

Alpha-Particle Emitting Atomic Generator (Actinium-225)-Labeled Trastuzumab (Herceptin) Targeting of Breast Cancer Spheroids: Efficacy versus HER2/neu Expression

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ABSTRACT

Purpose: The humanized monoclonal antibody, trastuzumab (Herceptin), directed against HER2/neu, has been effective in the treatment of breast cancer malignancies. However, clinical activity has depended on HER2/neu expression. Radiolabeled trastuzumab has been considered previously as a potential agent for radioimmunotherapy. The objective of this study was to investigate the efficacy of trastuzumab labeled with the α -particle emitting atomic generator, actinium-225 (^{225}Ac), against breast cancer spheroids with different HER2/neu expression levels. ^{225}Ac has a 10-day half-life and a decay scheme yielding four α -particles.

Experimental Design: The breast carcinoma cell lines MCF7, MDA-MB-361 (MDA), and BT-474 (BT) with relative HER2/neu expression (by flow cytometry) of 1:4:18 were used. Spheroids of these cell lines were incubated with different concentrations of ^{225}Ac -trastuzumab, and spheroid growth was measured by light microscopy over a 50-day period.

Results: The activity concentration required to yield a 50% reduction in spheroid volume at day 35 was 18.1, 1.9, and 0.6 kBq/ml (490, 52, 14 nCi/ml) for MCF7, MDA, and BT spheroids, respectively. MCF7 spheroids continued growing but with a 20–30 day growth delay at 18.5 kBq/ml. MDA spheroid growth was delayed by 30–40 days at 3.7 kBq/ml; at 18.5 kBq/ml, 12 of 12 spheroids disaggregated after 70, days and cells remaining from each spheroid failed to form colonies within 2 weeks of being transferred to adherent dishes. Eight of 10 BT spheroids failed to regrow at 1.85 kBq/ml. All of the BT spheroids at activity concentra-

tions 3.7 kBq/ml failed to regrow and to form colonies. The radiosensitivity of these three lines as spheroids was evaluated as the activity concentration required to reduce the treated to untreated spheroid volume ratio to 0.37, denoted DVR_{37} . An external beam radiosensitivity of 2 Gy was found for spheroids of all three of the cell lines. After α -particle irradiation a DVR_{37} of 1.5, 3.0, and 2.0 kBq/ml was determined for MCF7, MDA, and BT, respectively.

Conclusion: These studies suggest that ^{225}Ac -labeled trastuzumab may be a potent therapeutic agent against metastatic breast cancer cells exhibiting intermediate to high HER2/neu expression.

INTRODUCTION

The humanized monoclonal antibody, trastuzumab, directed against HER2/neu, particularly in combination with chemotherapy, has been effective in the treatment of breast cancer malignancies overexpressing HER2/neu (1–4). This work examines a treatment approach using trastuzumab labeled with the α -particle emitting atomic generator, actinium-225 (^{225}Ac), to eradicate breast cancer metastases expressing variable levels of HER2/neu.

The HER2/neu oncogene encodes a transmembrane protein (p185^{HER2}) with extensive homology to the epidermal growth factor receptor. Amplification and overexpression of HER2/neu have been documented in many human tumors, most notably in breast cancer (5, 6). The expression of HER2/neu is relatively stable over time and is generally congruent at different metastatic sites (5, 7). However, HER2/neu protein has also been identified on cell membranes of epithelial cells in the gastrointestinal, respiratory, reproductive, and urinary tract, as well as in the skin, breast, and placenta. HER2/neu expression levels in these normal tissues are similar to the levels found in nonamplified, nonoverexpressing breast cancers cells (6). Approximately 30% of breast cancer patients have tumors overexpressing the HER2/neu receptor. Trastuzumab treatment has been limited to these patients because of the cross-reactivity with normal tissues noted above. HER2/neu has been considered previously as a target for radioimmunotherapy against breast cancer. The radionuclides, ^{131}I , ^{125}I , ^{186}Re (8, 9), the positron-emitter, ^{86}Y (10), and also ^{212}Pb (11), of which the daughter, ^{212}Bi , decays by α -particle emission, have been labeled to antibodies targeting HER2/neu and investigated in animal models.

The α -particle emitting atomic generator, ^{225}Ac , has a 10-day half-life, and each decay of ^{225}Ac leads to the emission of four α particles (Fig. 1), greatly increasing its efficacy over previously considered α -particle emitters (12–14). Studies, *in vitro* and in animal models, have shown that this radionuclide is ~1000-fold more effective per unit radioactivity than ^{213}Bi , a first generation α -emitter that is currently under clinical inves-

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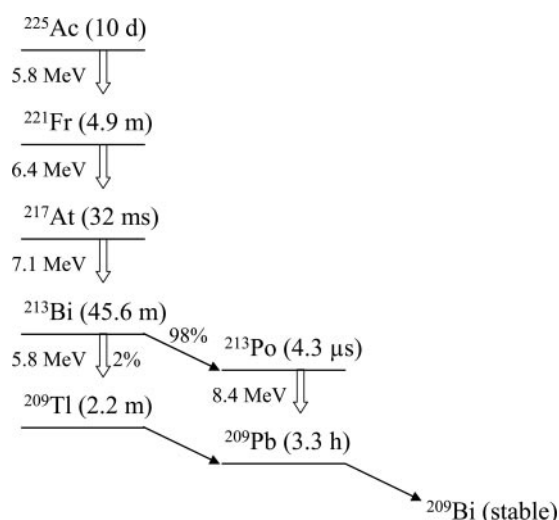


Fig. 1 Simplified decay scheme for actinium-225 (^{225}Ac). The arrows designate decay by α -particle emission; the average energy of emitted α -particles is shown next to each arrow.

