ABSTRACT

The efficacy of monoclonal antibodies (mAbs) as single agents in targeted cancer therapy has proven to be limited. Arming mAbs with a potent toxic drug could enhance their activity. Here we report that conjugating geldanamycin (GA) to the anti-HER2 mAb Herceptin improved the activity of Herceptin. The IC_{50}s of the immunoconjugate H-GA were 10–200-fold lower than that of Herceptin in antiproliferative assays, depending on the cell line. The H-GA mode of action involved HER2 degradation, which was partially lactacystin sensitive and thus proteasome dependent. The linkage between GA and Herceptin remained stable in the circulation, as suggested by the pharmacokinetics of Herceptin and conjugated GA, which were almost identical and significantly different from that of free GA. Tumor uptake of Herceptin and H-GA were similar (52 ± 7 and 43 ± 7% of the initial injected dose per gram tissue, respectively; \( P = 0.077 \)), indicating no apparent damage attributable to conjugation. Therapy experiments in xenograft-bearing mice consisted of weekly i.p. doses, 4 mg/kg for 4 months. H-GA showed a greater antitumor effect than Herceptin because it induced tumor regression in 69% of the recipients compared with 7% by Herceptin alone. Median survival time was 145 days as opposed to 78 days, and 31% of the recipients remained tumor free 2 months after therapy was terminated versus 0% in the Herceptin group. Enhancement of Herceptin activity could be of significant clinical value. In addition, the chemical linkage and the considerations in therapeutic regimen described here could be applied to other immunoconjugates for targeted therapy of a broad spectrum of cancers.

INTRODUCTION

Monoclonal antibodies (mAbs) are steadily becoming a new modality of cancer therapy. This is because of their selective tumor-targeting potential, combined with relatively low toxicity (1). However, with the accumulation of clinical experience, it has become apparent that the anticancer efficacy of many mAbs is limited. One approach to solving this problem is to arm mAbs with potent cytotoxic agents, with a design of the linkage that allows efficient release of the conjugated drug intracellularly (2–4). Several such immunoconjugates have been developed and advanced to clinical trials, and one, Mylotarg, has been approved by the Food and Drug Administration for the treatment of acute myeloid leukemia (5).

The experience with such constructs has highlighted a few critical requirements. One is the efficient internalization of the mAb. Another is the use of high-potency drugs, because usually only two to three drug molecules can be directly attached to an IgG molecule without damaging its functions (4). Other factors that impact the activity of an immunoconjugate involve the mechanism of drug release, biodistribution, and pharmacokinetics as well as tumor vasculature features. These factors are only partially understood and thus are still poorly controlled or manipulated (1).

Here we report our studies with an immunoconjugate composed of the mAb Herceptin and the cytotoxic antibiotic geldanamycin (GA). Herceptin (trastuzumab) targets HER2, a protein kinase receptor whose overexpression is strongly associated with poor prognosis and resistance to some chemotherapy (6, 7). HER2 is a member of the epidermal growth factor receptor family, functioning essentially as a co-receptor (8, 9). Herceptin binds to the juxtamembrane region of HER2 and induces cross-linking, followed by internalization and down-regulation of HER2 (10). The clinical benefit of Herceptin as a single agent is modest and is limited to carcinomas with an extremely high overexpression of HER2 (7, 11).

To enhance Herceptin efficacy, we have armed it with GA, a highly cytotoxic ansamycin benzoquinone antibiotic that exerts its toxicity by binding to the protein chaperone heat shock protein 90 (hsp90; Ref. 12). The inhibition of hsp90 by GA destabilizes several key enzymes that regulate essential cellular functions. Upon dissociation from hsp90, these proteins are ubiquitinated and rapidly degraded by the proteasomes. Thus, the cytotoxic effect of GA ultimately depends on intact proteasomal activity (13).

The anticancer potential of GA has long been recognized, but its nonselective, severe toxicity was prohibitive. Recently, a derivative with lower toxicity, 17-allylamino-17-demethoxy geldanamycin (17-AAG) was tested in phase I trials (14). Although this drug is more tolerable than GA itself, it still induces severe dose-limiting hepatotoxicity. A water-soluble, orally available analogue, 17-desmethoxy-17-N,N-dimethylaminoethylamino geldanamycin, has been shown to induce gastrointestinal tract complications in rats and dogs.3 Because of these adverse reactions, the next trials are set to test the clinical value of GA derivatives in low doses combined with other therapy modes.

GA derivatives represent “first-in-class” in the development of hsp90 targeting chemotherapy. They are particularly effective in down-regulating HER2, which may be explained by their ability to induce degradation of both the nascent and the mature forms of the receptor (15, 16). Consistent with that, tumor cells whose proliferation depends on HER2 overexpression exhibit particular sensitivity to GA (12, 15, 16). We therefore chose GA for the conjugation with Herceptin and designed an immunoconjugate that could deliver high concentrations of GA locally and specifically into HER2 overexpressing cells. In previous studies, we showed that such conjugation enhanced the antiproliferative activity of the mAb without compromising its specificity (17). That enhancement correlated with the induction of substantial HER2 down-regulation, suggesting that the GA moiety was released intracellularly in an active form. H-GA extended the survival of tumor-bearing mice beyond the time that was achieved with Herceptin alone (18). In the majority of the recipients, a transitory tumor regression was noted. However, most of the tumors kept growing at a slow yet steady rate, even in the face of continuous treatment. In the studies presented here, we examined further the in vivo behavior of H-GA to better understand factors affecting its antitumor activity. Pharmacokinetics and biodistribution data were

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Requests for reprints: Raya Mandler, 6701 Rockledge Dr., Room 5217, MSC 7840, Bethesda, MD 20892-7840. E-mail: rayam@mail.nih.gov.

