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# Synthesis and Evaluation of Antiproliferative Activity of a Geldanamycin–Herceptin™ Immunoconjugate

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**Abstract**—Geldanamycin was modified with 1,4-diaminobutane to introduce a primary amine that was subsequently employed to provide a maleimide for protein linkage. Monoclonal antibody Herceptin™ was then derivatized to generate thiol groups that reacted with the maleimide derivative to produce the immunoconjugate. The product showed antiproliferative activity greater than native Herceptin™. 

The advent of monoclonal antibody (mAb) technology raised hopes that cancer therapy would become a reality with the ability to develop high specificity agents that could target tumor-associated markers.[1] However, the clinical trials with unconjugated, or unmodified mAbs have generally yielded only partial responses because most mAbs possess minimal antitumor activity.[2] To compensate, toxins have been fused with, or drugs have been conjugated to mAbs. Trials with such immunoconjugates resulted in promising outcomes but have also been fraught with adverse complications.[3] Currently, one of the most promising targets for immunotherapy is the membrane receptor HER2, a member of the epidermal growth factor receptor family. While only marginally detected in adult tissues,[4,5] it is over-expressed in approximately 30% of human gastric, lung, and breast carcinomas.[4,6–8] HER2 over-expression in breast carcinomas is inversely related to estrogen receptor expression and is correlated with poor prognosis.[9]

Blocking HER2 activity or interfering with its expression has been shown to inhibit proliferation and reduce tumor growth.[4,6,10] Thus, several anti-HER2 mAbs have entered clinical trials in the past 6 years,[11–15] and the humanized anti-HER2 antibody Herceptin™ is currently used clinically in metastatic breast cancer therapy.[11,13] However, the objective response rate is relatively low when Herceptin is the sole therapeutic agent. We, therefore, set out to augment its activity by forming an immunoconjugate with the highly cytotoxic drug geldanamycin (GA).

GA (Fig. 1) is a benzoquinoid ansamycin produced by the actinomycete *streptomyces hygroscopicus* and is related to herbimycin A.[16] It binds with high affinity to the cytosolic protein chaperone hsp90 and disrupts its ability to protect cellular enzymes from proteasomal degradation.[17] Since HER2 stability depends on interaction with hsp90, GA causes accelerated elimination of this receptor.[18–20] and disruption of the proper processing of newly synthesized HER2 molecules into the endoplasmic reticulum.[21]

While the antitumor potential of GA has long been recognized, clinical use was not pursued due to its severe toxicity and difficulties with aqueous formulation. Thus, numerous derivatives have been synthesized and studied in an effort to develop more selective agents with antitumor activity.[22] These studies indicated that modest modifications at the 17-position on the quinone ring maintain cytotoxicity at nanomolar range. Although GA itself cannot be directly linked to proteins, after introduction of a primary amine group at the 17-position, a hetero-bifunctional cross-linking reagent could be employed to conjugate GA to mAbs. One such derivative, 17-(3-aminopropylamino)-GA (17-APA-GA) with a maleimide linker added (17-GMB-APA-GA) (Fig. 1), has been conjugated to the anti-HER2 mAb

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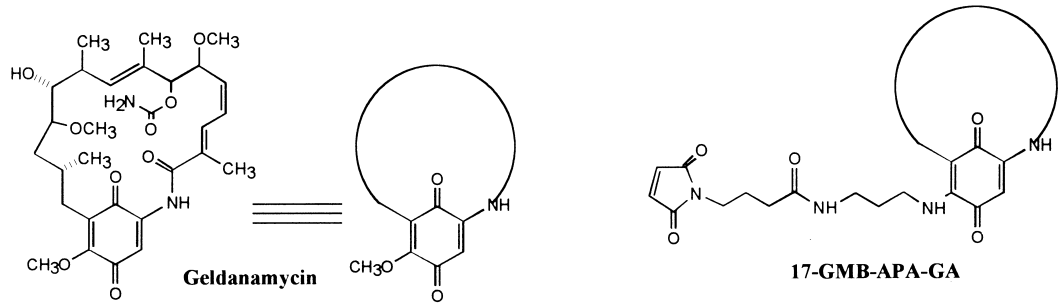


**Figure 1.**

e21 and its ability to augment both the anti-proliferative effect of e21 and its elimination of HER2 has been demonstrated.[23] However, preparation of this derivative has been problematic. Preparation of 17-APA-GA, as described in the literature,[22] produced a complex mixture of products.[24] Exposure to silica catalysed formation of a seven-membered imine ring from the distal amine and the quinone carbonyl precluding use of this medium for isolation of the desired primary amine. Employment of other chromatographic media has not yielded satisfactory results.[24] While milligram amounts of 17-APA-GA have been successfully obtained, the immunoconjugate formed with this derivative contained higher molecular weight by-products necessitating purification of the immunoconjugate by preparative HPLC to ensure purity (vida infra).

