ARTICLES

Immunoconjugates of Geldanamycin and Anti-HER2 Monoclonal Antibodies: Antiproliferative Activity on Human Breast Carcinoma Cell Lines

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Background: **HER2 is a membrane receptor whose overexpression is strongly associated with poor prognosis in breast carcinomas. Inhibition of HER2 activity can reduce tumor growth, which led to the development of Herceptin, an anti-HER2 monoclonal antibody (MAb) that is already in clinical use. However, the objective response rate to Herceptin monotherapy is quite low. HER2 activity can also be inhibited by the highly cytotoxic antibiotic geldanamycin (GA). However, GA is not used clinically because of its adverse toxicity. Our purpose was to enhance the inhibitory activity of anti-HER2 MAb by coupling it to GA. *Methods:* We synthesized 17-(3-aminopropylamino)GA (17-APA-GA) and conjugated it to the anti-HER2 MAb e21, to form e21:GA. The noninternalizing anti-HER2 MAb AE1 was used as a control. Internalization assays and western blot analyses were used to determine whether the anti-HER2 MAbs and their immunoconjugates were internalized into HER2-expressing cells and reduced HER2 levels. All statistical tests were two-sided. *Results:* The immunoconjugate e21:GA inhibited the proliferation of HER2-overexpressing cell lines better than unconjugated e21 (concentration required for 50% inhibition = 40 versus 1650 μg/mL, respectively). At 15 μg/mL, e21:GA reduced HER2 levels by 86% within 16 hours, whereas unconjugated e21, 17-APA-GA, or AE1:GA reduced HER2 levels by only 20%. These effects were not caused by release of 17-APA-GA from the immunoconjugate because immunoconjugates containing [^{3}H]GA were stable in serum at 37 °C. Furthermore, e21:GA did not significantly inhibit proliferation of the adult T-cell leukemia cell line HuT102, which is HER2 negative yet highly sensitive to GA. *Conclusions:* Our findings suggest that conjugating GA to internalizing MAbs enhances the inhibitory effect of the MAbs. This approach might also be applied in cellular targeting via growth factors and may be of clinical interest. [J Natl Cancer Inst 2000;92:1573–81]**

Use of monoclonal antibodies (MAbs) with specificity toward tumor-associated markers is a relatively new and exciting modality in cancer therapy. Such MAbs may have an adequate antitumor activity as sole agents, and they can also be used to deliver conjugated cytotoxic agents, such as chemotherapeutic drugs, toxins, and radionuclides, to the tumors *(1–6)*. Several MAbs have already been advanced into clinical trials, and two— Herceptin (anti-HER2) and Rituxan (anti-CD20)—have been approved by the U.S. Food and Drug Administration *(7–10)*. The potential of immunotherapeutic approaches is clear, but experi-

mental and clinical data indicate that the strategies of selecting the appropriate tumor-associated marker and the targeting MAb need further definition.

One of the most promising targets for immunotherapy is the membrane receptor HER2, whose overexpression is strongly associated with poor-prognosis breast carcinomas. HER2, the product of the proto-oncogene ERBB2, is a 185-kd transmembrane receptor with protein tyrosine kinase activity. HER2 is believed to function as a modulator of other receptors in the epidermal growth factor receptor family because it forms heterodimers with these receptors and augments their proliferative activity. This receptor is only marginally expressed in adult tissues *(11,12)*, but it is overexpressed in approximately 30% of human gastric, lung, and breast carcinomas *(11,13–16)*. When overexpressed, HER2 appears to play an active role in the induction of neoplastic transformation, and blocking its activity has been shown to inhibit tumor cell proliferation *(11,13,17)*. Currently, HER2 serves as a tumor-targeting marker for the humanized anti-HER2 antibody Herceptin in the treatment of patients with metastatic breast carcinomas *(7,18,19)*. Herceptin and other anti-HER2 MAbs that possess antitumor activity induce HER2 homodimerization and internalization. However, when administered as the sole therapeutic agent, these MAbs do not eradicate established tumors *(2,4–6,20–24)*. The best outcomes for patients were achieved when the anti-HER2 MAbs were given in combination with other cytotoxic agents.

We have conjugated an anti-HER2 MAb to a derivative of the highly cytotoxic antibiotic geldanamycin (GA) to determine whether this immunoconjugate will have an enhanced antiproliferative activity compared with that of the native MAb. GA is a benzoquinoid ansamycin related to herbimycin A (Fig. 1) and
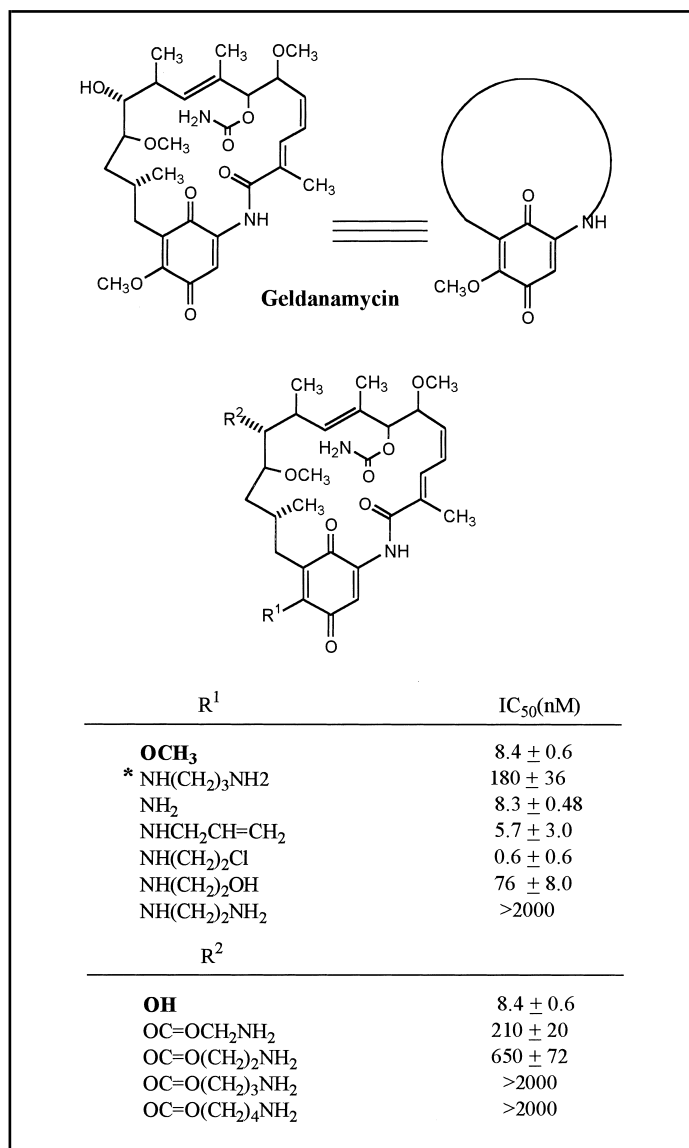
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Fig. 1. Geldanamycin (GA) derivatives and their effects on antiproliferative activity. GA is a naturally occurring antibiotic produced by the actinomycete *Streptomyces hygroscopicus*. It is a benzoquinoid ansamycin related to herbimycin A. The two-ring structure is shown schematically on the **right.** GA was modified on either position 17 (quinone ring) or position 11 (ansa ring), which are labeled R^1 and R^2, respectively. In native GA, $R^1 = OCH_3$ and $R^2 = OH$. For each derivative, only the modified site is shown. 17-(3-Aminopropylamino)GA (17-APA-GA) is marked by an **asterisk.** The antiproliferative activity of each derivative was tested after a 24-hour incubation by use of the [^{3}H]thymidine incorporation assay and MDA-361/DYT2 cells as the target cells. The concentration required for 50% inhibition (IC_{50}) values were obtained from dose–response curves of at least three experiments and are given as average ± 95% confidence intervals.

