antibody conjugate was achieved without alterations in specificity of targeting. Animal studies demonstrated that the conjugate targets HER2-expressing tumors.

## INTRODUCTION

Tumor-targeted drug delivery can enhance the effectiveness of chemotherapeutics while decreasing the systemic toxicity of these drugs. The use of monoclonal antibodies (mAbs) with specificity toward tumor markers is a well-established immunological strategy in cancer therapy. Monoclonal antibodies against various antigens have been used to deliver chemotherapeutic drugs (*1*, *2*), potent plant and bacterial toxins (*3*), and radionuclides (*4*) to tumors. The carrier capacity of antibodies can be increased significantly by attaching drug reservoirs such as nanoparticles, liposomes, and polymers. This approach potentially allows the delivery of a higher payload of drugs in a cell-specific manner.

Human epidermal growth factor receptor-2 (HER2) is one of the most promising targets in immunotherapy. The expression of the HER2 protein has been observed in a variety of tumors, in particular, breast and ovarian cancers (*5*), and has been associated with poor clinical outcomes (*6*). The overexpression of HER2 receptors in tumor cells provides an opportunity for development of HER2-targeted drug delivery systems. In addition, the presence of an extracellular domain makes the HER2 receptor an ideal target for receptor-mediated drug delivery in tumors. Monoclonal antibodies directed against HER2 offer a potential strategy for HER2-targeted delivery. Trastuzumab (Herceptin), a recombinant humanized monoclonal anti-HER2 antibody which has been approved for clinical use is reported to bind the HER2 receptor (*7*) with nanomolar affinity (*8*, *9*) and inhibits the growth of HER2-expressing breast cancer cells (*10*, *11*). The main objective of the present study was to investigate the utility of anti-HER2 mAb−PAMAM dendrimer conjugates as targeted drug delivery vectors. Antibody−dendrimer conjugates have been utilized in targeting prostate-specific membrane antigen (PSMA) (*12*) and CD14 antigen (*13*), delivery of boron (*14*), and tumor imaging applications (*15*). There has been growing interest in the synthesis of anti-HER2 mAb conjugates for gene delivery (*16*), tumor targeting (*17*), and imaging applications (*18*).

PAMAM dendrimers are biocompatible (*19*), nonimmunogenic (*20*), and water soluble and have been attached to many biological molecules such as proteins, synthetic drugs, and small molecules (*21*, *22*). In this study, we synthesized anti-HER2−mAb−dendrimer conjugate and studied its binding and internalization in HER2-overexpressing tumor cells. The specific targeting was clearly demonstrated in cell lines overexpressing HER2 by flow cytometry analysis. In addition, preincubation of HER2-overexpressing cells with free antibody prevented binding and uptake of antibody-conjugated dendrimers. This observation suggested that binding of the conjugate is mediated by HER2 on the surface of the analyzed cells. The cellular uptake of the conjugate as well as cellular distribution pattern was studied by confocal laser scanning microscopy. The antibody conjugated to dendrimer was shown to internalize in the cells faster and more efficiently than the antibody alone.

## EXPERIMENTAL PROCEDURES

**General.** G5−PAMAM dendrimer was prepared at the Michigan Nanotechnology Institute for Medicine and Biological sciences, University of Michigan, and analyzed extensively by $^{1}H$ and $^{13}C$ NMR, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, high-performance liquid chromatography (HPLC), gel-permeation chromatography (GPC), and polyacrylamide gel electrophoresis (PAGE). The molecular weight of synthesized dendrimer was found to be 26 530 g/mol by GPC, and the average number of primary amino groups was estimated to be 108 by potentiometric titration (*23*). The heterobifunctional cross-linkers sulfosuccinimidyl 3-(2-pyridyldithio)propionate (sulfo-LC-SPDP) and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) were obtained from Pierce (Rockford, IL). Prepacked Sephadex G-25 PD-10 columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and equilibrated with degassed eluting buffer before sample introduction.

**Synthesis of G5−Ac, 1.** G5 amine dendrimer (0.265 g, 0.0099 mmol) and triethyl amine (0.088 g, 0.8635 mmol) were dissolved in 30 mL anhydrous MeOH and allowed to stir for 30 min. To this solution, acetic anhydride (0.076 g, 0.744 mmol) in anhydrous MeOH (15 mL) was added dropwise while stirring. The reaction mixture was allowed to stir overnight at room temperature. After evaporation of the solvent, the residue was dissolved in $H_2O$ and dialyzed in 10 000 MWCO regenerated

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cellulose dialysis bags initially against PBS buffer followed by water. The partially acetylated dendrimer was lyophilized to give a colorless powder (0.270 g, 91.2%). The average number of acetyl groups (*80*) was determined on the basis of a $^1$H NMR calibration curve drawn by plotting a ratio of acetyl protons and the sum of all methylene protons vs degree of actetylation (*24*).