tigation (15, 16). Studies in animals, however, have also shown that depending on the administration route, target, and chelation chemistry, it is also substantially more toxic (17–19). The increased efficacy arises because ^{225}Ac has a longer half-life (10 days *versus* 45.6 min for ^{213}Bi), increasing the total number of decays per unit of radioactivity, allowing prolonged irradiation of targeted cells, and because its decay leads to the release of three α -particle emitting daughters. The toxicity arises because chelate conjugated antibody delivery of this radionuclide can only retain, within the chelate, the first of the four α -emitting atoms. The chelation of the radionuclide is disrupted upon transformation of the parent atom, and emission of the first α . Subsequent α -emitting daughter atoms are, therefore, free to possibly distribute elsewhere in the body and potentially irradiate normal organs. This will be mitigated if the radiolabeled antibody is internalized, because charged daughter atoms produced intracellularly are retained within the cell (18).

Such a treatment strategy has been investigated, *in vitro*, using the spheroid model to represent rapidly accessible, intravascularly distributed tumor cell clusters (20). In anticipation of variable HER2/neu expression in a particular population of breast carcinoma cells, efficacy against cells with different HER2/neu expression levels has been examined. In contrast to traditional radioimmunotherapy with β -particle emitters, which kill cells over a large, multi-mm range, α -particles can kill individual cells; therefore, antigen density on the target cell will play an accordingly greater role in efficacy.

MATERIALS AND METHODS

Cells. The breast carcinoma cell lines MCF7, MDA-MB-361 (MDA), and BT-474 (BT) were purchased from the American Type Culture Collection (Manassas, VA). MCF7 monolayer cultures were incubated in MEM with NEAA (Memorial Sloan-Kettering Cancer Center Media Lab, New York, NY), MDA in L-15 (Memorial Sloan-Kettering Cancer Center Media

Lab), and BT in RPMI with 10 mM HEPES, 1 mM NA pyruvate, 2 mM L-glutamine, 1.5g/liter bicarbonate, and 4.5g/liter glucose (MSKCC Media Lab). The medium for all of the cell lines was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cell cultures were kept at 37°C in a humidified 5% CO_2 and 95% air incubator.

Spheroids. Spheroids were initiated using the liquid overlay technique of Yuhas *et al.* (21) and Ballangrud *et al.* (22). Approximately 10^6 cells, obtained by trypsinization from growing monolayer cultures, were seeded into 100-mm dishes coated with a thin layer of 1% agar (Bacto Agar; Difco, Detroit, MI) with 15 ml of medium. The medium used was the same as for monolayer cultures. After 5–7 days, spheroids with approximate diameters of $200 \pm 20 \mu\text{m}$ were selected under an inverted phase-contrast microscope with an ocular scale using an Eppendorf pipette. The selected spheroids were transferred to 35-mm bacteriological Petri dishes in 2-ml medium for treatment.

Spheroids selected for disaggregation were centrifuged at $100 \times g$ for 1 min to remove medium. The pellet was then suspended and gently mixed in preheated (37°C) PBS contain-

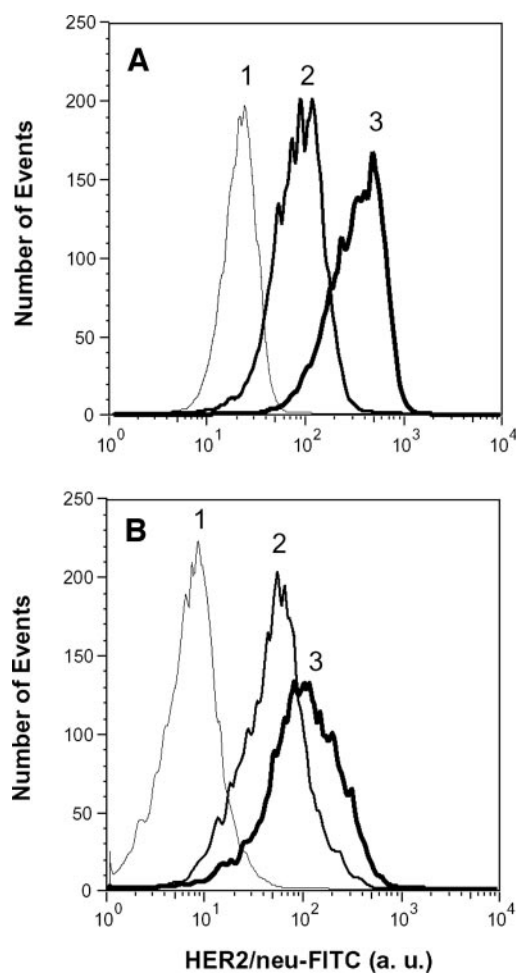


Fig. 2 Expression of HER2/neu as determined by flow cytometry of (A) cells from monolayer culture and (B) cells from disaggregated spheroids. Traces 1, 2, and 3 correspond to MCF7, MDA-MB-361, and BT-474 cells, respectively.