is produced by the actinomycete *Streptomyces hygroscopicus (25)*. GA binds with high affinity to the 90-kd cytosolic protein chaperone hsp90, causing the accelerated degradation of a number of key signal-transducing protein tyrosine kinases, including HER2 *(26,27)*. Within a few hours of exposure to GA, HER2 levels are reduced and the proper processing of newly synthesized HER2 molecules is interrupted *(28,29)*.

Although the antitumor potential of GA has long been recognized, this drug could not be used clinically because of severe toxicity *in vivo* and difficulties in formulating it in aqueous solutions. An effort to develop potent and selective agents with antitumor activity has led to the synthesis of several GA derivatives. Some are novel compounds, and they are described in this article. In its native form, GA cannot be easily linked to proteins. However, a modification at position 17 of the quinone ring introduced a primary amino group through which a linkage was established. We have conjugated this derivative, 17-aminopropylamino-GA (17-APA-GA), to the anti-HER2 MAb e21 and have assessed the ability of this construct to augment the antiproliferative effect of e21 and the elimination of HER2. Our results highlight the potential value of GA derivatives in specific tumor-targeted therapeutic modalities.

MATERIALS AND METHODS

Anti-HER2 Antibodies

The anti-HER2 MAbs used in the internalization studies were from three sources. The HER series (HER66–158) was raised in the laboratory of E. S. Vitetta (University of Texas, Southwestern Medical Center, Dallas). These MAbs were generated by immunizing BALB/c mice with a recombinant form of the extracellular domain of HER2. The series of e21, e23, e94, and e1 MAbs was raised by C. R. King (Georgetown University, Washington, DC). These antibodies were raised in mice that were challenged with a membrane preparation of HER2-transfected 3T3 cells *(4,23)*. AE1 was generated by N. F. Landolfi at Protein Design Laboratories (Fremont, CA) by immunizing BALB/c mice with HER2-transfected 3T3 cells that had been fixed with paraformaldehyde. AE1 and e21 were chosen for the conjugation studies because their ability to bind to HER2 was high, whereas they differed in internalization efficiency, as described below. Both are immunoglobulin G1 (IgG1) MAbs and were affinity purified from BALB/c ascites fluid. Humanized anti-Tac, which interacts with the α chain of the interleukin 2 receptor (human CD25), was prepared by Hoffmann-La Roche, Inc. (Nutley, NJ), as described previously *(30)*.

Cell Lines and Tissue Culture

Three human HER2-overexpressing cell lines were used: the breast carcinoma SKBr3 and the gastric carcinoma N87 (American Type Culture Collection, Manassas, VA) and MDA-361/DYT2, a tumorigenic subclone of the human breast carcinoma MDA-MB-361 *(31)*. Tumorigenicity of N87 and MDA-361/DYT2 was maintained by periodic *in vivo* passage in athymic mice. The HER2-negative cell line HuT102 was originally derived from a patient with adult T-cell leukemia and has been maintained in our laboratory. N87 and HuT102 were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Biofluids, Rockville, MD), penicillin G (250 U/mL), streptomycin (250 μg/mL), and glutamine (300 μg/mL) (all from BioWhittaker, Inc., Walkersville, MD). SKBr3 cells were maintained in McCoy's 5A medium supplemented with 10% FCS and the same concentrations of antibiotics as N87 and Hut102 cells. MDA-361/DYT2 cells were grown in improved minimum essential medium (IMEM) zinc option (Richter's modification) medium and were supplemented with 10% FCS and antibiotics as above and also with 0.01 m*M* nonessential amino acids and 1 m*M* sodium pyruvate. All culture medium supplements were purchased from Life Technologies, Inc. (GIBCO BRL), Rockville, MD.

Radiolabeling of Anti-HER2 MAbs

We screened 12 anti-HER2 MAbs for their ability to be internalized by HER2-expressing cells. The MAbs were radiolabeled with ^{125}I by using the Iodo-Beads method and following the instructions of the manufacturer (Pierce Chemical Co., Rockford, IL). To measure labeling efficiency, a sample of the labeled MAbs was precipitated with 10% (vol/vol) trichloroacetate. Labeled MAbs were adjusted to a concentration of approximately 3 μg/mL and a specific activity of 8 μCi/μg. (Slight variations in specific activity were noticed between different MAbs.)

Internalization Assay

The ability of the radiolabeled anti-HER2 MAbs to bind and to be internalized by cells was measured in HER2-overexpressing SKBr3 and N87 cells. Cultures in the logarithmic phase of growth were harvested and incubated with the labeled

MAbs at approximately 0.4 μCi per 5×10^5 cells. Cells were incubated in the presence or absence of excess unlabeled MAbs. Binding to cell-surface HER2 was measured after a 30-minute incubation at 4 °C. The cells were then transferred to a 37 °C incubator for an additional 4 hours to allow for internalization of the bound MAbs. At 1, 2, and 4 hours, aliquots were tested for internalization. In these tests, surface-bound (external) ^{125}I-labeled MAbs were removed by a weak acid wash (i.e., 50 m*M* acetate, 80 m*M* NaCl, and 5 m*M* KCl [pH 3.4]), and the cell-associated (internalized) and soluble (free) radioactivity was measured.

Cellular Proliferation Assay

The antiproliferative activity of GA derivatives and immunoconjugates was tested on three HER2-overexpressing cell lines (SKBr3, MDA-361/DYT2, and N87). The findings were similar in all three cell lines. To avoid repetition, we present data primarily obtained from MDA-361/DYT2 cells, because this cell line is the preferred line for future xenograft therapy studies. Cells were seeded in 96-well, flat-bottom tissue culture plates (Corning Costar Corp., Cambridge, MA) and were allowed to recover and adhere overnight. Reagents and antibodies were added to wells from fresh 10× stock solutions and were then serially diluted 1 : 3 in the wells. Incubation time was 24 hours, unless otherwise specified. Six hours before termination of the incubation, [^{3}H]thymidine (Amersham Life Science Inc., Arlington Heights, IL) was added at 1 μCi per well. Immediately before harvesting, the medium was removed and the cells were detached in 0.05% trypsin–EDTA (50 μL/well; Life Technologies, Inc.) for 20 minutes at 37 °C. Cultures were then harvested with a Tomtec Harvester Mach 296 (Tomtec, Orange, CT), and radioactivity was measured in a 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland). When HuT102 cells were used, trypsinization was not necessary.