3 Personal communication, CTEP meeting, Early Drug Development, National Cancer Institute.
analyzed, and the therapy regimen was revised, resulting in a more sustained antitumor efficacy.

**MATERIALS AND METHODS**

**Reagents.** GA (NSC-122750) was supplied by the Developmental Therapeutics Program of the National Cancer Institute (NCI, Frederick, MD). The 3,14,24,25-tetramethyl-1\(^{3}\)H[G]GA (GAtrit) was prepared at the Research Triangle Institute (Research Triangle Park, NC) under Public Health Service Contract N01CM97022 from the National Cancer Institute, NIH. \(^{3}\)H was incorporated into these four methyl groups on the ansa ring so that subsequent derivatization at the 17-position did not alter the \(^{3}\)H content. This radiolabeled preparation was converted to 17-GMB-APA-GAtrit in our laboratory, as described below, and was stored at 4°C before conjugation with Herceptin. Herceptin was provided by Genentech Inc. (San Francisco, CA).

**Cell Lines and Tissue Culture.** The cell lines N87, NT-5, and A431 were purchased from American Type Culture Collection (Manassas, VA). The HER2 transfectant cell line, NIH 3T3/HER2, was provided by Dr. S. Aroson, NCI (19). The HuT102 cell line was originally derived from a patient with adult T-cell leukemia and was maintained in our laboratory (20). The LS-174-T cell line was established at the NCI from a patient with colon adenocarcinoma (21) and was maintained in our laboratory. The SHAW cell line was originated from a patient with pancreatic carcinoma (22) and was maintained in these laboratory cells. These cell lines were grown in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS (Gemini Bioproducts, Calabasas, CA), 250 units/ml penicillin G, 250 \(\mu\)g/ml streptomycin, and 300 \(\mu\)g/ml glutamine (all from BioWhittaker, Inc., Walkersville, MD). The cell lines MDA-MB-453, BT-20, HCC1922, TOV 112D, OV-90, MDA-MB-231, and MCF7 were also purchased from American Type Culture Collection. The cell line MDA-361/DTY2 was provided by Dr. Dajan Yang (Georgetown University, Washington, DC; Ref. 17). These cell lines were grown in improved MEM zinc option (Richter's modification IMEM from Biofluids, Rockville, MD), supplemented with FCS and penicillin/streptomycin as above and also with 0.01 mM non-essential amino acids and 1.0 mM sodium pyruvate (Life Technologies).

**Mice.** Beige, athymic, severe combined immunodeficient (BNX) female mice and athymic female mice, 5–6 weeks of age, were purchased from NCI, Division of Cancer Treatment (Frederick, MD). They were housed according to the NIH regulations as put forth by the Animal Care Committee and were provided with autoclaved water and mouse chew pellets ad libitum.

**Conjugation of GA to Herceptin.** Synthesis of 17-GMB-APA-GA and the subsequent conjugation to mAb have been described previously (18). In brief, GA was first converted to a terminal amine carbamate-protected analog, 17-BOC-APA-GA. After acidic deprotection with trifluoroacetic acid, reaction with N-hydroxysuccinimidyl 4-hydroxymethyl 6-methyl-diethylenetriamine-penta-acetic acid (BMV-DTPA). Typically, such reaction resulted in between 0.3 and 0.4 14BM-DTPA per mAb. The proteins were then reacted with \(^{111}\)In (800 \(\mu\)Ci) in 0.3 mM acetic acid buffer at pH 5.5 for 15 min at room temperature, as described previously (24). Labeling of both Herceptin and H-GA with \(^{125}\)I was performed by a previously described modified Chloramine-T method (25). The N\(^{\text{3}}\)-125I- (600 \(\mu\)Ci) was mixed with 6 \(\mu\)g of Chloramine-T, dissolved in 0.05 M phosphate buffer pH 7.4, and then reacted with 1.0 \(\mu\)g H-GA for 30 min at room temperature. The radiolabeled products were purified using a PD-10 column without reducing agents (Pharmacia, Uppsala, Sweden). The specific activities of the radiolabeled Herceptin and H-GA were 7–9 \(\mu\)Ci/mg for both \(^{111}\)In and \(^{125}\)I labeling. Radioactivity of all radiolabeled agents was >98% as determined by both PAGE and by size-exclusion high pressure liquid chromatography using a TSK G2000SW HPLC column (0.067 M PBS, pH 7.4, 1 ml/min; Tosohaas, Philadelphia, PA).