Published data suggested that the 17-(4-aminobutylamino)-GA (17-ABA-GA) (Fig. 2) might be a better derivative for the production of adequate amounts of a biologically active GA immunoconjugate. The biological activity of 17-ABA-GA was expected to be mildly lower than that of 17-APA-GA, if one extrapolates from the $IC_{50}$ data against SKBr-3 cells for 17-ABA-GA, 17-*N,N*-dimethylABA-GA, and similar chain length derivatives.[22] However, the synthesis and isolation of 17-ABA-GA was not expected to be hindered by imine cyclization to form an eight-membered ring.

Treatment of GA with 1,4-diaminobutane (Fig. 2) produced a dark-purple solid that was isolated by precipitation with hexane.[25] Thin-layer chromatography indicated several products and after careful column chromatography on silica gel, 17-ABA-GA was successfully isolated in 84% yield. The product distribution was highly dependent upon both reaction time and the addition of excess diamine. Optimal conditions appeared to be 1–1.5 equiv of diamine and 3–5 days of reaction time while carefully following the progress of the reaction by TLC.[25] Use of larger excesses of diamine tended to significantly increase production of a compound that appeared to have added 2 equiv of diamine as determined by mass spectrometry, presumably due to formation of an imine on the quinone.

The 17-ABA-GA was then treated with the heterobifunctional cross-linking reagent GMB to introduce a maleimide.[26] Isolation of this product by column chromatography was uncomplicated and produced a reagent suitable for conjugation to a monoclonal antibody, 17-GMB-ABA-GA (73%).

In order to perform the conjugation, 17-GMB-ABA-GA was dissolved in DMSO (2 mg/mL) prior to addition to the conjugation reaction. Thiolation of the mAb with Traut's reagent was carried out as previously described[26] and excess reagent was removed by buffer exchange into conjugation buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA pH 7.0). The mAb was reacted with 17-GMB-ABA-GA and the reaction mixture was kept in the dark at 25 °C for 1 h and then dialyzed extensively (1 L×3 during 48 h) against PBS without $Ca^{+2}/Mg^{+2}$ at 4 °C. The presence of the GA moiety on the mAb was confirmed by spectrophotometric reading at $A_{334}$.

The purity of the immunoconjugate was found to be significantly improved compared with that of 17-GMB-APA-GA. The analytical size-exclusion HPLC[27] chromatograms clearly indicated that the 17-GMB-ABA-GA immunoconjugate was essentially congruent with Herceptin™, while the previously prepared analogous 17-APA-GA immunoconjugate contained significant levels of by-products (Fig. 3). These results were confirmed by SDS-PAGE as well (data not shown).