Conjugation of 17-APA-GA to MAbs

Synthesis of derivatives of GA modified at position 17 or 11 was carried out essentially as described previously *(32)*. For the synthesis of 17-APA-GA (NSC 687297), GA was dissolved in chloroform and mixed dropwise with diaminopropane. The mixture was tested at regular intervals by thin-layer chromatography for the formation of 17-APA-GA. When the reaction was completed, the product was precipitated, filtered, dried, and kept in the dark at 4 °C until used. By reacting 17-APA-GA with the linker *N*-(γ-maleimidobutyl-ryloxy)-sulfosuccinimide ester (S-GMBS), we have obtained the compound 17-[3-(γ-maleimidobutylamido)propylamino]GA (17-GMB-APA-GA). This step was carried out according to the instructions of the manufacturer (Pierce Chemical Co.). Briefly, S-GMBS and 17-APA-GA were stirred in chloroform at room temperature. The reaction mixture was partitioned between chloroform and water, and 17-GMB-APA-GA in the water-insoluble fraction was separated into aliquots and concentrated to dryness. The compound 17-GMB-APA-GA contains a reactive maleimide group and was stored lyophilized in the dark at 4 °C. It was dissolved in dimethyl sulfoxide just before it was added to the conjugation reaction.

Before conjugating 17-GMB-APA-GA to e21, AE1, or humanized anti-Tac, MAbs were brought to 5 mg/mL in thiolation buffer (i.e., 50 m*M* HCO$_3$, 150 m*M* NaCl, and 10 m*M* EDTA [pH 8.6]). Free thiol groups were added to the MAbs by interaction with Traut's reagent (2-iminothiolane) (Fluka Chemical Corp., Ronkonkoma, NY) for 30 minutes at 25 °C. The molar ratios of Traut's reagent to MAb were determined empirically for each MAb to obtain approximately two thiol groups per MAb. Traut's reagent was removed by extensive buffer exchanges into conjugation buffer (i.e., 50 m*M* HEPES, 150 m*M* NaCl, and 10 m*M* EDTA [pH 7.0]), and the molar ratio of thiol groups per protein molecule was established by the Ellman's reaction. The MAbs were then reacted with 17-GMB-APA-GA in the dark at 25 °C for 1 hour and dialyzed extensively (three 1-L changes over a 48-hour period) against phosphate-buffered saline (PBS) at 4 °C to remove unbound 17-GMB-APA-GA. The presence of the GA moiety on the MAb was confirmed spectrophotometrically by obtaining the absorbance at 334 nm (A_{334}) (HP diode ray spectrophotometer model 8450A [Hewlett Packard, Palo Alto, CA]). The A_{334} peak was routinely detectable in the MAbs' solutions after, but not before, conjugation at an A_{334}/A_{280} ratio of approximately 1 : 10. If the MAb was not thiolated, such a peak was not detectable after incubation with 17-GMB-APA-GA.

The immunoconjugates are referred to as e21 : GA, AE1 : GA, and anti-Tac : GA to indicate the linkage of the 17-APA-GA to the respective native MAbs.

Stability of the Immunoconjugate in *In Vitro* Conditions

The stability of the chemical linkage between GA and the MAb was measured by use of ^{3}H-labeled GA. This compound was prepared at the Research Triangle Institute (Research Triangle Park, NC) under Public Health Service contract N01CM97022 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. The ^{3}H was incorporated into four methyl groups on the ansa ring (Taylor GF, Foarde KK, Weber TD, Kepler JA: personal communication) and thus did not interfere with derivatization. This reagent was derivatized to the linkable compound 17-GMB-APA-GA as described above and was used to synthesize ^{3}H-labeled immunoconjugates. The labeled immunoconjugate was then mixed with IMEM/FCS and was incubated at 37 °C. Serum pH remained unchanged at pH 7.4 during the experiment. Aliquots containing 30 μL of immunoconjugate (12 000 cpm) were taken at 0, 2, 5, 10, 20, and 24 hours and were analyzed by high-pressure liquid chromatography (HPLC) using a size-exclusion HPLC Shodex® protein KW-802.5 column (Thomson Instruments, Chantilly, VA). Material was eluted with PBS (pH 7.2) at 1 mL/minute, and 2-mL fractions were collected. Under these conditions, the IgG peak was eluted between 6 and 8 minutes. Each sample (i.e., each time-point aliquot) was collected in 10 vials (total collection in 20 minutes) to account for smaller size molecules (i.e., cleaved GA) as well. The samples were collected into scintillation vials to which 10 mL of Hydrofluor™ scintillation liquid was then added (National Diagnostics, Atlanta, GA), and the radioactivity was measured in an LS 5801 liquid scintillation counter (Beckman Instruments, Inc., Columbia, MD).

Western Blot Analysis

As for the cell proliferation tests, these studies also were carried out in all three HER2-positive cell lines, and the results were similar in all three lines. The data presented are from MDA-361/DYT2 cells. Cells were plated in six-well plates. When cells were 70%–80% confluent, the cultures were washed and incubated with fresh medium containing the drugs or antibodies. Treatment was terminated at the specified times by washing the wells with ice-cold PBS. Cells were immediately lysed *in situ* with lysis buffer (i.e., 10 m*M* Tris–HCl, 140 m*M* NaCl, 2 m*M* EDTA, 5 m*M* iodoacetamide, and 1% Nonidet P-40 [pH 8.8]) containing protease inhibitors at 250 μL per well, as described previously *(28)*. Lysates were boiled with reducing Tris–sodium dodecyl sulfate buffer at pH 6.8, and the proteins were separated by polyacrylamide gel electrophoresis in 6.5% gels and transferred onto Immobilon P membranes (Millipore Corp., Bedford, MA). The antibodies used for the detection of HER2, protein phosphotyrosine, and vinculin were c-neu #3 (Oncogene Science, Inc., Cambridge, MA), 4G10 (Upstate Biotechnology, Lake Placid, NY), and clone hVIN-1 (Sigma Chemical Co., St. Louis, MO), respectively. Antibody signals were measured by use of the enhanced chemiluminescence method (reagents from Pierce Chemical Co.). The membranes were exposed to x-ray film (Kodak, Rochester, NY) and the film was developed according to the manufacturer's specifications.