**Immunoreactivity of the Radiolabeled Herceptin and H-GA.** To confirm that the conjugation did not compromise its ability to bind, H-GA was compared to native Herceptin in binding assays, as described (25, 26), using the HER2-transfectant cell line NIH 3T3/HER. This cell line expressed a high level of HER2 and was used as a positive control. The antibodies used for detection of HER2 levels were Herceptin or H-GA labeled with either \(^{111}\)In or \(^{125}\)I. Both labeled antibodies were incubated with NIH/3T3 cells in the presence of increasing concentrations of unlabeled Herceptin or H-GA (2\(\text{ng/100 \muL}\) each or 10\(\mu\)g/ml each) or 17-APA-GA at 10\(\mu\)g/ml each for 24 h. The radiolabeled products were purified using a PD-10 column without reducing agents (Pharmacia, Uppsala, Sweden). The specific activities of the radiolabeled Herceptin and H-GA were 7–9 \(\mu\)Ci/mg for both \(^{111}\)In and \(^{125}\)I labeling. Radioactivity of all radiolabeled agents was >98% as determined by both PAGE and by size-exclusion high pressure liquid chromatography using a TSK G2000SW HPLC column (0.067 M PBS, pH 7.4, 1 ml/min; Tosohaas, Philadelphia, PA).

**Internalization of Herceptin and H-GA.** Internalization assays were performed as described previously (26, 27). In brief, N87 cells were plated in 6-well plates, and the medium was replaced 24 h later with 2 ml of ice-cold medium containing 100 ng/ml of [\(^{125}\)I]- or [\(^{111}\)In]-labeled reagents (i.e., Herceptin or H-GA). After 30 min incubation at 4°C the non-cell-bound radioactivity was removed by washing twice with 1 ml of cold PBS. One set of plates was kept at 4°C, while the other was placed at 37°C. After 2, 6, and 24 h, the medium was removed and counted to determine the non-cell-bound...
fraction. The levels of cell surface-bound mAb were calculated from the radioactivity that was removable by a brief wash in dilute acid (1 ml 0.1 n acetic acid containing PBS, pH 2.5 at 37°C for 5 min). In this step, the [111In]-labeled reagents served as the internal controls. A successful acid wash removed the surface-bound reagents and brought the 125I cpm down to negligible values. The cells were then harvested and centrifuged, and radioactivity was measured in both the pellets and the supernatants. Each data point was obtained in triplicate. The internalized fraction was defined as the amount of the surface-bound radioactivity at time 0 (4°C) that remained cell-associated and acid wash resistant at 37°C.

Pharmacokinetic Studies in Mouse Circulation. In these studies, the curves depicting blood clearance of four reagents were generated for confirmation of linkage stability in the circulation. The reagents were [125I]-labeled Herceptin, [125I]-labeled H-GA, H-GAtrit, and GAtrit. The [125I]-labeled reagents were prepared as described above and were injected i.v at 2 μCi/mouse in 200 μl. The [131I]-labeled reagents were injected i.v at 150,000 cpm/mouse in 200 μl. Blood samples were drawn from five mice in each group, starting 5 min after injection and then at 30 min, 1, 2, 4, 6, 10, 20, 24, 30, and 48 h. Radioactivity of the [125I]-labeled serum samples (10 μl) were read in an automated gamma counter (Perkin-Elmer 1480 Wizard 3). The [131I]-labeled samples (100 μl) were added into vials containing scintillation fluid (National Diagnostics, Atlanta, GA) and were read by a beta counter (Beckman LS6000IC, Fullerton, CA). A 2-μl sample of each reagent in its original solution (i.e., PBS or DMSO) was read simultaneously to determine cpm of the total injected dose.

Biodistribution of Radiolabeled H-GA and Herceptin in Tumor-Bearing Mice. Athymic female mice were injected s.c. in the dorsal flank with 3 × 106 N87 cells/100 μl. When the tumors reached 3 mm in diameter, the mice were divided into two treatment groups receiving either Herceptin or H-GA.

Herceptin preparation was made of two solutions, mixed at a ratio of 3 μCi of [111In]-labeled Herceptin and 1 μCi of [125I]-labeled Herceptin and was brought to a final protein concentration of 5 μg/200 μl by adding unlabeled Herceptin. H-GA was prepared in the same way. The xenograft-bearing mice were injected i.v. with the labeled mixtures of H-GA or Herceptin. On days 1, 2, and 4, the mice (five per group) were euthanized, and blood samples as well as various organs were taken for measurement of radioactivity levels. The organs were individually weighed, and the data were expressed as the percent of the injected dose per gram of tissue (%ID/g)

The studies presented here demonstrate some of the considerations underlying the design of targeted therapy with immunocugates. As a model, we chose Herceptin conjugated to GA with the rationale that Herceptin targets a subset of carcinoma cells that tend to be highly sensitive to the toxic activity of GA (16, 28). Our data demonstrate that the anticancer efficacy of Herceptin could, indeed, be significantly improved by conjugation with GA.

