Cancer Research

Targeting HER2-Positive Breast Cancer with Trastuzumab-DM1, an Antibody–Cytotoxic Drug Conjugate

Gail D. Lewis Phillips, Guangmin Li, Debra L. Dugger, et al.

Cancer Res 2008;68:9280-9290.

Targeting HER2-Positive Breast Cancer with Trastuzumab-DM1, an Antibody–Cytotoxic Drug Conjugate

Gail D. Lewis Phillips,[1] Guangmin Li,[1] Debra L. Dugger,[1] Lisa M. Crocker,[1] Kathryn L. Parsons,[1] Elaine Mai,[1] Walter A. Blättler,[2] John M. Lambert,[2] Ravi V.J. Chari,[2] Robert J. Lutz,[2] Wai Lee T. Wong,[1] Frederic S. Jacobson,[1] Hartmut Koeppen,[1] Ralph H. Schwall,[1] Sara R. Kenkare-Mitra,[1] Susan D. Spencer,[1] and Mark X. Sliwkowski[1]

[1]Genentech, Inc., South San Francisco, California and [2]ImmunoGen, Inc., Waltham, Massachusetts

Abstract

HER2 is a validated target in breast cancer therapy. Two drugs are currently approved for HER2-positive breast cancer: trastuzumab (Herceptin), introduced in 1998, and lapatinib (Tykerb), in 2007. Despite these advances, some patients progress through therapy and succumb to their disease. A variation on antibody-targeted therapy is utilization of antibodies to deliver cytotoxic agents specifically to antigen-expressing tumors. We determined *in vitro* and *in vivo* efficacy, pharmacokinetics, and toxicity of trastuzumab-maytansinoid (microtubule-depolymerizing agents) conjugates using disulfide and thioether linkers. Antiproliferative effects of trastuzumab-maytansinoid conjugates were evaluated on cultured normal and tumor cells. *In vivo* activity was determined in mouse breast cancer models, and toxicity was assessed in rats as measured by body weight loss. Surprisingly, trastuzumab linked to DM1 through a nonreducible thioether linkage (SMCC), displayed superior activity compared with unconjugated trastuzumab or trastuzumab linked to other maytansinoids through disulfide linkers. Serum concentrations of trastuzumab-MCC-DM1 remained elevated compared with other conjugates, and toxicity in rats was negligible compared with free DM1 or trastuzumab linked to DM1 through a reducible linker. Potent activity was observed on all HER2-overexpressing tumor cells, whereas nontransformed cells and tumor cell lines with normal HER2 expression were unaffected. In addition, trastuzumab-DM1 was active on HER2-overexpressing, trastuzumab-refractory tumors. In summary, trastuzumab-DM1 shows greater activity compared with nonconjugated trastuzumab while maintaining selectivity for HER2-overexpressing tumor cells. Because trastuzumab linked to DM1 through a nonreducible linker offers improved efficacy and pharmacokinetics and reduced toxicity over the reducible disulfide linkers evaluated, trastuzumab-MCC-DM1 was selected for clinical development. [Cancer Res 2008;68(22):9280–90]

Introduction

The HER2 (ErbB2) receptor tyrosine kinase is a member of the epidermal growth factor receptor family of transmembrane receptors. These receptors, which also include HER3 (ErbB3) and HER4 (ErbB4), are known to play critical roles in both development and cancer (1, 2). Importantly, amplification and overexpression of HER2 occur in 20% to 25% of human breast cancer and are predictive of poor clinical outcome (3, 4). Because of the role of HER2 in breast cancer pathogenesis and the accessibility of the extracellular portion of the receptor, HER2 was recognized as a potential candidate for targeted antibody therapy. The humanized HER2 antibody, trastuzumab (Herceptin), was approved by the Food and Drug Administration in 1998 for use in metastatic breast cancer and has subsequently shown clinical benefit when used, in combination with cytotoxic chemotherapy, as first-line or adjuvant therapy (5, 6). Importantly, trastuzumab improves overall survival in early breast cancer after chemotherapy compared with observation alone (6). Increased survival after only 2 years of follow-up is impressive in breast cancer. Tamoxifen is the only other breast cancer treatment that is reported to offer a survival benefit in this short-time period (6).