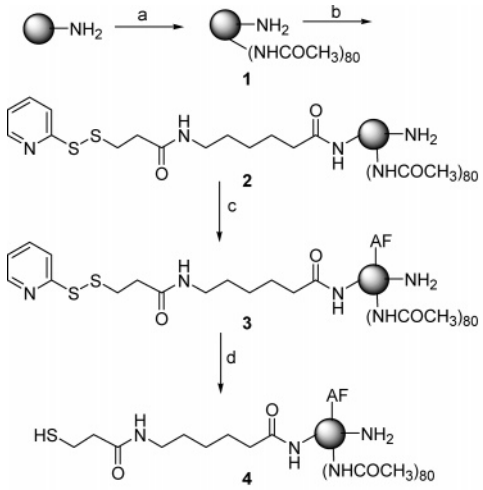
**Synthesis of G5−Ac−SPDP, 2.** To a solution of G5−Ac, **1**, (0.042 g, 0.0014 mmol) in PBS buffer (pH 7.4, 10 mL), Sulfo-LC-SPDP (0.0052 g, 0.0098 mmol) dissolved in 2 mL PBS buffer was added dropwise, and the reaction mixture was allowed to stir for 3 h. The unreacted reagents and byproducts were separated by ultrafiltration using a 10K MWCO Pelicon device washing initially with PBS and then with DI water. The conjugate was lyophilized to give 0.036 g, 85.7% of conjugate as a white powder. The compound was characterized by HPLC and $^1$H NMR spectroscopy. The number of SPDP linker was determined by pyridine-2-thione assay as described in the manufacturer's protocol. Briefly, conjugate **2** was dissolved in PBS/EDTA buffer (pH 7.4, 1 mg/mL), and the absorbance was recorded at 343 nm in comparison to a PBS−EDTA blank. To this solution, 10 $\mu$L of 15 mg/mL DTT was added and stirred for 15 min. After exactly 15 min, the absorbance at 343 nm of the reduced sample was recorded. The experiment was carried out in triplicate. On the basis of the change in absorbance, a molar ratio of 3.6 SPDP linkers per mole of dendrimer was calculated.

**Synthesis of G5−Ac−SPDP−AF, 3.** AlexaFluor-NHS ester (0.003 g, 0.0046 mmol) dissolved in DMSO was added to a solution of dendrimer disulfide conjugate, **2** (0.030 g, 0.00096 mmol) in PBS buffer (pH 7.4) while stirring. The solution was allowed to stir for 18 h at room temperature. Concentration of the reaction mixture by membrane filtration and further purification on a G-25 Sephadex column gave the dendrimer conjugate, which was further purified by membrane filtration and lyophilized to give an orange powder (0.027 g, 90.0%). The purified conjugate was characterized by $^1$H NMR and UV−vis spectroscopy and HPLC.

**Synthesis of G5−AF−HN, 7.** The disulfide bond on dendrimer conjugate **3** was reduced with 10 mM DTT solution in degassed PBS−EDTA buffer (pH 7.4) at room temperature (RT) under nitrogen to provide dendrimer−thiol, **4**. This conjugate was purified by gel filtration eluting with PBS−EDTA buffer (pH 7.4) under nitrogen on a PD-10 column to remove excess reagents and byproducts and was used immediately for antibody conjugation. A thiol-reactive maleimide group was introduced in anti-HER2 mAb, **5** (0.002 g, 0.00013 mmol), by reacting with sulfo-SMCC (0.0006 g, 0.00136 mmol) at room temperature for 2 h to give modified antibody **6**. The excess reagent was removed by gel filtration on a Sephadex G-25 column. Conjugate **6** was then concentrated on a microcon YM100 and immediately reacted with dendrimer thiolate (0.004 g, 0.00013 mmol) for 2 h at room temperature in PBS−EDTA buffer. After 2 h, *N*-ethylmaleimide (0.003 g, 0.024 mmol) was added to the reaction mixture. The final conjugate (G5−Ac−AF−HN, **7**) was purified by ultrafiltration (MWCO 100 000). The dendrimer−antibody conjugate was analyzed by HPLC, PAGE, and UV−vis spectroscopy.

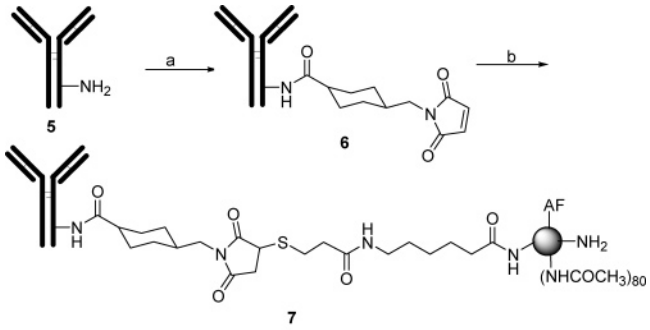
**Cell Culture and in Vitro Microscopy Experiments.** MCA 207 control and −HER2 cells were a generous gift from Dr. Kevin McDonagh (University of Kentucky). SK-BR-3 cells were obtained from American Type Tissue Collection (Manasass, VA) and were grown as a monolayer at 37 °C and 5% $CO_2$ in McCoy's 5A medium supplemented with 15% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 $\mu$g/mL). The standard fluorescence of the dendrimer solutions was quantified using a Beckman spectrofluorimeter. For flow

**Scheme 1. Synthesis of Dendrimer Thiol, 4**




(a) $Ac_2O$, $Et_3N$, MeOH, rt, 24 h; (b) Sulfo-LC-SPDP, PBS, rt, 3 h; (c) AF-NHS ester, DMSO/PBS, 24 h; (d) DTT, PBS-EDTA, 2h

**Scheme 2. Conjugation of Dendrimer Thiol with Anti-HER2 mAb**




a) Sulfo-SMCC, PBS, rt, 2h b) **4**, PBS EDTA, rt, 2h, N-ethylmaleimide

cytometric analysis of the uptake of the targeted polymer, cells were trypsinized and suspended in PBS containing 0.1% bovine serum albumin (PBSB) and analyzed using a Becton Dickinson FACScan analyzer. The FL1 fluorescence of 10 000 cells was measured, and the mean fluorescence of gated viable cells was quantified. For confocal experiments, cells were seeded at a density of $5 \times 10^5$ cells/plate on glass-bottom culture dishes (Mattek, Ashland, MA) 2 days prior to the experiment. Before each experiment, cells were washed three times in Dubelco's phosphate buffered saline (pH = 7.4) and incubated with 30 nM G5−HN−AF 488 or HN−AF 488 for 60 min at 37 °C except where noted. Preincubation experiments were carried with 3 $\mu$M HER2−mAb solution. For quenching experiments, anti-Alexa 488 (Molecular Probes, Eugene, OR) 600 nM was applied. The cells were fixed with 2% *p*-formaldehyde for 15 min at room temperature, washed, and mounted with a coverslip using Prolong Gold (Molecular Probes, Eugene, OR). In some cases, Prolong gold with DAPI was used to counterstain the nuclei of the cells. Cells were imaged on an Olympus FluoView 500 laser scanning confocal microscope with a 60×, 1.4 NA objective and pinhole settings resulting in an optical section thickness of 0.5 $\mu$m.