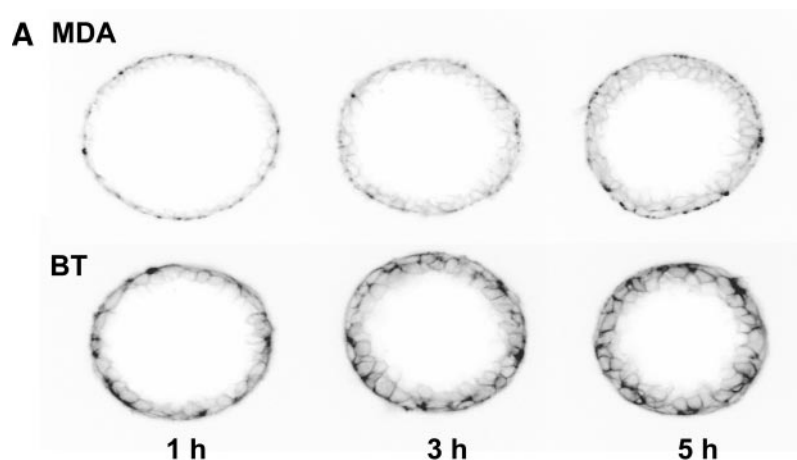
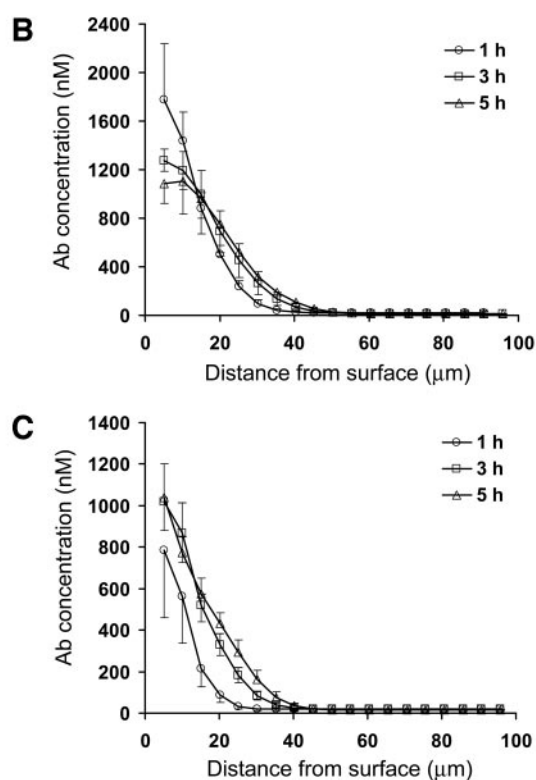


Fig. 3 A, confocal microscopy images of spheroids ($\phi \sim 200$ μm) after 1-, 3-, and 5-h incubation with 10 $\mu\text{g}/\text{ml}$ trastuzumab-FITC. The *black* or *gray* regions reflect presence of trastuzumab. Individual cells are clearly outlined in the surface layer of MDA-MB-361 (*MDA*) and BT-474 (*BT*) spheroids, consistent with cell-surface localization of *HER2/neu*. At 10 $\mu\text{g}/\text{ml}$ trastuzumab-FITC, no uptake of trastuzumab was observed in MCF7 spheroids. Also shown, trastuzumab concentration profiles across the spheroid equator after 1-, 3-, and 5-h incubation with 10 $\mu\text{g}/\text{ml}$ trastuzumab-FITC for (B) MDA and (C) BT spheroids. The mean of five individual spheroid measurements are depicted; bars, \pm SE.



ing 0.25% trypsin and 1 mM EDTA. Light microscopy was used to monitor the mixture for spheroid dissociation and membrane blebbing as an early indicator of membrane rupture. Dissociation normally occurred within 2 min, during which blebbing of cells was minimal. The suspension was immediately centrifuged at $75 \times g$ for 45 s to remove trypsin and the pellet resuspended in PBS for flow cytometry.

Flow Cytometry. The relative level of *HER2/neu* expression for the three cell lines was determined using the Becton-Dickinson FACSCalibur Analyzer (Franklin Lakes, NJ). *HER2/neu* expression was determined for cells from both monolayer culture and from disaggregated spheroids. All of the

washes and incubations were performed in FACS buffer (PBS + 0.5% BSA + 0.02% NaN_3). Cells were washed twice and resuspended at $1\text{--}2 \times 10^6$ cells/ml. A 100- μl aliquot was incubated with trastuzumab for 0.5 h on ice. Cells were again washed twice and resuspended in 100 μl buffer. The secondary, fluorescently tagged antibody, against the F_c portion of human IgG (Sigma; F-9512), was added and the suspension incubated on ice for 0.5 h. After a final two washes, cells were resuspended in 2 ml of cold buffer and analyzed. A total of 10,000 events were collected for each cell line.

Antibodies. Trastuzumab (anti-*HER2/neu*; Genentech, Inc., South San Francisco, CA) was used as the specific anti-

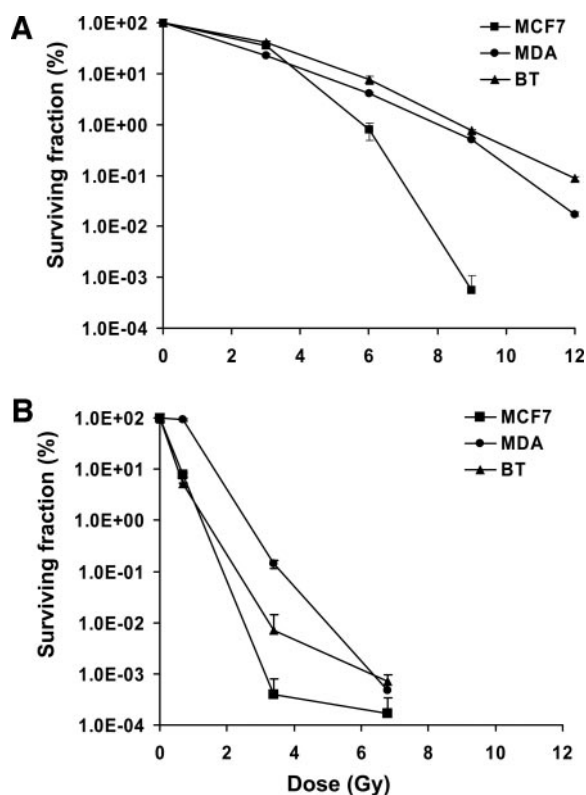


Fig. 4 Surviving fraction of MCF7, MDA-MB-361 (MDA), and BT-474 (BT) cells in monolayer cultures are shown after (A) acute doses of external beam radiation and (B) 24-h incubation with 3.7, 18.5, and 37 kBq/ml actinium-225-labeled nonspecific antibody, corresponding to absorbed doses of 0.7, 3.4, and 6.8 Gy, respectively; bars, \pm SE.