Although the mechanisms for response to trastuzumab are not completely understood, clinical benefit is attributed to interference with signal transduction pathways, impairment of extracellular domain (ECD) cleavage, inhibition of DNA repair, decreased angiogenesis; as well as induction of cell cycle arrest, and antibody-mediated cellular cytotoxicity (7, 8). Despite these diverse mechanisms of action, a significant proportion of patients treated with trastuzumab either do not respond initially or relapse after experiencing a period of clinical response (5, 9). Progression through trastuzumab-containing therapy is attributed to aberrant activation of signaling pathways, such as the phosphatidylinositol 3-kinase pathway (10–12), activation of compensatory signaling either through up-regulation of the insulin-like growth factor-I receptor (13, 14) or ErbB/HER ligands (15, 16) or generation of a constitutively active truncated form of HER2, designated p95HER2 (17, 18).

Direct covalent coupling of cytotoxic agents to monoclonal antibodies is an alternative to naked antibody-targeted therapy. To date, antitumor antibodies have been linked to cytotoxic agents, such as the calicheamicins, auristatins, maytansinoids and derivatives of CC1065 (19–22). Currently, only one such conjugate, anti-CD33 conjugated to calicheamicin (gemtuzumab ozogamicin or Mylotarg), has been granted marketing approval for the treatment of relapsed acute myeloid leukemia (23).

Maytansinoids are derivatives of the antimitotic drug maytansine. These agents bind directly to microtubules in a manner similar to the *Vinca* alkaloids (24, 25). Antibody-maytansinoid conjugates directed toward cancer antigens, such as CanAg (cantuzumab mertansine and IMGN242), prostate-specific membrane antigen (MLN2704), CD56 (IMGN901), CD33 (AVE9633), and

Note: Current address for H. Koeppen: M. D. Anderson Cancer Center, Houston, TX 77030.

Requests for reprints: Gail D. Lewis Phillips, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Phone: 650-225-2201; Fax: 650-225-1411; E-mail: gdl@gene.com or Mark X. Sliwkowski, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Phone: 650-225-1247; Fax: 650-225-5770; E-mail: marks@gene.com.

doi:10.1158/0008-5472.CAN-08-1776

CD44v6 (bivatuzumab mertansine) are in early stages of clinical testing (20, 26–28). Because HER2 is highly differentially expressed on breast tumor cells (1–2 million copies per cell) compared with normal epithelial cells, HER2 represents an ideal target for antibody-drug conjugate (ADC) therapy. Numerous preclinical and clinical studies indicate that trastuzumab combines extremely well with microtubule-directed agents (29–32). Given the mechanism of action and potency of maytansine, it was deemed to be a particularly attractive cytotoxic agent to conjugate to trastuzumab. Herein, we describe the efficacy, pharmacokinetic properties, and safety of several trastuzumab-maytansinoid conjugates, with particular emphasis on the chemical nature of the linker.

Materials and Methods

Cell lines and reagents. Tumor cell lines (breast carcinoma BT-474, SK-BR-3, MCF7, MDA-MB-468, MDA-MB-361, HCC1954, lung carcinoma Calu 3, and ovarian carcinoma line SK-OV-3) and MCF 10A breast epithelial cells were obtained from American Type Culture Collection. The breast tumor line KPL-4 was obtained from Dr. J. Kurebayashi (33), and MKN7 gastric carcinoma cells were from Mitsubishi Corp. Cells were maintained in Ham's F-12: high glucose DMEM (50:50) supplemented with 10% heat-inactivated fetal bovine serum and 2 mmol/L L-glutamine (all from Invitrogen Corp.). Normal human cell lines [human mammary epithelial cells (HMEC) and normal human epidermal keratinocytes (NHEK)] and the corresponding culture medium (MEGM and KGM, respectively) were obtained from Cambrex. The BT474-EEI cell line was derived by subculturing BT-474 tumors grown *in vivo* in the absence of estrogen pellet supplementation (exogenous estrogen independent) and is resistant both *in vitro* and *in vivo* to trastuzumab treatment.

Active agents used for cell culture and animal studies were the antibody trastuzumab (Genentech, Inc.), trastuzumab-maytansinoid ADC (ImmunoGen, Inc.), and the control ADC, anti–IL-8-MCC-DM1. The maytansinoid, DM1, was conjugated to trastuzumab through SPP, SMCC, or SPDP linkers (Fig. 1; refs. 24, 34); the thiol-containing maytansinoids, DM3 and DM4, which have methyl groups adjacent to their sulfhydryl group were linked to trastuzumab with the SSNPP linker (ImmunoGen, Inc.). All trastuzumab ADCs had an average molar ratio of 3 to 3.6 maytansinoid molecules per antibody. The drug-antibody molar ratio for trastuzumab-MCC-DM1 and trastuzumab-SPP-DM1 was 3.2 for cell culture and xenograft studies, 3.6 for trastuzumab-SPP-DM1 used in the rat toxicity study, and 3.8 for anti–IL-8-MCC-DM1.