**Animal Model.** MCA207 control and −HER2 xenograft tumors were developed in 7-week-old female SCID mice by subcutaneous inoculation of 50 000 cells in the left and right flank areas. The cells were rinsed and injected as a 0.1 mL suspension using sterile PBS. Mice were housed under sterile conditions, and the tumors were allowed to reach 0.7−0.8 cm in diameter before analysis. A solution of 2.4 nmol of the conjugate in 0.1 mL of PBS or a control solution of PBS alone

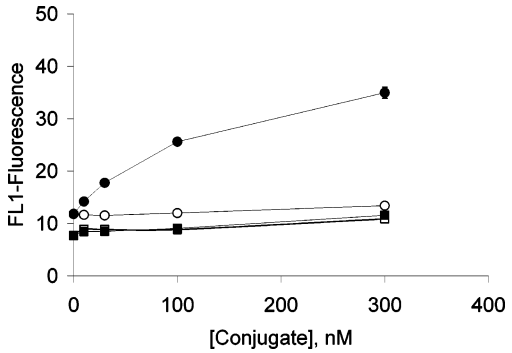



**Figure 1.** In vitro binding of the control conjugate G5−AF (open symbols) and the targeted conjugate G5−AF−HN (filled symbols) in control MCA207 cells (square symbols) and HER2-expressing (circle symbols) MCA207 cells. The cells were exposed to different concentrations of the conjugates for 1 h at 37 °C, rinsed, and the fluorescence measured by flow cytometry. Shown is the mean ± SE of three cell samples from an experiment. Similar results were obtained in an independent experiment.

was administered through the tail vein. Fifteen hours later, at the end of the study, the mice were anesthetized with isoflurane, and the tumors were excised and frozen in Optimum Cutting Temperature solution (Sakura Finetechnical, Tokyo, Japan) by immersing in methylbutane/dry ice, as we have described before (*25*). The tumors were subsequently sectioned on a cryostat into 10 $\mu$m sections which were thawed, rinsed three times with DPBS, and fixed in 2% *p*-formaldehyde for 15 min at 4 °C. The sections were then rinsed and mounted with coverslips using Prolong gold with DAPI. Images were taken on an Olympus FluoView 500 laser scanning confocal microscope using a 20×, 0.7 NA objective and an optical section thickness of 1.0 $\mu$m, unless otherwise indicated.

## RESULTS

**Conjugate Synthesis and Characterization.** The dendrimer terminal group modification was done as shown in Scheme 1. In order to improve targeting efficacy and reduce the nonspecific interactions, amine-terminated G5 dendrimers were partially surface modified with acetic anhydride (75 mol equiv) in the presence of triethylamine as base. The surface modification leaves some unreacted amines on the surface while considerably reducing the nonspecific binding to cells. The partially acetylated G5 dendrimer (G5−Ac, **1**) was purified by repeated dialysis initially using pH 7.4 PBS and subsequently using water. The purity of compound **1** and the extent of acetylation were measured and monitored by $^1$H NMR, which shows a distinct signal for the terminal $NHCOCH_3$ protons of the dendrimer at $\delta$1.85. $^{13}$C NMR spectrum also showed a new peak at $\delta$174.4 for the carbonyl carbon of the acetyl group. The degree of acetylation was measured by comparing the ratio of $NHCOCH_3$ protons with the sum of all methylene protons in the dendrimer