body. HuM195 (anti-CD33; Protein Design Laboratories, Inc., Sunnyview, CA) and J591 (anti-PSMA; generously supplied by Dr. Neil Bander, Department of Urology, New York Presbyterian Hospital-Weill Medical College of Cornell University and Ludwig Institute for Cancer Research, New York, NY) were used as nonspecific controls.

Confocal Microscopy. Spheroids of diameter 200 μ m were incubated with 10 μ g/ml FITC- (F7250; Sigma, St. Louis, MO) conjugated trastuzumab for 1, 3, and 5 h and imaged by confocal microscope (Zeiss LSM 510; Carl Zeiss, Inc. Oberkochen, Germany) while still in incubation medium. A 3- μ m-thick optical section was acquired at the center of each spheroid. Five spheroids were imaged for each time point. Antibody concentration as a function of radial distance was obtained using MIAU, a software package developed in-house (23). The method has been described previously (24). Briefly, an erosion element is used to follow the exterior contour of each spheroid, and the average pixel intensity in each ring is converted to antibody concentration by calibration with the known external concentration of antibody. The antibody concentration as a function of distance from the rim of the spheroid was corrected for light attenuation as described previously (24).

²²⁵Ac. ²²⁵Ac was obtained from the Department of Energy (Oak Ridge National Laboratory, Oak Ridge, TN) and was supplied as a dried nitrate residue. The ²²⁵Ac activity was

measured with a Squibb CRC-17 Radioisotope Calibrator (E.R. Squibb and Sons, Inc., Princeton, NJ) set at 775 and multiplying the displayed activity value by 5. The ²²⁵Ac nitrate residue was dissolved in 0.1 ml of 0.2 M Optima grade HCl (Fisher Scientific, Pittsburgh, PA). Metal-free water used for this and all of the other solutions was obtained from a Purelab Plus system (United States Filter Corp., Lowell, MA) and was sterile filtered.

Radiolabeling. Details regarding the radiolabeling methodology are described in reference (13).

The first step in construct preparation was the ²²⁵Ac-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA)-neocarzinostatin chelation reaction. The bifunctional isothiocyanato-derived 2B-DOTA, 2-(*p*-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid was obtained from Macrocyclics (Dallas, TX). ²²⁵Ac dissolved in 0.2 M HCl was mixed with 200–500 mg of 10 g/liter DOTA-neocarzinostatin in metal-free water, 0.015–0.020 ml of 150 g/liter stock *l*-ascorbic acid, and 0.025–0.150 ml of 2 M tetramethylammonium acetate. The mixture was then heated to 60°C for 30–45 min.

The second step in construct preparation was the ²²⁵Ac-DOTA-neocarzinostatin reaction with the IgG. The ²²⁵Ac-DOTA-neocarzinostatin chelation reaction was mixed with 0.5–1.0 mg of the IgG, 0.015–0.020 ml of 150 g/liter stock *l*-ascorbic acid, and 0.025–0.150 ml of a 1 M carbonate buffer. The reaction mixture was then heated to 36°C for 30–60 min. At the end of the reaction period, the mixture was treated with a 0.020-ml addition of 10 mM diethylenetriaminepentaacetic acid to complex any free metals during the size exclusion chromatographic purification using a 10 DG size exclusion column with a 1% human serum albumin as the mobile phase.

The radiochemical purity of ²²⁵Ac-DOTA-trastuzumab was >90% as determined by instant TLC methods, and the immunoreactivity of the labeled product was between 70% and 80% as determined by cell-based assay methods (25).

Radiosensitivity. The radiosensitivity of the different cell lines was determined in monolayer cultures using the colony-forming assay (26). Depending on the radiation dose, between 10³ and 10⁷ cells were plated in monolayer cultures. External beam radiosensitivity was determined after exposure to acute doses of 3, 6, 9, or 12 Gy photon irradiation using a cesium irradiator at a dose rate of 0.8 Gy/min (Cs-137 Model 68; JL Shepherd and Associates, Glendale, CA.). The absorbed dose required to yield a 37% survival in the log-linear portion of the surviving fraction curve (*i.e.*, the D_0 value) was obtained by fitting a monoexponential function to this portion of the curve.

Table 1 Dose, D_0 , required to reduce surviving fraction of cells in monolayer cultures following external beam and α -particle irradiation to 0.37

Cell line	External beam D_0 (Gy)	α -Particle D_0 (Gy)
MCF7	0.76 \pm 0.07	0.27 \pm 0.02
MDA ^a	1.38 \pm 0.01	0.53 \pm 0.03
BT	1.73 \pm 0.01	0.37 \pm 0.05

^a MDA, MDA-MB-361; BT, BT-474.