The Immunconjugate H-GA Shows Enhanced Antiproliferative Activity Compared with Herceptin, and Its Specificity Toward HER2-Overexpressing Cells Was Retained. The linkage between Herceptin and 17-APA-GA has been described previously and is shown schematically in Fig. 1. The linkage did not damage the binding affinity of the mAb or its ability to internalize into HER2 overexpressing cells. Scatchard plot analysis revealed that the binding affinities of Herceptin and H-GA to HER2 were very similar (Kₘ of 3.8 × 10⁻⁹ and 4.2 × 10⁻⁹ m, respectively), and the internalization rate into HER2-overexpressing cells was almost identical (Fig. 2).

The in vitro activity of H-GA is shown in Fig. 3 in a series of dose-response curves from studies with two HER2-overexpressing cell lines: N87, a human gastric carcinoma; and MDA-MB-453, a human breast carcinoma. IC₅₀s of H-GA were 20 and 7.0 μg/ml in these cell lines, respectively, whereas IC₅₀s of Herceptin were approximately 1000 μg/ml. Furthermore, H-GA almost totally suppressed cell proliferation at high concentrations with IC₅₀s of 130 and 60 μg/ml for N87 and MDA-MB-453, respectively. In contrast, the inhibitory activity of Herceptin plateaued and failed to achieve an IC₅₀ even at 1000 μg/ml. Similar observations were reported by others (29). The antiproliferative activity of H-GA was tested in similar assays in a panel of 15 different cell lines, including OV90, BT-20, MDA-MB-361DYT2, and the HER2 transfectant NIH 3T3/HER2. The activity of H-GA was found to be consistently higher than that of native Herceptin in cells that overexpressed HER2 and could internalize Herceptin, with IC₅₀s 10–200-fold lower than that of Herceptin (data not shown). Similar findings were reported with another Herceptin conjugate (30, 31) as well as with another anti-HER2 immunocoujugate, e21:GA, in our previous studies (17).

Fig. 1. A schematic presentation of the linkage between 17-APA-GA and the mAb. The GA derivative 17-APA-GA was conjugated to lysine residues on the mAb molecule with a GMB linker through a thioether bond. The conjugation conditions restricted the molar ratio of GA to mAb to two or three.
have been confirmed in two sets of studies. In the first, the intracellular activity of H-GA was deduced from Western blot analysis of HER2 levels in the treated cells. In the second study, stability in the circulation was confirmed by pharmacokinetic studies.

The Intracellular Mode of H-GA Action Resembles That of GA and Involves Proteasomal Degradation of HER2. GA and its derivatives inhibit cellular proliferation by binding to the N-terminus of hsp90 and interfering with the protective mechanism of this protein chaperone. As a result, hsp90 protein clients are ubiquitinated and subsequently degraded by the proteasomes (12, 13). Thus, the cytotoxic effects of GA ultimately depend on intact proteasomal activity. H-GA was designed to release the GA moiety intracellularly and, thus, was expected to display a mode of action similar to that of GA. We verified this by analysis of Western blots, such as the one shown

![Graph showing internalization rates of Herceptin and H-GA into HER2-overexpressing cells.](image)

**Fig. 2.** Internalization rates of Herceptin and H-GA into HER2-overexpressing cells. N87 cells were reacted with [111 In]-labeled Herceptin (squares) or H-GA (circles) at 4°C. The [111 In]-labeled compounds were used to measure the bound and internalized fractions. As internal controls, [125 I]-labeled reagents were used to indicate the level of surface-bound reagents and their removal by acid wash. Cpm readings were corrected by subtracting the values obtained with the [125 I]-labeled controls from those obtained with [111 In]. The points shown in the graphs represent the adjusted values that were derived from such calculations. The cultures were then incubated at 37°C (open symbols), and internalization was measured at the indicated time points. Control cultures were kept at 4°C (closed symbols), but the cells did not survive for 24 h. Internalized labeled mAb gave rise to cell-associated radioactivity that was resistant to acid wash. All points were run in triplicate; bars, SD. Binding and internalization of H-GA were essentially identical to those of Herceptin.

![Graph showing antiproliferative activity of Herceptin and H-GA in cells with various HER2 expression levels.](image)

**Fig. 3.** Antiproliferative activity of Herceptin and H-GA in cells with various HER2 expression levels. HER2 levels are approximately 10^6, 0.2 x 10^6, and <10^4 receptors/cell in N87 cells (squares), MDA-MB-453 cells (circles), and A431 cells (triangles), respectively. The cultures were incubated with Herceptin (open symbols) or H-GA (filled symbols) at the indicated concentrations. Cultures treated with PBS only served as controls, and their growth was referred to as 100% proliferation. To determine cell proliferation, the cells were fixed, and cellular proteins were stained with Crystal Violet. The dye was eluted, and absorbance at 540 nm was measured by an ELISA reader. All points were run in duplicate, and the data are expressed as percentages of untreated controls. Bars, SD.