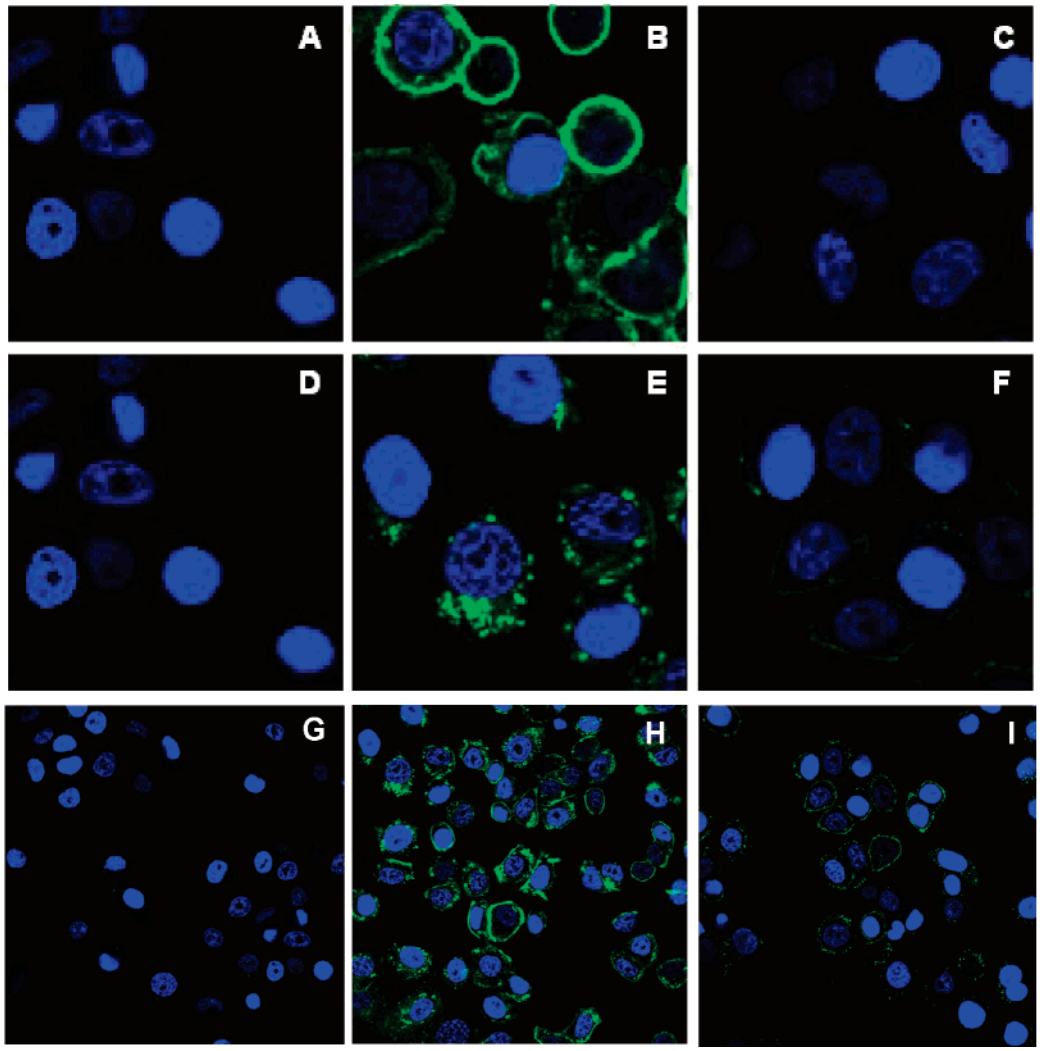



**Figure 2.** The blocking of HN−AF (A−C) and G5−HN−AF (D−F) by free anti-HER2 mAb in SKBR-3 cells. (A,D) Unstained cells. (B,E) Cells stained with 30 nM compound. (C,F) Cells blocked with 3 $\mu$M free anti-HER2 mAb before the addition of 30 nM compound. Panels G, H, and I show the lower-magnification images of D, E, and F, respectively.

to a calibration curve as described previously (*24*). In order to introduce a disulfide group on the dendrimer, heterobifunctional cross-linking agent sulfo-SPDP was conjugated to the partially acetylated dendrimer (G5−Ac, **1**) to provide a protected thiol in the form of a disulfide (G5−Ac−SPDP, **2**) as described previously (*13*). The extent of disulfide modification was monitored by UV spectroscopy, using the pyridine-2-thione assay as described in the manufacturer's protocol. Briefly, DTT was added to a measured quantity of dendrimer, and absorbance of the released 2-thiopyridine at 343 nm was recorded. On the basis of this measurement, an average of three disulfide groups per dendrimer in **2** were calculated, which is in agreement with values obtained by $^1$H NMR by comparing the integral values of the heteroaromatic signals of the pyridine and the aliphatic signals of the dendrimer.

Compound **2** was reacted with alexaFluor NHS ester (5 molar excess) to give alexaFluor-labeled conjugate **3**. The $^1$H NMR spectrum of the conjugate shows overlapping signals in the aromatic region for both the alexaFluor and the pyridine ring from the disulfide linker apart from the expected aliphatic signals for the dendrimer. The number of dye molecules was calculated to be ∼3 based on UV−vis spectroscopy. The reduction of disulfide bonds on dendrimer conjugate **3** was carried out using dithiothreatol, DTT, in PBS−EDTA buffer. The resultant dendrimer thiol, **4**, was carefully purified under an inert atmosphere in degassed PBS−EDTA buffer.

A thiol-reactive maleimide group was introduced on the antibody, **5**, with water-soluble sulfo-SMCC using standard protocols (*13*), and the resultant conjugate, **6**, was purified by gel filtration. To minimize the free antibody, a tenfold molar excess of dendrimer conjugate was used in the antibody−dendrimer coupling reaction; the unreacted thiols were quenched with *N*-ethylmaleimide to minimize the dimer formation due to cross-linking of dendrimer to antibodies. Free unreacted dendrimer was removed by filtration with a 100K MWCO microcon. The resultant conjugate, **7** (Scheme 2), was analyzed using PAGE, which shows a band above the antibody band that confirms the conjugation and an absence of a band for the free dendrimer. The fluorescence of the conjugates gives indirect proof of conjugation, as the fluorescent label was attached to the dendrimer.

**In Vitro Targeting Studies.** The cellular uptake of dendrimer−anti-HER2−mAb conjugates was measured in MCA207 control and MCA207−HER2 cells by flow cytometry (*25*). As shown in Figure 1, the conjugate G5−AF−HN bound the HER2-expressing cells but not MCA 207 control cells that do not express the receptor. The receptor specificity of the conjugates was further tested in SKBR-3 cells, which have been shown to express the HER2 receptor by confocal microscopy (*17*). SKBR-3 cells stained with HN−AF or G5−AF−HN (30 nM) were washed and fixed with *p*-formaldehyde; the nuclei were counterstained with DAPI. It is evident from the appearance of fluorescence in Figure 2B,E that conjugate **7** binds to the cell surface receptors and internalizes, whereas HN−AF reveals a different pattern with a higher signal on the cell surface and more uniform distribution of staining, suggesting a different internalization pattern. The images of cells stained only with DAPI (A,D) did not show autofluorescence within the instrumental parameters used. As shown in Figure 2C,F, pretreatment of cells with a 100-fold excess of free anti-HER2−mAb completely blocked fluorescence. The flow cytometry and confocal microscopic analysis suggest that anti-HER2−mAb dendrimer conjugate not only binds the cell surface HER2 receptor but is also internalized in the cells overexpressing the HER2 receptor.