Cell viability and cell death assays. The effects of trastuzumab and trastuzumab-maytansinoid conjugates on tumor cell viability were assessed using Cell Titer-Glo (Promega Corp.). Cells were plated in black-walled 96-well plates (20,000 per well for BT-474; 10,000 cells per well for all other lines) and allowed to adhere overnight at 37°C in a humidified atmosphere of 5% CO_2. Medium was then removed and replaced by fresh culture medium containing different concentrations of trastuzumab, trastuzumab ADC, or free DM1, and the cells incubated for varying periods of time. After each time point, Cell Titer-Glo reagent was added to the wells for 10 min at room temperature and the luminescent signal was measured using a Packard/Perkin-Elmer TopCount. For measurement of apoptosis, BT-474 and SK-BR-3 were exposed to trastuzumab or trastuzumab-MCC-DM1 for 48 h. Caspase activation was assessed by adding Caspase-Glo 3/7 reagent (Promega Corp.) for 30 min at room temperature, and the luminescence was recorded using a Packard TopCount. Induction of cytotoxicity was assessed in cells treated with trastuzumab or trastuzumab-MCC-DM1 for 72 h using ToxiLight Bioassay kit (Cambrex/Lonza). This assay measures release of the intracellular enzyme adenylate kinase as a result of cell lysis.

Normal HMEC and NHEK were plated in clear 96-well plates at densities of 10,000 and 8,000 cells per well, respectively, and allowed to adhere overnight. Cells were treated with trastuzumab or trastuzumab-MCC-DM1 for 72 h. Alamar Blue reagent (Trek Diagnostics Systems) was added to all wells, plates were incubated for 3 h at 37°C, and fluorescence was measured on a SpectraMax 190 (Molecular Devices) using 530-nm excitation and 590-nm emission. Because the normal cell lines were not healthy when grown in black multiplates (which is necessary for use of Cell Titer-Glo), Alamar Blue was used as the proliferation read-out. For all cellular assays, dose-response curves were generated using Kaleidagraph 4.0 (Synergy Software) four-parameter curve fitting.

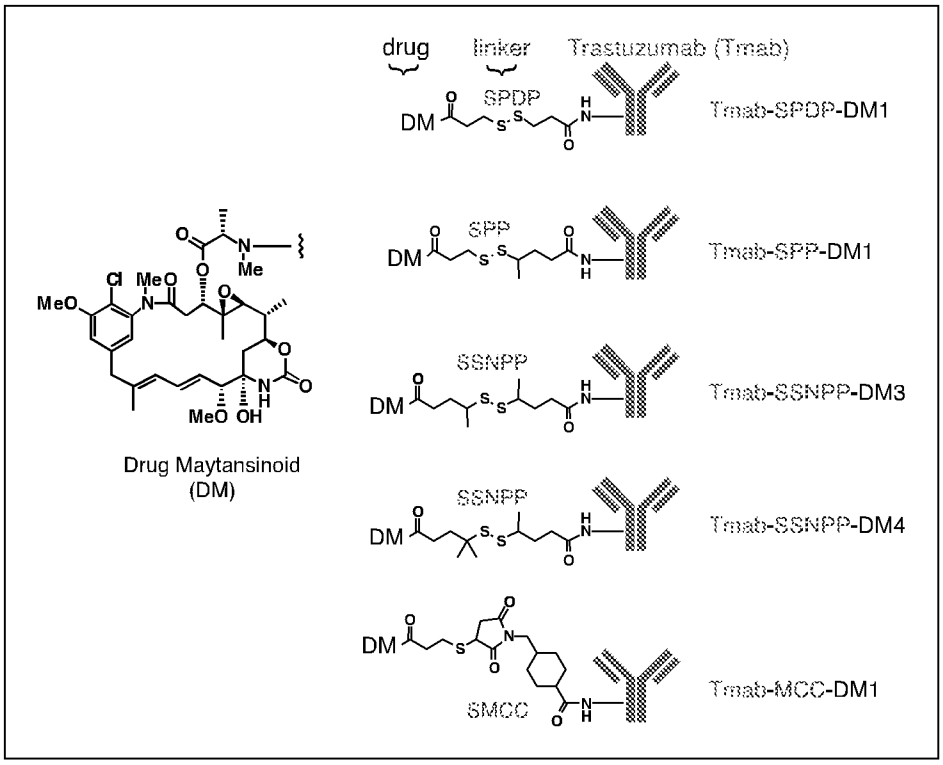

Figure 1. Structure of trastuzumab (Tmab)–maytansinoid conjugates (stability of linker, least to greatest): Tmab-SPDP-DM1, Tmab-SPP-DM1, Tmab-SSNPP-DM3, Tmab-SSNPP-DM4, and Tmab-SMCC-DM1 (nonreducible).