![Graph showing H-GA and 17-APA-GA reduce cellular HER2 levels in a proteasome-dependent manner.](image)

**Fig. 4.** H-GA and 17-APA-GA reduce cellular HER2 levels in a proteasome-dependent manner. The Western blot shows HER2 levels in cells that were treated with 3 μM 17-APA-GA (Lanes 2 and 4), 1 mg/ml H-GA (Lanes 5 and 6), or Herceptin (Lane 7) with or without the proteasomal inhibitor lactacystin (Lanes 4 and 6). Lane 1 is of untreated cells. Lane 3 is of cells treated with 50 μM lactacystin alone. After 3 h incubation, the cells were lysed, and lysate samples were separated by PAGE and blotted onto a membrane. The membrane was first reacted with anti-HER2 mAb and after thorough washing with anti-vinculin mAb. Signals were developed on X-ray film by the chemiluminescence method. When adjusted for slight loading variations (see vinculin lanes), the level of HER2 was 100% in the control (Lane 1) and did not decrease in Herceptin-treated cells (Lane 7, 109%). However, HER2 was markedly depleted in 17-APA-GA- and H-GA- treated cells (Lanes 2 and 3, 6% and 27%, respectively), and in both cases lactacystin protected from such depletion (79 and 47%, respectively).

Fig. 2. Internalization rates of Herceptin and H-GA into HER2-overexpressing cells. N87 cells were reacted with [111 In]-labeled Herceptin (squares) or H-GA (circles) at 4°C. The [111 In]-labeled compounds were used to measure the bound and internalized fractions. As internal controls, [125 I]-labeled reagents were used to indicate the level of surface-bound reagents and their removal by acid wash. Cpm readings were corrected by subtracting the values obtained with the [125 I]-labeled controls from those obtained with [111 In]. The points shown in the graphs represent the adjusted values that were derived from such calculations. The cultures were then incubated at 37°C (open symbols), and internalization was measured at the indicated time points. Control cultures were kept at 4°C (closed symbols), but the cells did not survive for 24 h. Internalized labeled mAb gave rise to cell-associated radioactivity that was resistant to acid wash. All points were run in triplicate; bars, SD. Binding and internalization of H-GA were essentially identical to those of Herceptin.

Fig. 3. Antiproliferative activity of Herceptin and H-GA in cells with various HER2 expression levels. HER2 levels are approximately 10^6, 0.2 x 10^6, and <10^4 receptors/cell in N87 cells (squares), MDA-MB-453 cells (circles), and A431 cells (triangles), respectively. The cultures were incubated with Herceptin (open symbols) or H-GA (filled symbols) at the indicated concentrations. Cultures treated with PBS only served as controls, and their growth was referred to as 100% proliferation. To determine cell proliferation, the cells were fixed, and cellular proteins were stained with Crystal Violet. The dye was eluted, and absorbance at 540 nm was measured by an ELISA reader. All points were run in duplicate, and the data are expressed as percentages of untreated controls. Bars, SD.

Fig. 4. H-GA and 17-APA-GA reduce cellular HER2 levels in a proteasome-dependent manner. The Western blot shows HER2 levels in cells that were treated with 3 μM 17-APA-GA (Lanes 2 and 4), 1 mg/ml H-GA (Lanes 5 and 6), or Herceptin (Lane 7) with or without the proteasomal inhibitor lactacystin (Lanes 4 and 6). Lane 1 is of untreated cells. Lane 3 is of cells treated with 50 μM lactacystin alone. After 3 h incubation, the cells were lysed, and lysate samples were separated by PAGE and blotted onto a membrane. The membrane was first reacted with anti-HER2 mAb and after thorough washing with anti-vinculin mAb. Signals were developed on X-ray film by the chemiluminescence method. When adjusted for slight loading variations (see vinculin lanes), the level of HER2 was 100% in the control (Lane 1) and did not decrease in Herceptin-treated cells (Lane 7, 109%). However, HER2 was markedly depleted in 17-APA-GA- and H-GA- treated cells (Lanes 2 and 3, 6% and 27%, respectively), and in both cases lactacystin protected from such depletion (79 and 47%, respectively).
in Fig. 4. HER2 levels were compared in cells that were treated with H-GA, Herceptin, or 17-APA-GA. As expected, 17-APA-GA induced almost complete degradation of HER2, bringing it down to 6% of control within 3 h (Fig. 4, Lanes 1 and 2). That degradation was effectively blocked in the presence of the proteasomal inhibitor lactacyustin (Fig. 4, Lanes 2 and 4). H-GA similarly induced a substantial reduction in HER2, which was partially blocked by lactacyustin (Fig. 4, Lanes 5 and 6; 27% versus 47% of untreated control).

The results also suggest that the conjugate acted, at least partially, through proteasome-independent pathway(s) as well. This is not surprising because HER2 is likely to be shuttled to the lysosomes. HER2 is engulfed by the endosomes as a result of interaction with Herceptin. Normally, a portion of the endocytosed receptor is subsequently shuttled into the lysosomes while the rest is recycled (8, 9, 32). It is possible that H-GA shifted the balance toward degradation rather than recycling. In studies with another anti-HER2 mAb, modifications of the IgG molecule (configuration and charge) were reported to greatly influence trafficking into the lysosomes (33). It is conceivable that the linkage chemistry in synthesizing H-GA may have affected aspects of endocytosis, recycling, and shuttling to lysosomes. Unlike the GA-armed mAb, native Herceptin did not down-regulate HER2 levels under the same conditions (1 mg/ml, 3 h) as demonstrated in Fig. 4, Lanes 1 and 7 (100 and 109%, respectively).