**In Vitro Internalization.** AlexaFluor 488 serves as a hapten that is recognized by specific rabbit, anti-Alexa 488 antibodies.

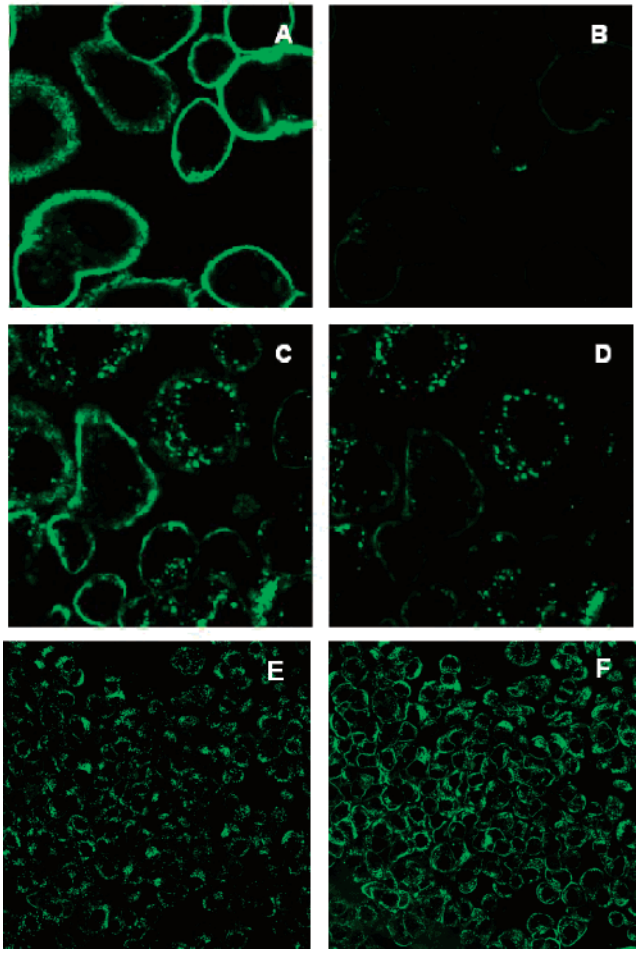



**Figure 3.** The quenching of surface-bound AlexaFluor with anti-Alexa 488 in SKBR-3 cells. (A,B) Cells stained with 30 nM HN−AF 488. The same field of view is shown before (A) and 10 min after (B) the addition of 600 nM anti-Alexa. (C,D) The same experiment using G5−HN−AF. (E,F) Lower-magnification images of C and D, respectively.

Upon binding, anti-Alexa 488 effectively quenches the fluorescence of AlexaFluor 488. Since anti-Alexa 488 does not cross cellular membranes, it quenches the fluorescence of surface-bound AlexaFluor-labeled conjugates, leaving aside the conjugates internalized within cells. Therefore, the decrease in fluorescence upon binding of anti-Alexa 488 to cells pretreated with HN−AF 488 or G5−AF−HN provides a useful index of the degree of internalization of these compounds. Figure 3A shows cells that were incubated with HN−AF for 1 h at 37 °C. In Figure 3B, after the addition of anti-Alexa 488, nearly all of the fluorescence was quenched, confirming that this compound is mainly surface-bound. In contrast, when the same experiment is performed with G5−AF−HN, a much smaller decrease in fluorescence is observed, indicating that antibody−dendrimer conjugate is largely internalized.

Comparison of the time course of the internalization of HN−AF and G5−AF−HN in SKBR-3 cells reveals that the antibody−dendrimer conjugate was rapidly internalized in HER2-expressing cells in comparison with antibody alone. As shown in Figure 4A,D, after 30 min at 4 °C, both conjugates were mainly localized in the cellular membrane. After 1 h incubation at 37 °C (panels B and E), HN−AF was still present in the plasma membrane, whereas a significant internalization was observed in the cells incubated with G5−AF−HN. After 6 h incubation at 37 °C (panels C and F), G5−AF−HN internalization was significantly higher as compared to HN−AF.