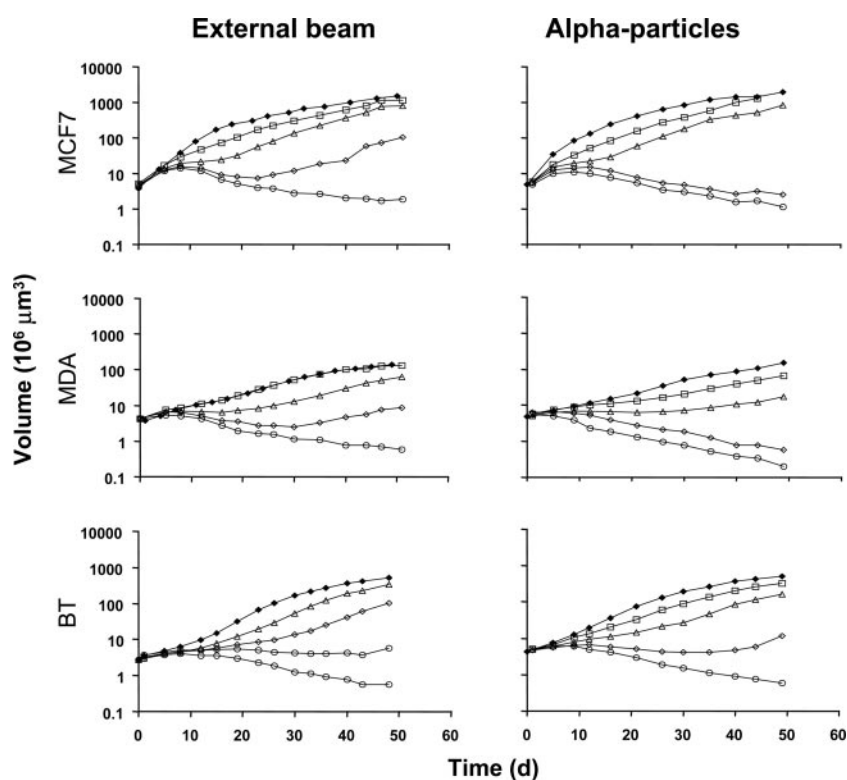


Fig. 5 Spheroid response to external beam irradiation (◆, untreated; □, 3 Gy; △, 6 Gy; ◇, 9 Gy; ○, 12 Gy) and increasing concentrations of actinium-225-labeled nonspecific antibody (◆, untreated; □, 1.85 kBq/ml; △, 3.70 kBq/ml; ◇, 9.25 kBq/ml; ○, 18.5 kBq/ml).

Monolayer cultures incubated with 3.7, 18.5, and 37 kBq/ml ^{225}Ac -labeled nonspecific antibody for 24 h were used to determine α -particle radiosensitivity. Over a 24-h period, 6.7% of the total number of ^{225}Ac atoms will have decayed. Because the longest-lived daughter, Bi-213, has a half-life of 45.6 min, all of the daughters generated during this period will also decay. Assuming, therefore, that each decay of ^{225}Ac deposits one-half (to account for cells settling to the bottom of the plate and, therefore, being irradiated only from one side) of the sum of all four of the α -particle energies, the mean absorbed dose is estimated to be 0.7, 3.4, and 6.8 Gy for each of the three concentrations, respectively.

The radiosensitivity of spheroids was evaluated as the activity concentration required to reduce the treated to untreated spheroid volume ratio to 0.37. Because this parameter depends on the day post-therapy, volume ratios from day 20 to day 45 after therapy were calculated for each spheroid, and the median value across this time period was used. By plotting this volume ratio *versus* activity concentration and fitting the log-linear portion of the curve to a monoexponential function, a radiosensitivity parameter may be derived from the slope. The inverse of the slope gives the dose that yields a volume ratio of 0.37. This value is denoted “DVR₃₇,” and it is loosely analogous to the D₀ in colony formation assays.

Treatment Protocol. The response to ^{225}Ac -labeled trastuzumab was evaluated by incubating spheroids with 0.37, 1.85, 3.70, or 18.50 kBq/ml ^{225}Ac on 10 $\mu\text{g}/\text{ml}$ trastuzumab (specific antibody) for 1 h. Spheroids exposed to 18.50 kBq/ml ^{225}Ac on 10 $\mu\text{g}/\text{ml}$ irrelevant antibody (radioactive control), 10 $\mu\text{g}/\text{ml}$ unlabeled trastuzumab (unlabeled antibody control), and un-

treated spheroids (control) were followed in the same manner. Twenty-four or 12 spheroids were used in each experiment. After incubation, the spheroids were washed three times by suspension in fresh medium and placed in separate wells of a 24-well plate. The medium in each well was replaced, and individual spheroid volume measurements were performed twice per week. An inverted phase microscope fitted with an ocular micrometer was used to determine the major and minor diameter d_{max} and d_{min} , respectively, of each spheroid. Spheroid volume was calculated as $V = \pi \cdot d_{\text{max}} \cdot d_{\text{min}}^2 / 6$. Volume monitoring was stopped once a spheroid diameter exceeded 1 mm or when the spheroid fragmented to individual cells or smaller (2–3-cell) clusters. The viability of such fragments was assessed in an outgrowth assay by plating the cell clusters on to adherent dishes, incubating for 2 weeks, and then evaluating for colony formation or outgrowth.

RESULTS

The relative HER2/*neu* cell-surface expression of MCF7, MDA, and BT cells derived from monolayer culture and from disaggregated spheroids is depicted in Fig. 2. The highest HER2/*neu* expressing cell line, BT, shows a decrease in the number of HER2/*neu* sites (relative to MDA) and also a greater variability in cell surface expression in cells derived from spheroids compared with cells from monolayer culture. The relative expression of HER2/*neu* in cells derived from monolayers is 1:4:18 (MCF7:MDA:BT); the corresponding expression ratios for cells derived from spheroids are 1:6:12.