Pharmacokinetic Pattern of H-GA Indicates Linkage Stability and No Accelerated Clearance of the Conjugate. Linkage stability in the circulation is necessary to assure effective drug delivery to the tumor. It is of particular importance because of toxicity considerations because immunoconjugates circulate in the vasculature for a relatively long time. A continuous release of the drug could eventually cause systemic toxicity, negating the very purpose for using immunoconjugates. To evaluate this issue, we compared the PK patterns of Herceptin in its native and conjugated forms. H-GA was radiolabeled with either $^{125}$I (to trace the IgG component) or with 17-GMB-APA-GAtrit (to establish the PK of the conjugated GA).

Fig. 5 depicts clearance from the circulation of $^{125}$I-labeled Herceptin, $^{125}$I-labeled H-GA, and H-GAtrit. The tracings of these three reagents showed substantial overlap with no significant differences ($P = 0.13$ for differences between Herceptin and H-GAtrit PK curves). In the terminal clearance phase, the $t_{1/2}$S for Herceptin and H-GAtrit were 75.1 and 77.6 h, respectively, indicating that the linkage chemistry did not cause accelerated clearance. As expected, unconjugated GAtrit showed a strikingly different PK. There was a rapid distribution phase (only 7% remained detectable in the circulation 5 min after injection), and $\sim 0.6\%$ of the initial dose was still lingering in the circulation after 18 h, with a $t_{1/2}$ of 30 h ($P < 0.0001$ between conjugated and free GAtrit). Taken together, the findings shown in Figs. 4 and 5 suggest that when conjugated, GA circulated in the blood, stably attached to the mAb. Once internalized, though, it was released and gained access to the cytoplasm, where it interacted with hsp90. That, in turn, initiated a cascade of events leading to HER2 degradation by the proteasomes.

Toxicity Studies of H-GA. The confirmation of the linkage stability was encouraging because it implied that the risk of systemic toxicity attributable to premature cleavage of GA was minimal. Toxicity was also evaluated in dose-escalation studies. The findings revealed only minimal toxicity, limited to the highest doses of free and conjugated 17-APA-GA. Unconjugated 17-APA-GA (0.15 $\mu$g/kg, every 3 days for 5 weeks) was more toxic than the conjugate. It induced mild hepatotoxicity (elevated glutamate oxaloacetic transaminase and lactate dehydrogenase), which was confirmed by histopathology. It also induced minimal chronic nephropathy and a decrease in monocytes and large WBCs. The dose given in these studies was ~40-folds lower than a therapeutic dose of a similar GA derivative, 17-AAG. For 17-AAG, in mice, a therapeutic effect was noted with 60 mg/kg two times every day for 5 days, and the treatment was associated with considerable toxicities, including hepatotoxicity and renal damage. In contrast, the conjugated form (H-GA, 12 mg/kg) did not induce histopathological findings and caused only mild glutamate oxaloacetic transaminase elevation as well as a decrease in monocyte count in 50% of the recipients. The dose used in the therapy studies (H-GA or Herceptin at 4 mg/kg) did not induce detectable hepatotoxicity or cardiotoxicity. This is of importance because in human subjects, these toxicities are the most serious adverse reactions associated with 17-AAG and Herceptin, respectively (1, 6, 9, 14, 31).

Next we examined the antitumor activity of the conjugate. In line with the marked augmentation of its activity in vitro, H-GA also possessed a superior antitumor activity in vivo when compared with native Herceptin. However, in earlier studies, this improvement appeared suboptimal compared with the substantial enhancement in in vitro cytotoxicity. Using a higher therapy dose (10 mg/kg as opposed to 4 mg/kg) or shorter treatment intervals (48 h as opposed to 72 h) did not improve the antitumor efficacy. This raised the questions of whether the linkage chemistry has a potential deleterious effect on tumor-specific uptake and whether the therapy regimen offered optimal conditions for tumor saturation. Obviously, the efficacy of the conjugate greatly depends on its ability to accumulate specifically in the tumor. Thus, biodistribution studies were performed to verify that such accumulation took place.

Conjugation to GA Did Not Alter Herceptin Biodistribution and Tumor-specific Uptake. In the biodistribution studies, we used a mixture of $^{125}$I- and $^{111}$In-labeled reagents (H-GA and Herceptin) that were injected i.v. into tumor-bearing mice. Metal ions such as $^{111}$In are attached to the antibody via the chelate 1B4M-DTPA and are retained within the cells upon internalization. In contrast, the $^{125}$I-labeled reagents allow measurements of the association between Herceptin and surface HER2 (26, 27). Fig. 6 shows the distribution of $^{111}$In-labeled Herceptin and H-GA in various organs as measured 4
The biodistribution studies were also instrumental in revealing subtle deterioration of H-GA with prolonged storage conditions. When stored at low concentrations (<0.5 mg/ml), the conjugate underwent changes with time that did not affect in vitro activity. However, when injected in mice, its hepatic uptake was significantly accelerated, reducing tumor accumulation and consequently the anti-tumor efficacy (data not shown). Storage at 10 mg/ml with 0.1% BSA accelerated, reducing tumor accumulation and consequently the anti-tumor efficacy (data not shown). Storage at 10 mg/ml with 0.1% BSA accelerated, reducing tumor accumulation and consequently the anti-tumor efficacy (data not shown). Storage at 10 mg/ml with 0.1% BSA accelerated, reducing tumor accumulation and consequently the anti-tumor efficacy (data not shown).