Densitometry

Exposed films were scanned with the use of a Umax Astra 1200S scanner (Umax Technologies, Inc., Fremont, CA). Band intensities were evaluated with Advanced Image Data Analyzer software (Raytest, Straubenhardt, Germany). Vinculin bands served as indicators of levels of protein loaded from each sample.

Statistical Analysis

The program StatView 4.02 by Abacus Concepts (Berkeley, CA) was used to obtain means, 95% confidence intervals (CIs), and statistical *P* values. In proliferation assays, the samples were set in triplicates, and statistical analysis was carried out after the mean ± 95% CIs for each point was established. IC$_{50}$ (concentration required for 50% inhibition) values were obtained by analyzing best-fit curves of each dose–response graph. The average ± 95% CIs of the IC$_{50}$ value for each reagent was then calculated from data of repeated experiments, as stated in the text. All *P* values are from two-sided statistical tests.

RESULTS AND DISCUSSION

In this study, we show that immunoconjugates containing the cytotoxic antibiotic GA and an anti-HER2 MAb retained both the antiproliferative activity and the specificity toward HER2-expressing neoplastic cells. Moreover, when compared with the

native MAb, the immunoconjugate exhibited enhanced antiproliferative activity.

We chose HER2 as the target for antitumor-directed immunoconjugates because extensive studies show promising results with HER2-directed cancer immunotherapy *(4,6,7,18–24)*. Blocking HER2 activity with MAbs reduced the tumor growth rate or even caused tumor regression [reviewed in *(11,13)*]. Experimental approaches in which the anti-HER2 MAbs were armed with an additional cytotoxic agent dramatically improved the *in vivo* efficacy. Nonetheless, clinical trials that followed these encouraging findings indicated that, although HER2-directed cancer immunotherapy is highly promising, it still needs to be improved. Thus, efforts to further optimize the composition and use of anti-HER2 immunoconjugates are well warranted. Here we report that coupling anti-HER2 MAb to GA can improve the antibody's activity.

Selecting Anti-HER2 MAbs for Conjugation With GA: Ability to Bind and to be Internalized by HER2-Expressing Cells

The ability of the MAb to be internalized was essential because the MAb had to deliver GA intracellularly so that it could exert its cytotoxic effect. We screened 12 anti-HER2 MAbs from three sources for their capacity to internalize into HER2-overexpressing cells lines. Of these MAbs, e21 and e1 were internalized best, with 10% (95% CI = 4%–16%) of total offered radioactivity internalized within 4 hours (observed in four experiments with freshly iodinated MAbs). This rate appears satisfactory because it is within the range of other anti-HER2 MAbs that have been reported to possess antitumor activities (e23 [3%] and Herceptin [20%]). We chose e21 for further studies because it was better characterized than e1; e21 is HER2 specific, does not cross-react with other epidermal growth factor receptor family members, and has been shown to reduce the growth rate of human HER2-positive xenografts in athymic mice *(23)*. This MAb acts as a partial agonist of HER2, since it induces rapid, yet transient, autophosphorylation of HER2 followed by a slight increase in turnover rate and elimination of the receptor *(23)*. Our studies *(2,23)* and studies by other investigators *(9–11)* suggest that antibodies with partial agonist activity have an advantage as inhibitors of cellular proliferation.

Anti-HER2 AE1 was chosen as the negative control in our studies. This antibody binds with high affinity ($K_d = 10^{-11}$ M) to HER2-expressing cells. However, it was poorly internalized (2% [95% CI = 1%–3%] of offered radioactivity in 4 hours), and it did not alter tumor xenograft growth in preclinical studies in a statistically significant manner *(2)*.

As an additional nonspecific control, we used the anti-Tac MAb (anti-human CD25), a humanized MAb directed against the α chain of the interleukin 2 receptor. Because this chain is not expressed on epithelial cells and HER2 is, conversely, not detected in hematopoietic cells, anti-Tac MAb was an appropriate control for nonspecific activity. The cellular target for anti-Tac was HuT102, a cell line that originated from a patient with adult T-cell leukemia. HuT102 cells bind anti-Tac well but internalize it poorly *(30,33)*.

Synthesis of a GA Derivative That Can be Linked to Protein Molecules

GA has long been recognized as a potent antitumor compound. However, it was never developed into a clinically useful

drug because it has considerable nonspecific toxicity and there have been difficulties in formulating an aqueous solution for it. Our studies show that both issues could be solved by conjugating GA to MAbs. We chose to link the two molecules through a thioether bond because such linkage has been shown to be relatively stable *in vivo* in circulation *(1,3,5)*. However, native GA does not have a suitable site for such linkage, and thus a "linkable" GA molecule had to be synthesized. We screened various GA derivatives that had a free amino group, as summarized in Fig. 1. The results indicated that position 17 on the quinone ring could best tolerate modifications without substantial compromise of its biologic activity. In fact, amino or allylamino groups in that position hardly altered the antiproliferative potency of GA [IC_{50} = 8.3 nM (95% CI = 7.8–8.8 nM) for 17-amino-GA, 5.7 ± 3 nM (95% CI = 2.7–8.7 nM) for 17-allylamino-GA, and 8.4 nM (95% CI = 7.8–9.0 nM) for native GA; Fig. 1 and *(32)*]. This site of GA has been shown by others as well to be the most dispensable one *(32)*. When bound to hsp90, GA fits tightly into the N-terminal active site groove with the quinone and the ansa rings being folded on top of each other. In that configuration, position 17 is facing outward, with no direct contact with the protein, and thus it can accommodate modifications *(34)*.

Despite their high antiproliferative activity, 17-amino-GA and 17-allylamino-GA were not suitable for conjugation; the former did not possess a primary amino group because of electronic resonance and the latter did not have an amino group at the end of the side chain. 17-APA-GA, the derivative we have chosen, displayed an acceptable combination of biologic activity (IC_{50} = 180 nM [95% CI = 144–216 nM]) and chemical suitability for conjugation through an amino group.

Conjugating 17-APA-GA to MAbs

The conjugation process is summarized schematically in Fig. 2. After synthesis of 17-APA-GA, this compound was reacted with the bifunctional linker S-GMBS to introduce a maleimide group. Free thiol groups were added on the MAb and were then reacted with maleimide groups of 17-GMB-APA-GA. In the course of these studies, we determined that the optimal GA/MAb ratio was between 1:1 and 3:1 because the biologic functions of the MAbs deteriorated substantially with the addition of more than three thiol groups. Three MAbs were conjugated with 17-GMB-APA-GA—i.e., e21, AE1, and anti-Tac.