**In Vivo Studies.** Tumors were developed in SCID mice by subcutaneous injection of the control and HER2-expressing

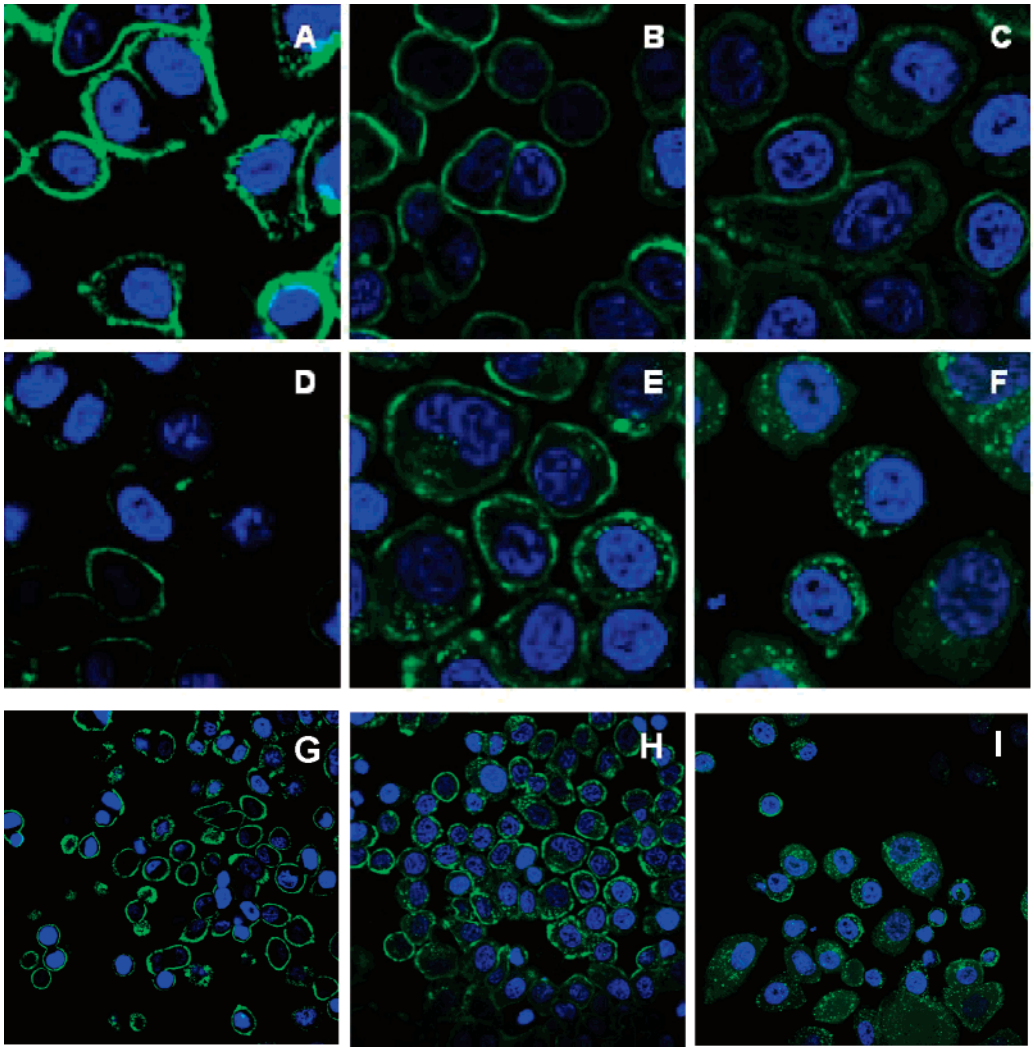



**Figure 4.** The time course of the internalization of HN−AF (A−C) and G5−HN−AF (D−F) in SKBR-3 cells. Cells were stained with 30 nM compound according to the following conditions: (A,D) 30 min at 4 °C; (B,E) 1 h at 37 °C; (C,F) 6 h at 37 °C; (G,H,I) lower-magnification images of D, E, and F, respectively.

MCA 207 cells, on left and right flank areas of the mice. The tumor uptake of G5−AF−HN or its vehicle was tested by injecting the conjugates into the bloodstream from the tail vein. As shown in Figure 5, the conjugate showed an increased binding to HER2-expressing tumor when compared with control tumor, whereas both PBS and control compound (G5−AF) did not show any preferential binding by flow cytometry.

We also analyzed the in vivo binding by confocal microscopy. Internalized fluorescence due to uptake of the conjugate can be clearly seen in HER2-expressing tumor cells (Figure 6A), whereas no fluorescence was observed in any of the control cells, including an HER2-negative tumor from the same animal (B) and HER2-positive tumors in animals where PBS (C) or the control conjugate G5−AF (D) was given.

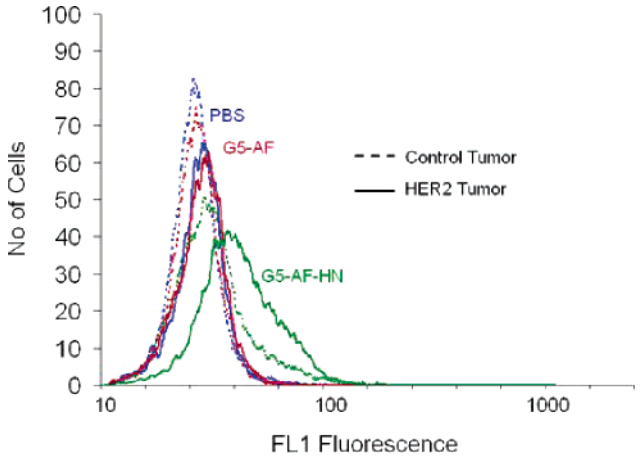



**Figure 5.** Binding of G5−AF−HN in MCA 207 control and HER2-expressing mice xenograft tumors in vivo. Tumors were developed in SCID mice by subcutaneous injection of the two cell lines on left and right flank areas, respectively, of the mice. When the tumors were about 0.7 cm in diameter, 2.4 nmols G5−AF−HN or its vehicle were injected intravenously. The tumors were isolated after 15 h, and the cells were separated by collagenase digestion and analyzed by flow cytometry.

## DISCUSSION

The major disadvantage of most antitumor drugs is their high toxicity, which leads to toxic side effects. Targeted cancer therapy aims to reduce or eliminate side effects by specifically delivering a cytotoxic pharmaceutical agent to tumor cells. Successful drug targeting can also result in a lower dosage required to obtain a therapeutic response, thus improving antitumor effects and again limiting systemic toxicity. Antibodies themselves can be used as drug carrier systems, but attaching drugs to an immunoglobulin may result in the loss of binding efficacy and decrease the solubility of the immunoconjugates. These problems can be overcome by attaching a water-soluble drug reservoir to the antibodies. The G5−PAMAM dendrimer is stable and nonimmunogenic and contains, on average, 110−

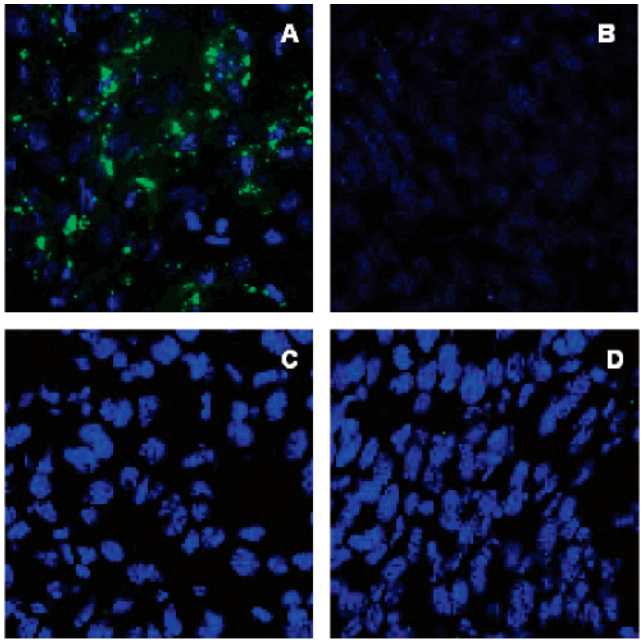



**Figure 6.** Images of tumor sections. (A,B) HER2+ (A) and HER2− (B) tumors from an animal injected with G5−HN−AF. (C,D) HER2+ tumors from an animal injected with PBS (C) or G5−AF (D).