Penetration of trastuzumab into spheroids was evaluated by

measuring FITC-labeled trastuzumab by confocal microscopy. Images acquired through the equator of 200- μm diameter spheroids incubated for 1, 3, and 5 h with 10 $\mu\text{g}/\text{ml}$ trastuzumab-FITC are shown for MDA and BT spheroids in Fig. 3A. The cells on the spheroid rim are clearly outlined, consistent with antibody localization to cell-surface HER2/neu. Trastuzumab has penetrated \sim 1, 2, and 3 cell layers after 1-, 3-, and 5-h incubation, respectively. FITC intensity was converted to antibody concentration as described in "Materials and Methods." The results are depicted in Fig. 3, B and C. After 1-h incubation, the concentration of antibody on the surface of BT spheroids is greater than twice that on the surface of MDA spheroids. At a depth of 20 μm , the antibody concentration in BT spheroids is 5-fold greater than in MDA spheroids.

To examine for a possible differential sensitivity to unlabeled trastuzumab antibody, spheroids of the three cell lines were incubated for 1 h in 10, 50, 100, and 500 $\mu\text{g}/\text{ml}$. No impact on spheroid growth was observed (data not shown).

To discriminate between inherent radiosensitivity of the different cell lines and increased targeting due to the differential expression of HER2/neu, the radiosensitivity of each cell line was determined in monolayer cultures as well as by following spheroid growth after external beam irradiation and after incubation with ²²⁵Ac-labeled nonspecific antibody. The surviving fraction of cells in monolayer culture is plotted *versus* mean absorbed dose for photons and α -particles in Fig. 4, A and B, respectively. The dose, $D_{0\%}$, required to yield a surviving fraction of 37% is listed in Table 1. MCF7 cells are 2-fold and 2.4-fold more sensitive to external beam radiation than MDA and BT cells, respectively. Although this cell line is also more sensitive to α -particle radiation than MDA and BT, the differences in radiosensitivity are less pronounced.

Spheroid response to 3, 6, 9, and 12 Gy external beam irradiation and increasing concentrations of ²²⁵Ac-labeled nonspecific antibody (24 h incubation) is depicted in Fig. 5. Fifty days after a 12 Gy external dose, outgrowth assays for MCF7 and BT spheroids showed viable cells, whereas no colonies were formed for MDA spheroids. At the two highest concentrations of ²²⁵Ac-labeled nonspecific radiolabeled antibody, outgrowth assays for MCF7 and MDA spheroids yielded no colonies; for BT the same result was obtained only at the highest radioactivity concentration used. The dose required to reduce the volume ratio of treated to untreated spheroids to 0.37, denoted DVR_{37} , was used as a measure of spheroid radiosensitivity and is listed in Table 2. The DVR_{37} results show no difference among spheroids of the three cell lines in sensitivity to external beam irradiation. Differences in volume response to α -particle irradiation are seen, however, with MCF7 almost a factor of 2 more sensitive than MDA.

Table 2 Dose required to reduce the treated to untreated spheroid volume ratio to 0.37 (DVR_{37})

Cell line	External beam DVR_{37} (Gy)	α -Particle DVR_{37} (kBq/ml)
MCF7	2.2 \pm 0.2	1.6 \pm 0.3
MDA ^a	2.1 \pm 0.1	3.0 \pm 0.8
BT	2.7 \pm 0.4	2.5 \pm 0.7

^a MDA, MDA-MB-361; BT, BT-474.

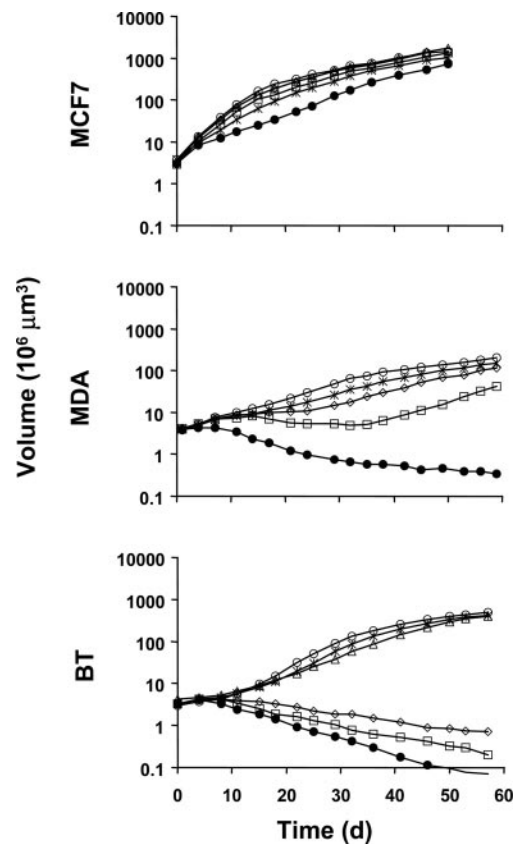


Fig. 6 Median growth curves for spheroids incubated 1 h with 0.37 (*), 1.85 (◇), 3.7 (□), and 18.5 (●) kBq/ml actinium-225 on 10 $\mu\text{g}/\text{ml}$ trastuzumab, or 18.5 kBq/ml on nonspecific antibody (radioactive control; ○).