Effect of Conjugation of GA to e21 on the Antiproliferative Activity of the MAb

In Fig. 3, the antiproliferative activity of native anti-HER2 MAbs is compared with the activities of their respective GA immunoconjugates. MAb e21 reduced proliferation of HER2-expressing cells only marginally; at a concentration as high as 1650 μg/mL (11 μM), it inhibited cell proliferation by less than 20%. In contrast, when conjugated to 17-APA-GA, e21 displayed substantially higher inhibitory activity. In repeated experiments, we observed that the activity of the immunoconjugate e21:GA was associated with the levels of the conjugated GA moiety more than the level of the delivering MAb. Therefore, IC_{50} values of e21:GA were calculated according to the molar concentrations of the GA moiety. On the basis of four separate experiments, the IC_{50} of e21:GA was 0.58 μM (95% CI = −0.82 to 1.98 μM) (i.e., 40 μg/mL when GA/e21 was approximately 2:1). This level of inhibition was only threefold lower
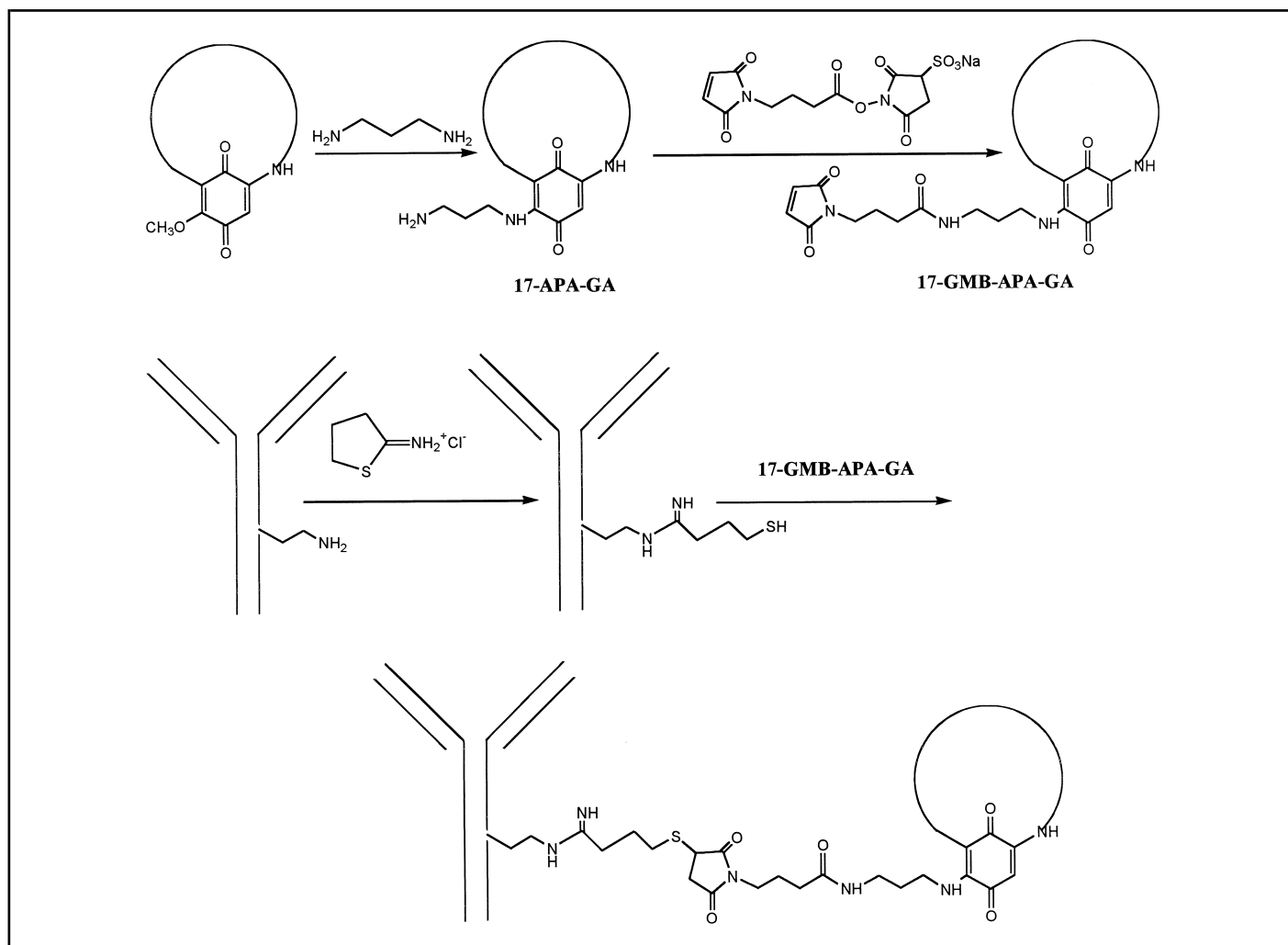
Fig. 2. Conjugation of 17-aminopropylamino-geldanamycin (17-APA-GA) to monoclonal antibodies (MAbs). The conjugation was a four-step process, marked by **arrows.** In the first step, diaminopropane was reacted with GA to form 17-APA-GA. The linker *N*-(γ-maleimidobutylryloxy)sulfosuccinimide ester (S-GMBS) was then reacted with 17-APA-GA to form 17-[3-(γ-maleimidobutyl-amido)-propylamino] geldanamycin (17-GMB-APA-GA), with a reactive ma-leimide group. Just before conjugation, the MAbs were thiolated on lysine residues with the use of Traut's reagent under conditions tailored for each MAb individually to ensure that one to three thiol groups were added per protein molecule. Immedi-ately afterward, 17-GMB-APA-GA was dissolved in dimethyl sulfoxide and con-jugated to the MAb through formation of thioether bonds.

than that of unconjugated 17-APA-GA (IC$_{50}$ = 0.18 μ*M; see* Fig. 1). Thus, 17-APA-GA is the first GA derivative to be de-scribed that could be linked to an MAb and maintain its cyto-toxic activity.

The acquisition of antiproliferative activity by the MAb ap-peared to be related to the MAb's inherent ability to be endo-cytosed. As shown in Fig. 3, AE1, which did not internalize well following binding to HER2-expressing cells, also did not acquire a substantial antiproliferative ability when conjugated to GA. At a concentration of 900 μg/mL (6 μ*M*), this immunoconjugate inhibited proliferation by only 24% (proliferation was 76% ± 6% of control cells).

Stability of the Linkage Between GA and the Antibody

A paramount concern in designing the conjugation chemistry was to ensure that the GA moiety will not be released from the immunoconjugates before it is internalized by target cells. Ex-tensive research has been reported in the last few years regarding the best strategy for such linkage. It appears that a thioether bond would be suited for *in vivo* delivery of immunoconjugated drugs because it imparts acceptable stability in plasma with prolonged distribution and elimination times *(1,3,5)*. We have verified the stability of the linkage by conjugating ^{3}H-labeled GA to the MAb and measuring MAb-associated radioactivity after a 24-hour incubation with FCS at 37 °C. In control samples without FCS, the radioactivity associated with the eluted MAb (HPLC aliquot at 6–8 minutes) was 97% (95% CI = 93%–101%). That level did not change substantially after incubation with FCS. At 0, 2, 5, 10, 20, and 24 hours, the values were 100%, 98%, 99%, 98%, 96%, and 97%, respectively. Furthermore, there was no detectable accumulation of radioactivity associated with smaller molecules (i.e., cleaved GA derivatives) throughout this incuba-tion period. Thus, there was no measurable dissociation of GA from the immunoconjugate after incubation with serum.