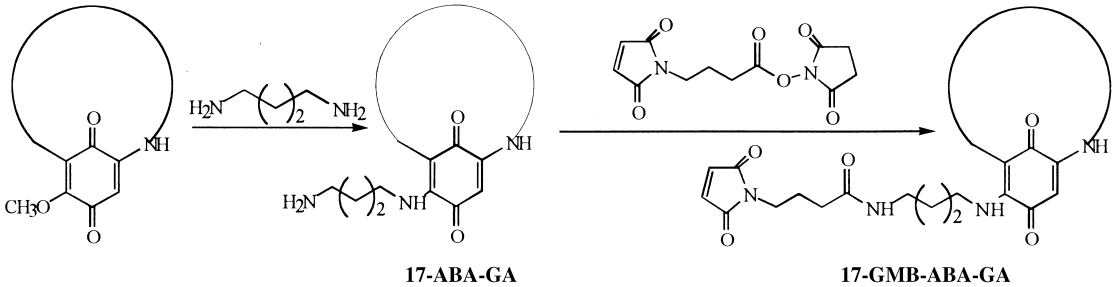


**Figure 2.**

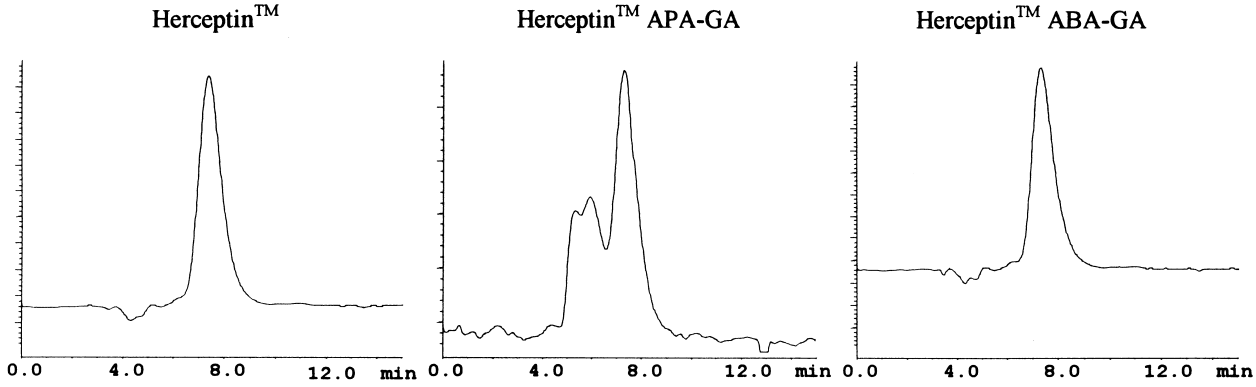


**Figure 3.**

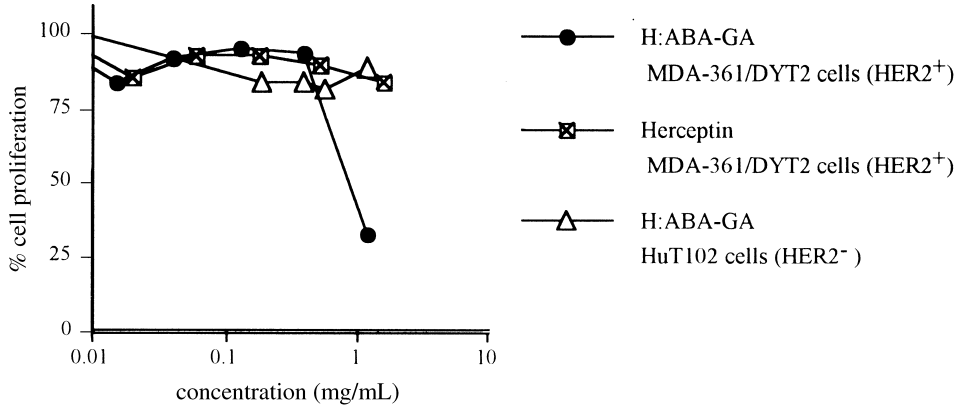


**Figure 4.**

The antiproliferative action of the immunoconjugate thus formed was evaluated by testing its activity on MDA-361/DYT2, a HER2-positive human breast carcinoma cell line.[28] The cells were seeded in 96-well, flat-bottom plates and allowed to adhere. Herceptin™, in either free or conjugated form, was added to the wells in serial dilutions (range of 1.5 to 0.01 mg/mL). The cells were allowed to proliferate until the untreated control cultures reached 80% confluency (usually 5 days). Cell proliferation was assessed by the crystal violet staining method after washing the cultures in PBS and fixing the cells with 80% ethanol.[29] As depicted in Figure 4, Herceptin™ had minimal antiproliferative activity, reducing cellular growth by only 30% in the HER2-positive cell line at 1.5 mg/mL.[30] In contrast, at the same concentration, the Herceptin™ 17-ABA-GA conjugate, H:ABA-GA, reduced proliferation by 85%. The $IC_{50}$ of the immunoconjugate was determined from four separate experiments to be 0.5±0.03 mg/mL. In contrast, $IC_{50}$ values for native Herceptin™ could not be determined as in the native form Herceptin™ maximal inhibition reached only 35%. Furthermore, the immunoconjugate did not inhibit the HER2 negative cells (Fig. 4).

In addition, the cell doubling time was measured in MDA-361/DYT2 cultures treated with 0.5 mg/mL Herceptin™ or H:ABA-GA. Cell doubling time for the control (PBS), Herceptin™, or H:ABA-GA treated cultures were 1.4, 1.6 and 6.2 days, respectively. Thus, H:ABA-GA caused approximately a 4-fold reduction in growth as compared to unmodified Herceptin™.

In this study we have shown that conjugation of the cytotoxic derivative of GA, 17-ABA-GA, to an anti-HER2 mAb was successful in retaining both the antiproliferative activity and the specificity towards HER2-expressing neoplastic cells. Furthermore, the immunoconjugate exhibited enhanced antiproliferative action when compared with that of the native mAb. Additionally, we have demonstrated that the insertion of a single methylene in the GA derivative significantly reduced the technical obstacles encountered in larger-scale production of a 17-APA-GA immunoconjugates. Ongoing biological experiments are under way to evaluate in vivo efficacy of these conjugates in animal xenograft model.