Tumor Uptake after Pretreatment with Herceptin. The biodistribution results also suggested that mAb retention in the tumor site had different kinetics as compared with that in the blood. Fig. 6, inset, shows that within 96 h, Herceptin was steadily cleared from the blood (53% in 3 days), whereas in tumors its levels did not change significantly. This indicated that the tumors were saturated even as blood levels were decreasing and that an additional mAb dose would have only a limited effect on the tumor. Therefore, in the next set of experiments we examined the fate of the mAb when given in repeated doses. Xenograft-bearing mice were divided into two groups and were injected i.v. with radiolabeled Herceptin, as in the previous studies. However, the mice in one group were pretreated 2 days earlier with 4 mg/kg of unlabeled Herceptin. This dose was based on clinical use of Herceptin, on our previous studies, and those reported by others (17, 29). Under these conditions, tumor uptake was markedly lower in the pretreated mice (Fig. 7). In naïve mice, tumor uptake reached 52% ± 17% ID/g, whereas in the pretreated mice it only reached 23% ± 4% ID/g (P < 0.001). This discrepancy in uptake between the first and second dose was unique to the tumor site and was not detected in other organs. In fact, accumulation of the second dose in tumors may have largely been nonspecific because it did not differ much from the levels detected in the blood (28% ± 2% ID/g), lungs (16% ± 2% ID/g), or kidney (13 ± 0.5% ID/g). These results suggest that the 4 mg/kg dose either heavily saturated or effectively down-regulated HER2 in the tumor site. This was further supported by measurements of [125I]-labeled Herceptin. The levels of that reagent specifically indicate membrane binding, and they were, indeed, reduced in the pretreated mice (data not shown). The implication of those results was that frequent dosing would probably offer only a limited advantage and perhaps ultimately contribute a negative effect.

Little is known concerning the dynamics between mAb retention in the tumor and accessibility for subsequent binding. It is clear though, that to optimize the therapeutic outcome these parameters have to be addressed. Our results suggest that the intervals between doses should be greater than 4 days to assure optimal delivery of GA into the tumors. We based the next therapy experiments on those observations.

In Xenograft-bearing Mice, H-GA Prolonged Survival More Than Herceptin by Retarding Tumor Growth and Inducing Complete Tumor Regression. Mice were inoculated s.c. with N87 cells, and treatment was initiated when the tumors reached volume of ~75 mm³. The treatment regimen consisted of 4 mg/kg i.p. injections of Herceptin and H-GA-labeled with chelated 111 In were injected i.v. into athymic mice bearing N87 s.c xenografts. On days 1, 2, and 4, the mice were euthanized, and the indicated organs were taken for measurements of radioactivity and total weight. Blood samples (0.5 ml) were measured as well. The figure shows the data collected on day 4 from Herceptin (stripes) and H-GA (dots) treated mice. Data are expressed as %ID/g (n = 5; bars, SD). Tumor uptake of Herceptin and H-GA was 52 ± 7 and 43 ± 7% ID/g, respectively, showing no significant differences (P = 0.077). The inset shows the changes in %ID/g on day 1 (thin stripes), day 2 (heavy dots), and day 4 (speckled lines) in blood and tumors.

Cardiac uptake was very low in all recipient mice (Fig. 6, 4 ± 0.6% ID/g and 4 ± 0.5% ID/g for H-GA and Herceptin, respectively). This observation is emphasized in light of the reported clinical cardiac toxicity of Herceptin (11).

These experiments also provided data for calculation of t₁/₂ in the circulation. Herceptin blood levels on days 1, 2, and 4 were 34, 29, and 18% ID/g (Fig. 6, inset). For H-GA, the values were similar (24, 19, and 14% ID/g). Accordingly, the calculated terminal phase t₁/₂ values were 3.2 and 3.46 days for Herceptin and H-GA, respectively, which were similar to those obtained in the PK studies.

The biodistribution studies were also instrumental in revealing subtle deterioration of H-GA with prolonged storage conditions. When stored at concentrations (<0.5 mg/ml), the conjugate underwent changes with time that did not affect in vitro activity. However, when injected in mice, its hepatic uptake was significantly accelerated, reducing tumor accumulation and consequently the anti-tumor efficacy (data not shown). Storage at 10 mg/ml with 0.1% BSA corrected that problem.
Herceptin or H-GA once a week for 4 months. PBS was used as control treatment. Both Herceptin and H-GA slowed tumor growth as compared with the PBS. However, H-GA was more effective than Herceptin at suppressing tumor growth, as shown in Table 1. After treatment for 1 month, the median tumor size in the H-GA-treated mice was only 9 mm$^3$ as compared with 144 mm$^3$ in the Herceptin group, with a significant difference in average size tumors in each group ($P = 0.012$). That difference was partially attributable to tumor regression, which was noted in 69% (9 of 13) of the H-GA recipients as opposed to only 7% (1 of 13) in the Herceptin-treated group.