If released into the medium, GA would have been expected to inhibit proliferation, regardless of the MAb interaction with the cells. However, our data indicate that this was not the case. The e21 : GA immunoconjugates inhibited only HER2-positive cells but not HER2-negative cells. As shown in Fig. 4, e21 : GA sub-stantially inhibited the proliferation of MDA-361/DYT2 cells
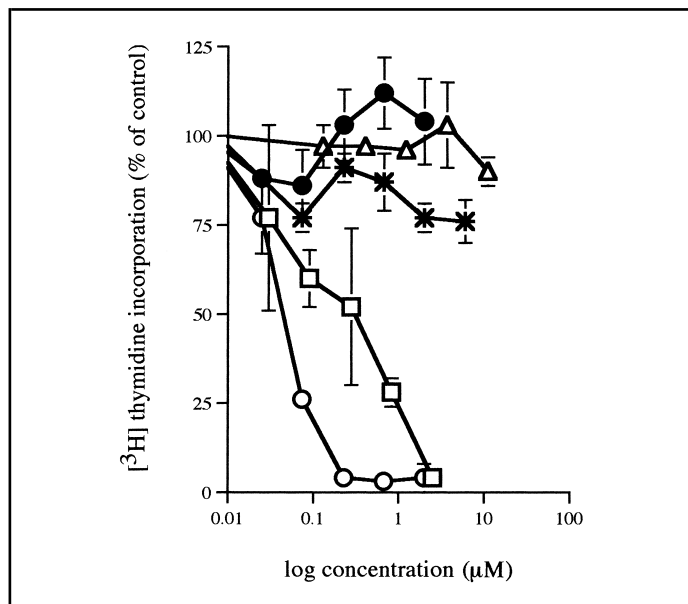
Fig. 3. Antiproliferative activity of native anti-HER2 monoclonal antibodies (MAbs) and their geldanamycin (GA) immunoconjugates. Antiproliferative activity was assessed by the [³H]thymidine incorporation assay after a 24-hour incubation with each MAb and its corresponding GA immunoconjugate. The data are from four experiments using the HER2-expressing cell line MDA-361/DYT2 as the target. e21 (**triangles**), e21 : GA (**squares**), AE1 (**solid circles**), AE1 : GA (**asterisks**), and unconjugated 17-aminopropylamino-GA (17-APA-GA) (**open circles**). For the conjugated MAbs, concentrations are given as the molar levels of GA. The total amount of radioactivity in untreated controls was 140×10^3 cpm. **Error bars** are 95% confidence intervals. When they are less than 2%, they do not show at this scale.

Fig. 4. Antiproliferative activity of geldanamycin (GA) immunoconjugates on HER2-positive versus HER2-negative cell lines. Antiproliferative activity was measured with the [³H]thymidine incorporation assay after a 24-hour incubation with the specified GA immunoconjugates or 17-aminopropylamino-GA (17-APA-GA). Concentrations refer to GA, and the monoclonal antibody (MAb) levels were adjusted according to the particular GA/MAb ratios in each immunoconjugate preparation. Shown are dose–response curves of e21 : GA in the HER2-positive cell line MDA-361/DYT2 (**open squares**) compared with the HER2-negative cell line HuT102 (**open triangles**) and anti-Tac : GA in HuT102 cultures (**solid triangles, broken line**). The dose–response curve for 17-APA-GA in HuT102 cells is shown for comparison (**circles**). Phosphate-buffered saline (**solid squares**) was added in the same manner as in the serial dilutions of anti-Tac : GA and anti-HER2 : GA to HuT102 cultures. **Error bars** are 95% confidence intervals.

but not that of HuT102 cells, which are HER2 negative. Even at the highest tested concentration (adjusted to 2.0 μM of conjugated GA) of anti-HER2 immunoconjugate, the effect was not different in a statistically significant manner from that of PBS alone ($P>.78$) or that of unconjugated anti-HER2 at the same protein concentration ($P>.69$). Likewise, there was no statistically significant difference between samples treated with 2.0 μM free versus conjugated anti-Tac ($P>.32$), whereas these cells were highly sensitive to free 17-APA-GA (IC$_{50}$ = 0.25 μM [95% CI = 0.13–0.37 μM]). Such selective activity toward HER2-positive cells would not have been expected if free GA were present in the medium. Furthermore, as discussed above, AE1 : GA lacked substantial inhibitory activity against HER2-expressing cells (Fig. 3). Likewise, the anti-Tac MAb, which did not internalize into HuT102 cells, gave rise to GA immunoconjugates with no substantial inhibitory activity against these cells (Fig. 4). Thus, our data indicate that 17-APA-GA was indeed stably bound and did not leak into the extracellular medium at a substantially detectable level.

e21 : GA maintained its activity for at least 6 months when stored in PBS at 4 °C and also after incubation in human serum for 16 hours at 37 °C and in FCS at 4 °C for at least 48 hours (data not shown).

Antiproliferative Activity of e21 : GA and Delivery of GA in the Conjugated Form Into the Cells

It could be argued that the enhanced antiproliferative activity of the immunoconjugates was the outcome of a synergy between e21 and unconjugated GA. More specifically, it could be argued
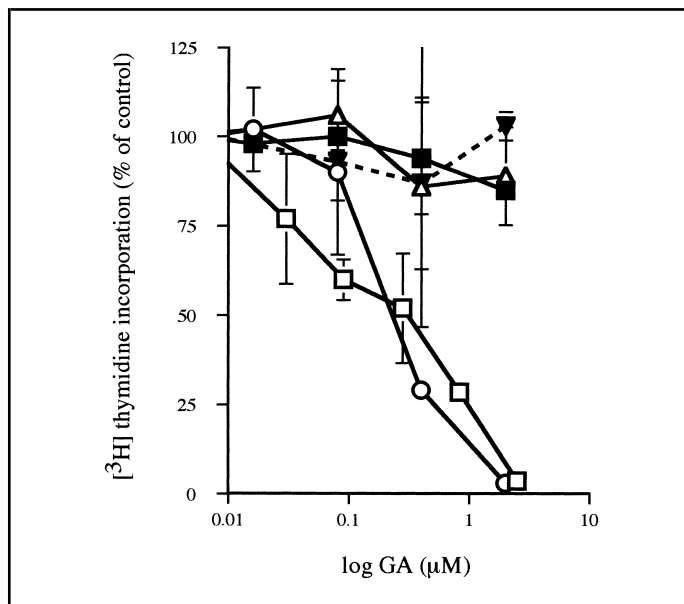
that trace amounts of 17-APA-GA, too low to evoke a detectable cellular response, leaked from the immunoconjugates and had a marked enhancement of antiproliferative effect in the presence of e21. According to such a scenario, GA is ultimately not delivered into the cells via the immunoconjugate but rather it requires the presence of the anti-HER2 MAb to exert an augmented activity. However, the findings shown in Fig. 5 rule out such a possibility. In these experiments, the inhibition of cellular proliferation was examined after treatment with 17-APA-GA alone or 17-APA-GA and free (unconjugated) e21. The concentration of e21 was maintained at 0.5 μM, which was within the range of the IC$_{50}$ value of e21 : GA. As shown in Fig. 5, the dose–response curves of 17-APA-GA alone or with free e21 were essentially identical, with IC$_{50}$ values of 0.28 and 0.27 μM, respectively, and no clear evidence that e21 enhanced the activity of 17-APA-GA.