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25. Typical synthesis of 17-GMB-ABA-GA: GA (0.5 g, 0.89 mmol) was dissolved in $CHCl_3$ (300 mL and 1,4-diaminobutane (66 mg, 0.75 mmol) was added. The reaction was left stirring shielded from light for at least 72 h during which the golden yellow of the solution was replaced with a deep purple. The reaction was monitored by TLC on silica (10% MeOH: $CHCl_3$, $R_f = 0.03$ and 10% MeOH:$CHCl_3$:1% $Et_3N$, $R_f = 0.2$–0.34). Additional diamine was added as needed and the reaction permitted to continue until TLC indicated near complete consumption of the GA. The solvent was removed with minimal heating to near dryness and the crude product precipitated with addition of ~1 L of hexane. The product was collected and dried. The 17-ABA-GA was isolated by flash chromatography on silica (2.5×35 cm column). Trace amounts of GA and several unidentified side products were eluted with increasing percentages of MeOH in $CHCl_3$ up to 10% MeOH:$CHCl_3$. Addition of 1% $Et_3N$ to that solvent then permitted clean elution of the product while subsequent increase of MeOH to 20% permitted elution of the presumed bis-amine adduct as determined by CI–MS. $^1$H NMR (300 MHz, $CDCl_3$) δ 0.899 (d, 3H, $J = 6.9$ Hz), 0.936 (d, 3H, $J = 6.9$ Hz), 1.504 (m, 1H), 1.667 (m, 2H), 1.735 (s, 3H), 1.960 (s, 3H), 2.356 (dd, 1H, $J = 13.2$, 9.9 Hz), 2.57.77 (m, 9H), 3.206 (s, 3H), 3.301 (s, 3H), 3.405 (m, 3H), 3.514 (m, 3H), 4.248 (d, 1H, $J = 9.6$ Hz), 4.769 (br. S, 2H), 5.124 (s, 1H), 5.75.90 (m, 2H), 6.380 (t, 1H, $J = 5.7$ Hz), 6.523 (t, 1H, $J = 14.4$ Hz), 6.895 d, 1H, $J = 11.7$ Hz), 7.204 (s, 1H), 9.127 (s, 1H); CI–MS or FAB–MS ($NH_3$ or nba) *m/e* 617 ($M^+ + 1$). Anal. calcd for $C_{32}H_{48}N_4O_8$: C, 62.30; H, 7.86; N, 9.09; found: C, 62.42; H, 8.04; N, 9.32. The 17-ABA-GA (250 mg, 0.406 mmol) was dissolved in EtOAc (30 mL) and treated with GMB (133 mg, 0.45 mmol). TLC on silica with 10% MeOH:$CHCl_3$ indicated one major component, (10% MeOH:$CHCl_3$, $R_f = 0.32$ and 10% MeOH:$CHCl_3$:1% $Et_3N$, $R_f = 0.73$). The solvent was removed without heating and the product isolated by flash chromatography on silica as above eluting with 2% MeOH/$CHCl_3$ (230 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ 0.900 (d, 3H, $J = 6.6$ Hz), 0.933 (d, 3H, $J = 6.9$ Hz), 1.605 (m, 1H), 1.657 (m, 2H), 1.732 (s, 3H), 1.872 (m, 4H), 1.963 (s, 3H), 2.096 (t, 2H, $J = 6.6$ Hz), 2.305 (t, 2H, $J = 6.9$ Hz), 2.50.78 (m, 4H), 3.206 (s, 3H), 3.264 (q, 2H, $J = 6.9$ Hz), 3.301 (s, 3H), 3.35.60 (m, 8H), 4.248 (d, 1H, $J = 9.6$ Hz), 5.125 (s, 1H), 5.75.90 (m, 2H), 5.944 (t, 1H, $J = 6.0$ Hz), 6.234 (t, 1H, $J = 5.1$ Hz), 6.523 (t, 1H, $J = 11.7$ Hz), 6.644 (s, 2H), 6.8992 (d, 1H, $J = 11.7$ Hz), 7.204 (s, 1H), 9.114 (s, 1H); CI–MS/FAB–MS ($NH_3$/nba) *m/e* 782 ($M^+ + 1$); HR–FAB–MS m/e = 913.3146 error = −1.8 ppm. Anal. calcd for $C_{40}H_{55}N_5O_{11}$: C, 61.43; H, 7.10; N, 8.96; found: C, 61.57; H, 7.23; N, 8.69.
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30. The error bars for the data in Figure 4 do not appear due their small size. The standard error for all of the data points therein did not exceed 5%.