In earlier studies, the effects of free 17-APA-GA and that of a mixture of unconjugated Herceptin and 17-APA-GA were examined as well. The schedule and amounts were kept identical to those given in the conjugated form. The free drug was considerably inferior to the conjugated counterpart. Unconjugated 17-APA-GA did not inhibit tumor growth by itself nor did it potentiate the efficacy of unconjugated Herceptin. Judging from the PK studies (Fig. 5) and preclinical data with 17-AAG, free 17-APA-GA probably never reached cytotoxic levels in the tumor and was cleared from the circulation very rapidly when given at equivalent dosing and schedule as used with the conjugate.

In the therapy studies shown here, tumors of 38% (5 of 13) of the H-GA-treated mice became undetectable after 3 months of treatment. The mice remained in apparent remission as long as therapy was continued. In contrast, Herceptin did not induce complete remission in any recipient. Fig. 8 depicts the survival outcome at the end of 4 months of this therapy. Herceptin prolonged survival of the mice compared with PBS treatment with a median survival time of 78 days as opposed to 55 days in the PBS group ($P < 0.0071$). However, at termination of therapy only 15% (2 of 13) in the Herceptin group were still alive, and both had visible tumors. In contrast, the majority of the H-GA recipients (9 of 13, 69%) were alive at that point, and 5 of these mice (38%) were tumor free. The survival pattern of H-GA-treated mice was significantly different from that of Herceptin- and PBS-treated groups ($P < 0.008$ and $P < 0.0001$, respectively).

The mice were observed for 2 months after treatment was terminated. At that point, survival in the Herceptin-treated group was 1 of 13, whereas in the H-GA-treated group, 6 of 13 were still alive, including 4 mice (31%) that remained clinically tumor free.

Another Herceptin conjugate has been described recently in which the maytansinoid derivative, DM1, was linked to Herceptin through a disulfide bond. This conjugate induced complete regression of xenografts and effectively controlled growth of murine F05 tumors that were derived from transgenic MMTV-HER2 donors (30, 31). However, the effects of Herceptin-DM1 were transitory, and tumor growth eventually resumed in a pattern that resembled that observed with H-GA. DM1 itself is over two orders of magnitude more cytotoxic than 17-APA-GA, and the therapy dose of Herceptin-DM1 was higher than that used in the H-GA studies (18 mg/kg versus 4 mg/kg). These parameters apparently were still not sufficient to induce substantially higher cure rates. In these studies, the dynamics of tumor saturation and biodistribution were not described. It will be of interest to examine how these parameters differ, if at all, from the data presented here.

The therapeutic potential of GA continues to be of burgeoning interest. Obviously, harnessing its cytotoxic activity in a tumor-selective fashion is of significant therapeutic potential. Here we show that through conjugation to tumor-targeting mAb, 17-APA-GA became more tumor specific, acquired longer circulation survival, and became less harmful to normal tissues. Efforts to conjugate this drug with another mAb have been reported as well (34). The linkage device was designed to yield a high molar ratio between the drug and the mAb, using the N-(2-hydroxypropyl)methacrylamide polymer. However, in that configuration, the high molar ratio was offset by a substantial reduction in GA activity, resulting in a conjugate with relatively low activity (IC$_{50} > 60$ μM). The approach of attaching GA derivatives to polymers for enhanced efficacy (but with no mAb-guided delivery) is being pursued further (35).

Enhancement of Herceptin activity, as shown in our studies, could be of significant clinical value because Herceptin targets a subset of the most common cancers. In addition, targeting GA through specific delivery constructs could be expanded to include other mAbs as well. A host of new mAbs are becoming available, and over 400 are already in clinical trials. Furthermore, the chemical linkage presented here is not limited to IgG molecules but could be applied to smaller ligand proteins, such as cytokines and growth factors or to artificial vehicles. Such conjugates may introduce new avenues for targeted therapy for a broad range of cancers.

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### REFERENCES


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Table 1: The effect of Herceptin and H-GA on tumor size and tumor regression

|Mice inoculated s.c with N87 tumor cells, and when the tumors reached 75–100 mm$^3$, the mice were divided into three groups, receiving the treatments as indicated. Therapy was given as a weekly i.p. dose of 4 mg/kg, and tumors were measured every 3 days. The table shows average and median tumor sizes and the percentage of recipients in each group whose tumors regressed. Data are from day 33 of treatment, when the first recipient reached the survival end point (in the PBS group). |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average size (mm$^3$)</th>
<th>Median size (mm$^3$)</th>
<th>Tumor regression (% of recipients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS ($n = 10$)</td>
<td>398 ± 220</td>
<td>360</td>
<td>0</td>
</tr>
<tr>
<td>Herceptin ($n = 13$)</td>
<td>129 ± 120$a$</td>
<td>144</td>
<td>7</td>
</tr>
<tr>
<td>H-GA ($n = 13$)</td>
<td>35 ± 35$b$</td>
<td>9</td>
<td>69</td>
</tr>
</tbody>
</table>

$a$ Significantly different from PBS-treated group ($P < 0.001$).

$b$ Significantly different from H-GA-treated group ($P = 0.012$).

$c$ Significantly different from PBS-treated group ($P < 0.001$).
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