These results confirmed that meaningful quantities of 17-APA-GA were not cleaved off prematurely and that, rather, 17-APA-GA conjugated to e21 was transported intracellularly.

Reduction of HER2 Levels by e21 : GA Immunoconjugates Versus the Effect of Either of the Unconjugated Components Alone

The effect of e21 : GA on HER2 levels is shown in Fig. 6. Compared with the control lane (lane 1), the HER2 band from e21 : GA (15 μg/mL)-treated cells had markedly lower intensity
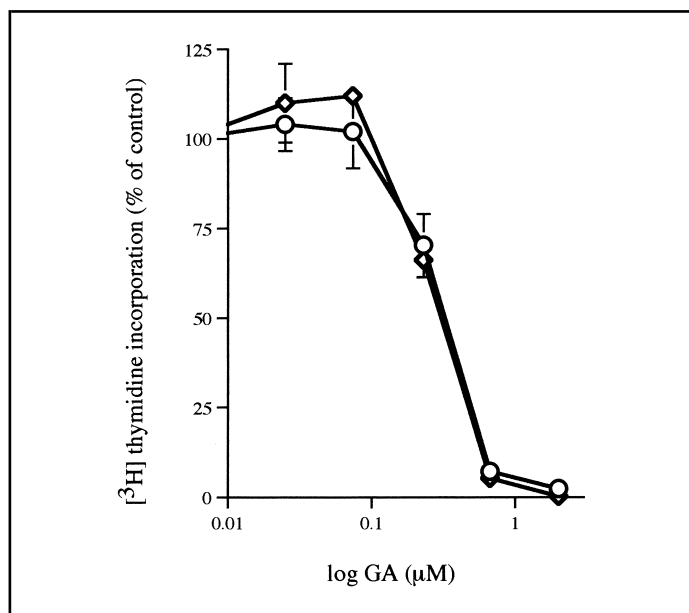
Fig. 5. Dose–response curves of 17-aminopropylamino-geldanamycin (17-APA-GA) with and without e21. MDA-361/DYT2 cells were treated with increasing concentrations of 17-APA-GA, with (**diamonds**) or without (**circles**) 0.5 μ*M* unconjugated e21. [³H]Thymidine incorporation was measured at the end of a 24-hour incubation. Concentrations required for 50% inhibition (IC$_{50}$ values) were 0.28 and 0.27 μ*M,* respectively.
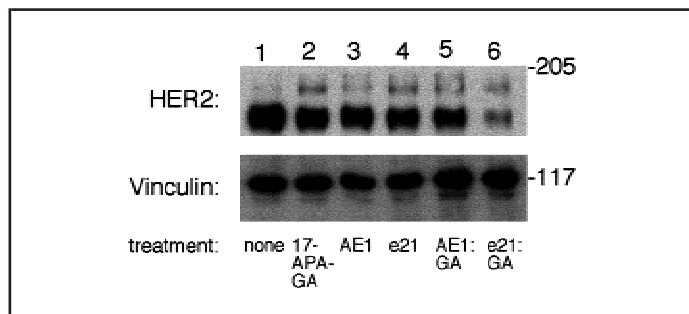

Fig. 6. Effect of geldanamycin (GA) immunoconjugates on HER2 protein levels. MDA-361/DYT2 cells were incubated for 16 hours with the indicated reagents. 17-Aminopropylamino-GA (17-APA-GA) was adjusted to 0.2 μ*M* whether free or in conjugated form. The unconjugated monoclonal antibody (MAb) concentrations were likewise matched with concentrations of the conjugated MAbs. At the end of the treatment, the cultures were harvested and lysed, and lysate samples were separated by polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane. The blots were probed first with anti-HER2 MAb, washed extensively, and then probed with anti-vinculin MAb. Bands were developed by the enhanced chemiluminescence method, and their intensities were measured by densitometry. An untreated control sample is in **lane 1.** When adjusted for slight loading differences (obtained from vinculin bands), the HER2 level in e21:GA-treated cells (**lane 6**) was 14% of control, and, in the other samples, it was 80%.

(lane 6), which corresponded to 86% reduction in HER2 level as evaluated by densitometry. The effect of e21:GA was, in fact, more pronounced than that induced by either 17-APA-GA or e21 alone (Fig. 6; compare lane 6 with lanes 2 and 4, respectively); these agents reduced HER2 levels by only 20% of control. The fact that e21:GA was more potent than native e21 at reducing HER2 levels was consistent with, and may partially explain, the higher antiproliferative activity of this immunoconjugate, as shown in Fig. 3.

Association Between Reduction of HER2 Levels by the Immunoconjugates and the Ability of the Anti-HER2 MAb to be Internalized

MAb AE1 could not be effectively internalized after binding to HER2, as discussed above, and its immunoconjugates AE1:GA were ineffective at reducing the levels of HER2 (Fig. 6). This is consistent with much lower antiproliferative activity of AE1:GA compared with that of e21:GA (Fig. 3). Thus, our data suggest that the antiproliferative effect of the immunoconjugates was the consequence, at least in part, of binding to HER2, reducing its levels and thus interfering with transduction of the receptor's proliferative signals. Furthermore, it appears that such an effect depends on the delivery of GA intracellularly in the conjugated form via endocytosis.