Western immunoblot analysis. SK-BR-3 cells were seeded at a density of 1 million per dish in 100 × 15 mm dishes and allowed to adhere for 2 d. The medium was then removed and replaced with fresh medium containing either trastuzumab, free DM1, or a range of concentrations of trastuzumab-MCC-DM1. After a 48-h incubation, floating cells were collected and combined with detached adherent cells. The total cell population was then centrifuged and resuspended in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1.0 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 10 mmol/L $Na_4P_2O_4$, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 1 μmol/L leupeptin, 0.3 μmol/L aprotinin, 1 μmol/L pepstatin A, 10 μmol/L bestatin, and 1.4 μmol/L E-64]. Lysates were cleared by centrifugation at 4°C for 15 min at 20,800 × *g* in a microcentrifuge, and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a polyclonal antibody against poly(ADP-ribose) polymerase (PARP), which recognizes intact 116-kDa PARP and the 23-kDa cleavage fragment (R&D Systems). Blotting was carried out in TBS containing 0.1% Triton X-100 and 5% nonfat dry milk, followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibodies (Amersham Biosciences). Proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences).

Measurement of total and phosphorylated HER2 and p95HER2 in transgenic tumors was performed as follows. Tumors from the founder 5 (Fo5; ref. 35) and founder 2#1282 (F2#1282) lineages of MMTV-HER2 transgenic mice (Genentech, Inc.) were excised from the animals, placed in lysis buffer containing protease inhibitors, and homogenized on ice. Tumor lysates were centrifuged, and total protein levels in the supernatant were determined using a BCA protein assay kit. HER2 was immunoprecipitated overnight at 4°C using the mouse monoclonal antibody Ab-15 (Lab Vision) complexed to protein A/G sepharose, with 1 mg total protein from at least three independent tumor lysates. Complexes were pelleted by centrifugation, washed twice with lysis buffer, resuspended in SDS sample buffer, and boiled. Samples were separated on a 4% to 12% Tris-glycine gel and transferred to nitrocellulose membranes. Blots were probed with mouse monoclonal antibody Ab-18 (Lab Vision) to detect the phosphorylated forms of HER2 and p95HER2 or with Ab-15 to detect total HER2 and p95HER2.

***In vivo* efficacy and pharmacokinetic studies.** Tumor tissue from Fo5 or F2#1282 HER2 transgenic mice was collected aseptically, rinsed in HBSS, and cut into pieces of ~2 × 2 mm in size. These pieces were surgically transplanted into the mammary fat pad of female nu/nu mice (Charles River Laboratories). For efficacy studies using BT474EEI cells, naive female beige nude XID mice (Harlan Sprague-Dawley) were inoculated in the mammary fat pad with 20 million tumor cells suspended in 50% phenol red–free Matrigel (Becton Dickinson Bioscience) mixed with culture medium. All animals were randomly assigned into treatment groups, such that the mean tumor volume for each group was 100 to 200 mm^3. Trastuzumab or trastuzumab-maytansinoid conjugates were given by either a single i.v. injection or injection once every 3 wk. Vehicle control was either PBS (for pharmacokinetic studies) or ADC formulation buffer [10 mmol/L sodium succinate, 0.1% polysorbate (Tween) 20, 20 mg/mL trehalose dihydrate (pH5.0)]. Similarly, KPL-4 human breast tumor cells were inoculated (3 million cells per mouse, in Matrigel) into the mammary fat pads of SCID beige mice (Charles River Laboratories). Trastuzumab (15 mg/kg) was given i.p. once per week for 4 wk; trastuzumab-MCC-DM1 (15 mg/kg) was given i.v. (single injection on treatment day 0). All treatment groups consisted of 6 to 10 animals per group, and tumor size was monitored twice weekly using caliper measurement. Mice were housed in standard rodent microisolator cages. Environmental controls for the animal rooms were set to maintain a temperature of ~70°F, a relative humidity of 40% to 60%, and an approximate 14-h light/10-h dark cycle.