128 primary amines on the surface. This provides an ample number of reactive sites for the construction of complex drug delivery systems by attaching multiple chemical moieties, such as radiopharmaceuticals, drugs, dyes, and contrast agents (*26*). The overexpression of HER2 protein in a high percentage of epithelial tumors and substantially greater expression of HER2 protein on cancerous cells will permit the selective targeting of malignant cells using anti-HER2−mAb. Therefore, in the present study, we synthesized a conjugate of HER2−mAb with PAMAM dendrimer as the drug carrier. Amine-terminated PAMAM dendrimers are reported to bind to the cells in a nonspecific manner owing to the positive charge on the surface and were also shown to be comparatively more toxic than charge neutral surfaces. The amine-terminated G5 dendrimer was partially surface modified with acetic anhydride in presence of triethylamine as base. The partial modification of the surface also improves the aqueous solubility and minimizes aggregate formation. The partially acetylated dendrimer was reacted with a heterobifunctional linker, sulfo-LC-SPDP to provide a 2-pyridylthiol group on the surface, which upon reduction with DTT gives a reactive thiol group. For detection of conjugates by flow cytometry or confocal microscopy, a detectable fluorescent probe is needed. We used AlexaFluor 488 (AF) as the fluorescent label, because it is significantly brighter than fluorescein conjugates (*27*) and is also much more photostable. The reduction of the 2-pyridylthiol group and conjugation to anti-HER2−mAb which was modified with maleimide linker gave the final dendrimer−mAB conjugate. The remaining thiol groups were capped with *N*-ethylmaleimide to prevent cross-linking. The HPLC analysis of the final conjugate shows a homogeneous peak, which indicates that under the present reaction conditions there was minimal cross-linking, possibly because of the steric hindrance posed by the relatively larger size of the antibody.

The dendrimer−anti-HER2−mAb bound HER2-expressing MCA 207 to the cell in a dose-dependent manner as shown by flow cytometery. As flow cytometry cannot distinguish between conjugate uptake and cell surface binding, confocal laser scanning microscopy was used to evaluate the cellular uptake of the synthesized dendrimer conjugate. By confocal microscopic analysis, it was shown that the dendrimer−HER2−mAb conjugate not only bound to the outer surface of the HER2-expressing cells but was also internalized. The conjugate uptake was further confirmed by quenching experiments carried out using anti-Alexa 488. The anti-Alexa is a noninternalizing antibody that interacts with surface-bound AlexaFluor and quenches its fluorescence. This experiment indicates that dendrimer−HER2−mAb conjugate was predominantly internalized into HER2-expressing tumor cells. A time course study of internalization demonstrated that the antibody-conjugated dendrimer internalized in the HER2-expressing cells more efficiently and rapidly. The in vivo experiments indicate that this conjugate may be capable of targeting imaging agents and/or chemotherapeutics to HER2-overexpressing tumors. In conclusion, we have synthesized anti-HER2−dendrimer conjugate that efficiently binds to HER2-overexpressing cells. From flow cytometery and confocal microscopic analysis, it was demonstrated that the anti-HER2−mAb−dendrimer conjugates not only bound the HER2-overexpressing cells but was also internalized in the cells. Preincubation experiments with unlabeled antibody, a lack of binding and uptake in control cells, and insignificant binding of control antibody−dendrimer conjugates all suggest that the conjugates internalize in HER2-overexpressing cells. The time course of internalization reveals a faster and more efficient internalization of the conjugate than anti-HER2 antibody alone. In vivo experiments indicate that this conjugate may be capable of targeting imaging agents and/or chemotherapeutics to HER2-overexpressing tumors.

## ACKNOWLEDGMENT

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**Supporting Information Available:** Potentiometric titration, pyridine-2-thione assay, and HPLC analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

## LITERATURE CITED

(1) Guillemard, V., and Saragovi, H. U. (2001) Taxane-Antibody Conjugates Afford Potent Cytotoxicity, Enhanced Solubility and Tumor Target Selectivity. *Cancer Res. 61*, 694−699.

(2) Baloglu, E., Miller, M. L., Roller, E. E., Cavanagh, E. E., Leece, B. A., Goldmacher, V. S., and Chari, R. V. J. (2004) Synthesis and biological evaluation of novel taxoids designed for targeted delivery to tumors. *Bioorg. Med. Chem. Lett. 14*, 5885−5888.

(3) Smallshaw, J. E., Ghetie, V., Rizo, J., Fulmer, J. R., Trahan, L. L., Ghetie, M. A., and Vitetta, E. S. (2003) Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice. *Nat. Biotechnol. 21*, 387−389.

(4) Seidl, C., Schrock, H., Seidenschwang, S., Beck, R., Schmid, E., Abend, M., Becker, K. F., Apostolidis, C., Nikula, T., Kremmer, E., Schwaiger, M., and Senekowitsch-Schmidtke, R. (2005) Cell death triggered by alpha-emitting Bi-213-immunoconjugates in HSC45-M2 gastric cancer cells is different from apoptotic cell death. *Eur. J. Nucl. Med. Mol. Imaging 32*, 274−285.