Median growth curves for MCF7, MDA, and BT spheroids incubated 1 h with 0.37, 1.85, 3.7, and 18.5 kBq/ml ²²⁵Ac on 10 $\mu\text{g}/\text{ml}$ trastuzumab or 18.5 kBq/ml on nonspecific antibody (radioactive control) are depicted in Fig. 6. At day 35, the median volume of spheroids treated with 18.5 kBq/ml ²²⁵Ac-trastuzumab relative to spheroids incubated for 1 h with ²²⁵Ac-labeled nonspecific antibody (radioactive control) was 52%, 1.4%, and 0.3% for MCF7, MDA and BT, respectively. The ²²⁵Ac activity concentration required to yield a 50% reduction in spheroid volume relative to the radioactive controls at day 35 was 18.1, 1.9, and 0.6 kBq/ml (490, 52, 14 nCi/ml) for MCF7, MDA, and BT spheroids, respectively. Growth of individual spheroids after 1 h of incubation with increasing concentrations of ²²⁵Ac on 10 $\mu\text{g}/\text{ml}$ trastuzumab are shown in Fig. 7. The variability in response of individual spheroids was minimal. At an activity concentration of 1.85 kBq/ml, 2 of 12 BT spheroids were viable; no colonies were observed at 3.7 and 18.5 kBq/ml for this cell line. Likewise, no colonies were observed for MDA spheroids treated at 18.5 kBq/ml. Fig. 8 depicts optical microscope images of MDA spheroids after ²²⁵Ac-trastuzumab treatment. By 21 days after incubation with 3.7 kBq/ml sloughing of cells may be observed; by 42 days, however, the spheroid appears to have recovered. At 18.5 kBq/ml, however, no such recovery is observed.

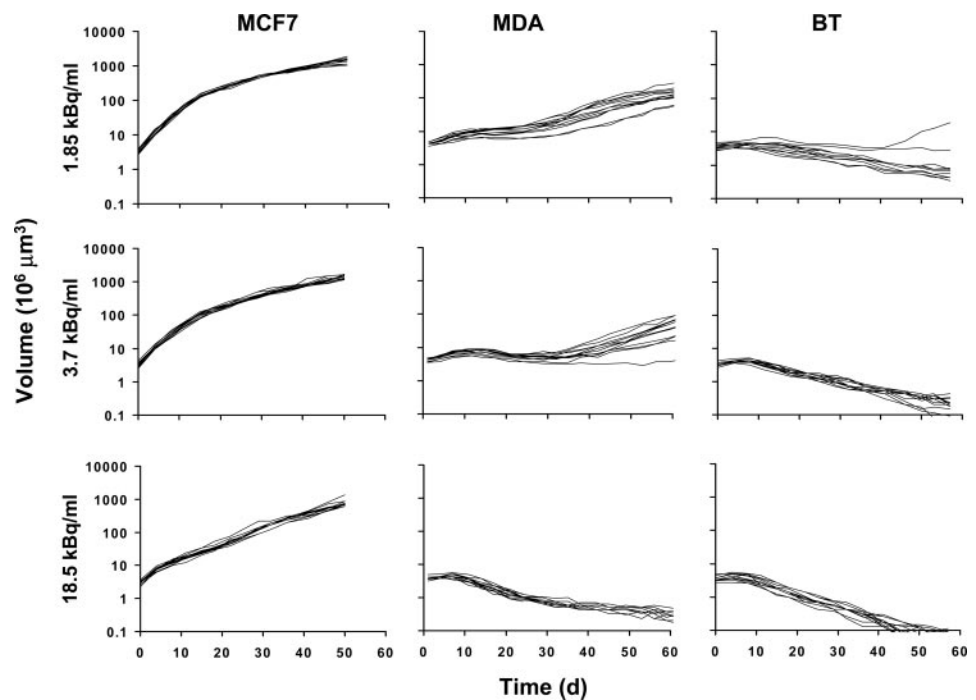


Fig. 7 Growth of individual spheroids after 1 h incubation with actinium-225-trastuzumab. Each curve corresponds to an individual spheroid. Twelve spheroids were used per experiment.

DISCUSSION

Trastuzumab-mediated targeting of ^{225}Ac to disseminated breast cancer will be a viable therapeutic approach in humans only if two fundamental problems are addressed. First, the high background expression of *HER2/neu* in normal tissues must be obviated, as this cross-reactivity is likely to lead to α -particle irradiation of normal tissues. Second, the potential toxicity associated with the distribution of free, α -particle emitting daughters resulting from the decay of ^{225}Ac must be overcome.

Both of these requirements may be met by targeting rapidly accessible micrometastatic disease in a treatment schedule in which i.v. administered ^{225}Ac -trastuzumab is allowed to distribute for several hours and is then cleared from the circulation, either by direct physical means such as plasmapheresis or immunoadsorption (27, 28). Extravasation of intact antibody into normal tissue parenchyma generally requires 24–48 h (29). By rapidly decreasing the concentration of circulating antibody, binding to normal cross-reactive tissues would be reduced sub-