For pharmacokinetic analysis of trastuzumab-maytansinoid conjugates, female beige nude mice (age 15–20 wk; Harlan Sprague-Dawley) were injected i.v. with 2 mg/kg of different trastuzumab ADCs (four mice per group). To assess circulating levels of total and conjugated antibody, blood was collected via cardiac puncture from three animals at 5 min and 1, 6, 24, 72, and 168 h postinjection. The samples were left at room temperature for 30 min until the blood coagulated. Subsequently, serum was obtained by centrifuging the samples at 10,000 × *g* for 5 min at 4°C, after which serum samples were stored at −70°C. Total trastuzumab concentration in the serum samples was measured as follows: 96-well ELISA plates were coated with HER2 ECD in 0.05 mol/L carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight. After removal of the coat solution, nonspecific binding sites were blocked by incubating with blocking solution [0.5% bovine serum albumin (BSA) in PBS] for 1 to 2 h. The plates were then washed with wash buffer (0.05% Tween in PBS), and standards or samples diluted in ELISA assay buffer [PBS containing 0.5% BSA, 0.05% Tween, 10 ppm proclin 300, 0.2% bovine γ-globulin, 0.25% CHAPS, 0.35 mol/L NaCl, 5 mmol/L EDTA (pH 7.4)] were added. After a 2-h incubation, plates were washed and HRP-conjugated goat anti-human Fc (The Jackson Laboratory) was added for an additional 2 h. Plates were then washed again, followed by the addition of tetramethyl benzidine substrate for color development. The reaction was stopped after 10 to 15 min by the addition of 1 mol/L phosphoric acid. Plates were read on a Molecular Devices microplate reader at a wavelength of 450 to 630 nm. The concentration of trastuzumab in the samples was extrapolated from a four-variable fit of the standard curve. For measurement of trastuzumab-maytansinoid concentration, wells were coated with anti-DM antibody (GNE DM1-3586) and serum samples added as above. After the 2-h sample incubation, the plates were washed, 60 ng/mL biotin-conjugated HER2 ECD was added to each well, and the plates were incubated for 1 h. Plates were then washed, and HRP-conjugated streptavidin (GE Healthcare) was added for an additional 30-min incubation. Color detection and measurement were performed as described above. Previous analyses of different preparations of trastuzumab-DM1 conjugates with drug-antibody ratios ranging from 1.9 to 3.8 showed that the conjugated antibody ELISA is not sensitive to drug load.

Circulating HER2 ECD levels from mice harboring Fo5 or F2#1282 xenograft tumors was measured, as previously described (35). Briefly, serum was diluted 1:50 with ELISA assay buffer (above) followed by 1:2 serial dilutions. HER2 ECD was captured using goat anti-HER2 polyclonal antibody (Genentech, Inc.) and detected with biotin-conjugated rabbit anti-Her2 polyclonal (Genentech, Inc.), followed by addition of streptavidin-HRP.

Rat toxicity studies. Female Sprague-Dawley rats weighing 75 to 80 g were obtained from Charles River Laboratories and allowed to acclimate for 5 d before study. Trastuzumab-SPP-DM1, trastuzumab-MCC-DM1, and free DM1 were diluted in PBS (Invitrogen Corp.) as vehicle and given as a single i.v. bolus tail-vein injection on day 1 at a dose volume of 10 mL/kg. Body weights were measured predose on day 1 and daily thereafter for 5 d.

Results

Linker optimization. Antibody-DM1 conjugates were originally designed with a disulfide-based linker for release of active drug by intracellular reduction (24). Recently, it was discovered that the endocytic pathway is oxidizing and that cleavage of the disulfide linker, SPP, is very inefficient (36). Thus, different trastuzumab ADCs were constructed to investigate the effect of disulfide linker hindrance on the biological activity of these conjugates (Fig. 1). A trastuzumab-DM1 conjugate made with the SPDP linker contains no methyl substitutions adjacent to the disulfide bond and is therefore the least hindered disulfide-containing design. Trastuzumab ADCs composed of SPP-DM1, SSNPP-DM3, or SSNPP-DM4 contain one, two, or three methyl groups, respectively, around the disulfide bridge and show increasing resistance to cleavage via thiol-disulfide exchange reactions.[3] DM3 and DM4 nomenclature

[3] ImmunoGen, Inc., unpublished data.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.