(5) Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich. A., and Press, M. F. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244*, 707−712.

(6) Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J. 19*, 3159−3167.

(7) Cho, H.-S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W. J., and Leahy, D. J. (2003) Structure of the extracellular region of Her2 alone and in complex with the herceptin Fab. *Nature (London) 421*, 756−760.

(8) Park, J. W., Hong, K., Carter, P., Asgari, H., Guo, L. Y., Keller, G. A., Wirth, C., Shalaby, R., Kotts, C., and Wood, W. I. (1995) Development of anti-p185her2 immunoliposomes for cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1327−1331.

(9) Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B. B., Henner, D., Wong, W. L. T., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. (1992) Humanization of an anti-p185her2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. U.S.A. 89*, 4285−4289.

(10) Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. (1999) Phase II study of weekly intravenous trastuzumab (herceptin) in patients with her2/neu-overexpressing metastatic breast cancer. *Semin. Oncol. 26*, 78−83.

(11) Vogel, C., Cobleigh, M. A., Tripathy, D., Gutheil, J. C., Harris, L. N., Fehrenbacher, L., Slamon, D. J., Murphy, M., Novotny, W. F., Burchmore, M., Shak, S., and Stewart, S. J. (2001) First-line, single-agent herceptin (trastuzumab) in metastatic breast cancer: a preliminary report. *Eur. J. Cancer 37*, 25−29.

(12) Patri, A. K., Myc, A., Beals, J., Thomas, T. P., Bander, N. H., and Baker, J. R. (2004) Synthesis and in vitro testing of J591 antibody−dendrimer conjugates for targeted prostate cancer therapy. *Bioconjugate Chem. 15*, 1174−1181.

(13) Thomas, T. P., Patri, A. K., Myc, A., Myaing, M. T., Ye, J. Y., Norris, T. B., and Baker, J. R. (2004) In vitro targeting of synthesized antibody-conjugated dendrimer nanoparticles. *Biomacromolecules 5*, 2269−2274.

(14) Wu, G., Barth, R. F., Yang, W. L., Chatterjee, M., Tjarks, W., Ciesielski, M. J., and Fenstermaker, R. A. (2004) Site-specific conjugation of boron-containing dendrimers to anti-EGF receptor monoclonal antibody cetuximab (IMC-C225) and its evaluation as a potential delivery agent for neutron capture therapy. *Bioconjugate Chem. 15*, 185−194.

(15) Kobayashi, H., Reijnders, K., English, S., Yordanov, A. T., Milenic, D. E., Sowers, A. L., Citrin, D., Krishna, M. C., Waldmann, T. A., Mitchell, J. B., and Brechbiel, M. W. (2004) Application of a macromolecular contrast agent for detection of alterations of tumor vessel permeability induced by radiation. *Clin. Cancer Res. 10*, 7712−7720.

(16) Chiu, S. H., Ueno, N. T., and Lee, R. J. (2004) Tumor-targeted gene delivery via anti-HER2 antibody (trastuzumab, Herceptin (R)) conjugated polyethylemmine. *J. Controlled Release 97*, 357−369.

(17) Wartlick, H., Michaelis, K., Balthasar, S., Strebhardt, K., Kreuter, J., and Langer, K. (2004) Highly specific HER2-mediated cellular uptake of antibody-modified nanoparticles in tumour cells. *J. Drug Targeting 12*, 461−471.

(18) Copland, J. A., Eghtedari, M., Popov, V. L., Kotov, N., Mamedova, N., Motamedi, M., and Oraevsky, A. A. (2004) Bioconjugated gold nanoparticles as a molecular based contrast agent: Implications for imaging of deep tumors using optoacoustic tomography. *Mol. Imaging Biol. 6*, 341−349.

(19) Duncan, R., and Izzo, L. (2005) Dendrimer biocompatibility and toxicity. *Adv. Drug Delivery Rev. 57*, 2215−2237.

(20) Roberts, J. C., Bhalgat, M. K., and Zera, R. T. (1996) Preliminary biological evaluation of polyamidoamine (PAMAM) starburst dendrimers. *J. Biomed. Mater. Res. 30*, 53−65.

(21) Patri, A. K., Majoros, I. J., and Baker, J. R. (2002) Dendritic polymer macromolecular carriers for drug delivery. *Curr. Opin. Chem. Biol. 6*, 466−471.

(22) Boas, U., and Heegaard, P. M. H. (2004) Dendrimers in drug research. *Chem. Soc. Rev. 33*, 43−63.

(23) Majoros, I. J., Thomas, T. P., Mehta, C. B., and Baker, J. R. (2005) Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. *J. Med. Chem. 48*, 5892−5899.

(24) Majoros, I. J., Keszler, B., Woehler, S., Bull, T., and Baker, J. R. (2003) Acetylation of poly(amidoamine) dendrimers. *Macromolecules 36*, 5526−5529.

(25) Thomas, T. P., Myaing, M. T., Ye, J. Y., Candido, K., Kotlyar, A., Beals, J., Cao, P., Keszler, B., Patri, A. K., Norris, T. B., and Baker, J. R. (2004) *Biophys. J. 86*, 3959−3965.

(26) Kukowska-Latallo, J. F., Candido, K. A., Cao, Z. Y., Nigavekar, S. S., Majoros, I. J., Thomas, T. P., Balogh, L. P., Khan, M. K., and Baker, J. R. (2005) Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Res. 65*, 5317−5324.

(27) Shukla, R., Thomas, T. P., Peters, J., Kotlyar, A., Myc, A., Baker, J. R. (2005) Tumor angiogenic vasculature targeting with PAMAM dendrimer−RGD conjugates. *Chem. Commun. 46*, 5739−5741.

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