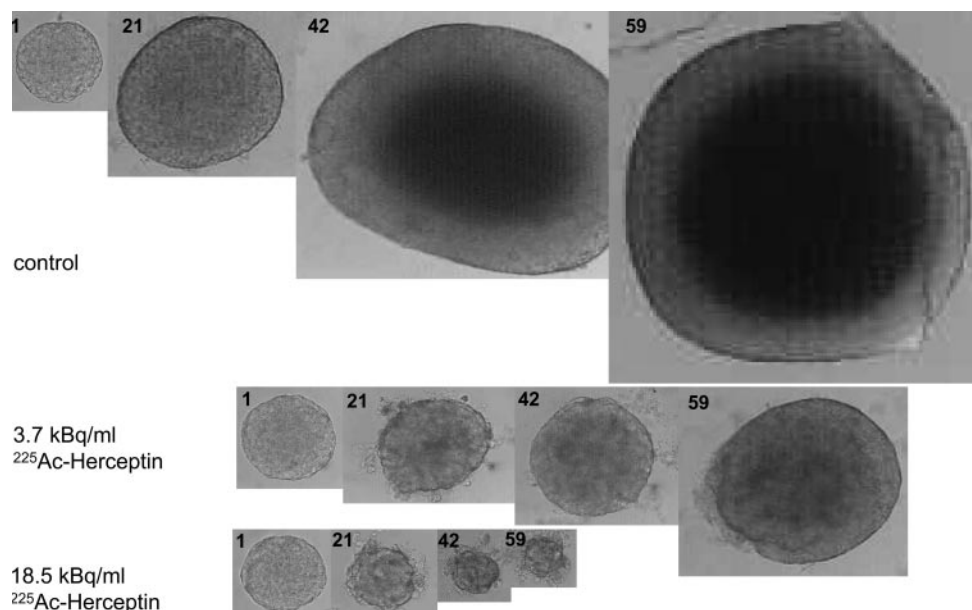


Fig. 8 Microscope images of two treated (3.7 kBq/ml and 18.5 kBq/ml) and one untreated (*control*) MDA-MB-361 spheroid after 1, 21, 42, and 59 days of 1-h incubation with actinium-225 (^{225}Ac)-trastuzumab.

stantially, whereas also reducing the ²²⁵Ac concentration in the circulation and, therefore, the subsequent concentrations of free daughters.

We have used the spheroid model as a preliminary *in vitro* model to examine the feasibility of targeting breast cancer micrometastases using trastuzumab labeled with the atomic α -particle generator, ²²⁵Ac. In particular, the efficacy against tumor cell clusters with different expression levels of HER2/neu was examined. As demonstrated by flow cytometry, the three cell lines considered approximated low (MCF7), intermediate (MDA MB-361), and high (BT-474) HER2/neu expressing metastases. The 1:4:18 relative cell-surface HER2/neu expression levels for MCF7:MDA:BT are of comparable magnitude to the 1:14:21 values calculated from data reported by Lewis *et al.* (30). Differences in the actual values may be explained by the different agents and incubation conditions used to perform the measurements. In a previous report (30), cells were incubated with the murine-derived anti-HER2/neu antibody 4D5 and also with a fluorescently labeled F(ab')₂ fragment of goat antimouse IgG. In the current studies, incubation was carried with the humanized version of 4D5, trastuzumab. The cells were subsequently incubated with a commercially available fluorescently tagged antihuman F_c antibody.

Antibody penetration relative to cell-surface antigen density was also examined. The trastuzumab concentration in BT spheroids after 1-h incubation was found to be a factor 2–3 higher than for the MDA spheroids, whereas the penetration depth into the spheroids was similar. The antibody concentration used is close to the average receptor concentration within the spheroid. Assuming 10⁶ receptors per cell (31) and $\sim 4 \times 10^{11}$ cells/liter (24), the concentration of receptor sites within the BT spheroid is ~ 660 nM. Because 10 μ g/ml intact antibody translates to ~ 670 nM, the concentration of antibody matches, and, due to the large antibody supply, would saturate available cell-surface receptor sites (27, 32). The confocal microscopy studies are consistent with this analysis.

Trastuzumab incubation in monolayer cultures is reported to result in increased cell doubling time leading to increased cell dormancy (30). This was not observed in spheroids where we found that a 1-h incubation with concentrations up to 500 μ g/ml trastuzumab had no effect on spheroid growth kinetics for the three cell lines tested. The absence of an effect on spheroids as opposed to monolayer cultures is probably the result of the very short incubation duration, the resulting incomplete penetration of the antibody and also possibly due to the increased resistance of spheroids *versus* monolayer cultures to growth inhibitory agents (33).

The differences in response of the three cell lines cannot be attributed to differences in radiosensitivity, because MCF7 spheroids, having the lowest response to ²²⁵Ac-trastuzumab, were also the most radiosensitive. In monolayer cultures, MDA and BT were approximately equivalent in photon radiosensitivity, whereas MCF7 was ~ 2 -fold more radiosensitive. MCF7 was also the line most sensitive to α -particle radiation, and MDA was the lowest in sensitivity to α . MCF7, MDA, and BT spheroids were found to have similar external beam radiosensitivity. Spheroids were found to have a greater differential sensitivity to α -particles than to external beam irradiation, although the opposite is true in monolayer cultures. It is important to note

that the radiosensitivity parameter defined in this work for spheroids is not a measure of cell sterilization but rather of volume reduction. Volume reduction encompasses a number of biological variables including the rates of cellular sterilization, removal of sterilized cells, and cellular proliferation.

As in other studies investigating the relationship between HER2/neu expression and growth inhibition using chemotherapeutic and biological agents (30, 34), the response of spheroids to ²²⁵Ac-trastuzumab was found to be highly dependent on HER2/neu expression. It was possible to sterilize spheroids with intermediate HER2/neu expression and to induce a growth delay in low HER2/neu-expressing spheroids by increasing the specific activity of the radiolabeled antibody. A very high specificity relative to the radioactive controls was observed. This is because targeted spheroids are exposed to the atomic α -particle generator for a prolonged time period due to binding and retention of the antibody. Longer radioactive control exposure durations such as the 24-h period used in the radiosensitivity measurements showed volume reductions similar to those obtained with the 1 h specific antibody incubation. The very high specificity seen with a short exposure time supports the clearing strategy outlined above.

In conclusion, we have demonstrated the ability to increase the efficacy of trastuzumab against clusters of tumor cells expressing intermediate levels of HER2/neu by labeling trastuzumab with the α -particle emitting atomic generator, ²²⁵Ac. These results suggest that an ²²⁵Ac concentration in the range 0.6–2 kBq/ml (20–75 nCi/ml) may be sufficient to achieve a substantial reduction in the number of tumor cells with intermediate HER2/neu expression. This translates to approximately 0.07–0.3 mCi for human administration. On the basis of animal studies, we expect that this activity concentration will be clinically implementable.

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