Rate of HER2 Elimination By e21:GA Immunoconjugates Versus That Induced by Unconjugated 17-APA-GA

The rate at which e21:GA eliminated HER2 was compared with that of 17-APA-GA and e21 by western blot analysis (Fig. 7). HER2 levels were measured in lysates of cells that were
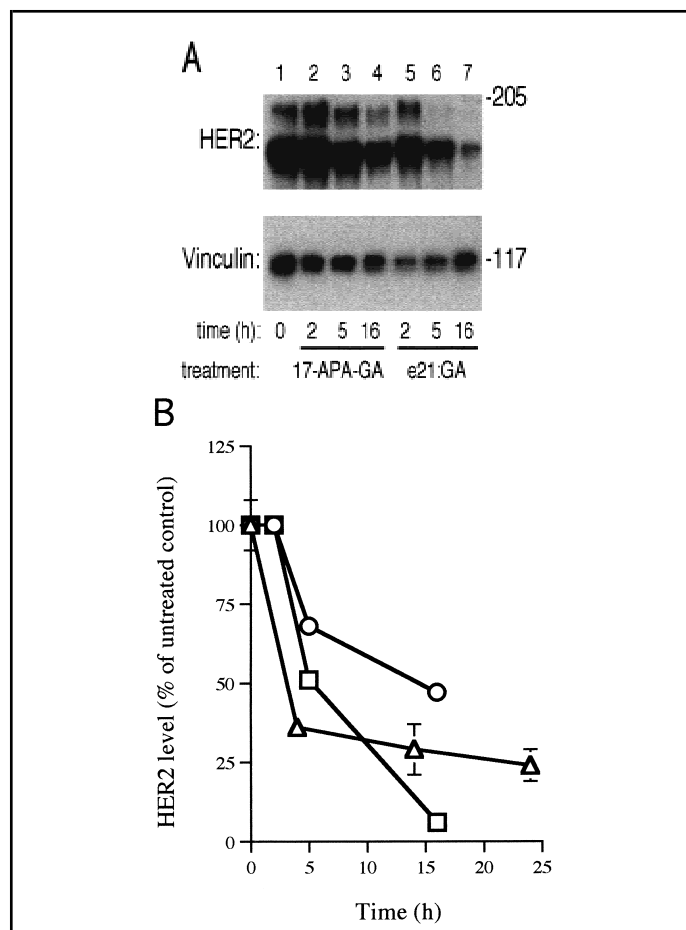

Fig. 7. Time course of HER2 elimination by e21: geldanamycin (GA), e21 and GA. **A**) MDA-361/DYT2 cells were treated with 0.2 μ*M* 17-aminopropylamino-GA (17-APA-GA) or e21:GA. The concentration of free e21 was adjusted to match that of conjugated e21. The cultures were harvested at the indicated time points, lysed, and processed for western blot analysis. The blot was probed first for HER2 levels, washed, and reprobed for vinculin. Signals were developed by enhanced chemiluminescence assay on x-ray film. **B**) HER2 levels were read by densitometry and adjusted according to the corresponding vinculin levels for each sample. e21 (**triangles**); e21:GA (**squares**); 17-APA-GA (**circles**). Error bars are 95% confidence intervals.

treated for 2, 5, and 16 hours with 0.2 μM e21:GA or 17-APA-GA. In e21:GA-treated cells (Fig. 7, A; lanes 5–7), HER2 levels dropped drastically after 16 hours, reaching 6% of control (Fig. 7, A; compare lanes 1 and 7). In contrast, 17-APA-GA induced only a 47% reduction (lane 4). Moreover, the half-life of the decline in HER2 levels in e21:GA-treated cells was only 5 hours compared with approximately 16 hours in 17-APA-GA-treated cells (lanes 6 and 4, respectively). These data demonstrate that e21:GA was more effective than 17-APA-GA at eliminating HER2; i.e., it caused more complete removal of the receptor at a faster rate. Native e21 and its immunoconjugate reduced HER2 but had different kinetics. Although e21:GA induced a continuous decline, eliminating HER2 almost entirely within 16 hours, e21 induced first a rapid decrease (half-life = 2.5 hours) followed by a new steady-state with HER2 levels at approximately 25% of control (Fig. 7, B).

Potential Intracellular Target Site(s)

The potent cytotoxicity of GA results from its interaction with the 90-kd chaperone protein hsp90 in the cytosol and in some cases also with glucose response protein, p94 (GRP94), in the endoplasmic reticulum. Binding of GA to hsp90 accelerates degradation of fully functional HER2 molecules and prevents proper maturation of nascent HER2 molecules *(26,27,29,35)*. However, when conjugated to an anti-HER2 MAb, GA may not be available in the cytosol but rather may be transported with the MAb and the endocytosed receptors into the lysosomes. Modification of the antibody's configuration has been reported to influence trafficking of the internalized receptor. Specifically, the fate of internalized HER2 was shown to depend greatly on the MAb characteristics and on the phosphorylation state of HER2 *(36)*. When in the lysosomes, 17-APA-GA can be cleaved because of the acidic pH and subsequently gain access into the endoplasmic reticulum, where it may interact with—and inhibit—GRP94. An alternative hypothesis is that the GA moiety is brought into close proximity with chaperone proteins when the immunoconjugate binds to HER2. In that case, the immunoconjugates may affect mainly the mature receptor molecules and are likely to induce degradation mainly by the proteosomal pathway.

Relevance of New Anti-HER2 Immunoconjugates

The effectiveness of e21:GA in eliminating HER2 is emphasized herein because of its potential relevance in the clinical setting. Reducing HER2 levels (and, consequently, HER2 activity) has been repeatedly demonstrated to heighten the sensitivity of cancer cells to chemotherapeutic drugs, specifically, to paclitaxel, cisplatin, or doxorubicin *(24,37)*. Today, the treatment of metastatic breast cancer with the humanized anti-HER2 MAb Herceptin is combined with paclitaxel chemotherapy *(7),* and the benefit from treatment with Herceptin is limited to carcinomas that overexpress HER2 with a very high score. This observation suggests that the cytocidal effect of Herceptin requires that many MAb molecules interact with the target cell. Of interest, in preliminary *in vitro* studies, we observed that Herceptin:GA immunoconjugates were effective against breast carcinoma cells that overexpressed only moderate levels of HER2 and were hardly inhibited by Herceptin alone (data not shown).

The combined treatment with Herceptin and doxorubicin has been associated with cardiac toxicity, raising the question of potential toxicity with GA as well. Cardiac toxicity in Herceptin-treated patients appears to be more severe with doxorubicin than with cisplatin or paclitaxel conceivably because of the particular adverse effects of doxorubicin itself on cardiac functions. Consequently, thorough *in vivo* studies are needed to assess any adverse reactions to anti-HER2:GA conjugates. It should be noted that 17-allylamino-GA currently is in phase I clinical trials for treatment of solid tumors. The results from these studies may be predictive in evaluating adverse reactions and, in particular, the cardiac toxicity of anti-HER2:GA immunoconjugates.

Apart from targeting HER2, it would be of interest to expand the repertoire of GA immunoconjugates and to direct them against a variety of other tumor-associated antigens. Targeted immunotherapy is expanding, using a host of MAbs, and some are already used clinically *(7–10,33)*. Furthermore, the chemical conjugation presented herein is not limited to antibodies. GA could also be conjugated to smaller protein molecules, such as hormones and growth factors, and such conjugates may present new avenues for cancer-directed therapy.

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