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

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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 (ErB2) 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 calicheamincins, 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 (cuntuzumab mertansine and IMGN242), prostate-specific membrane antigen (MLN2704), CD56 (IMGN901), CD33 (AVE9633), and...
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 maytansinoid, 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 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% CO2. 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. Alammar 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), Alammar 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 MgCl2, 1.0 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 10 mmol/L Na3PO4, 1 mmol/L Na2VO4, 50 mmol/L NaF, 1 μmol/L leupeptin, 0.3 μmol/L aprotinin, 1 μmol/L peptatin 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 enhanced chemiluminescence reagents (Amersham Biosciences). 

Measurement of total and phosphorylated HER2 and p95HER2 in transgenic mice was performed as follows. Tumors from the founder 5 (Fo5; ref. 35) and founder 2 (Fo2) transgenic mice (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 Fo2 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, surgically transplanted into the mammary fat pad of female nu/nu mice (Charles River Laboratories). For efficacy studies using BT474EEI cells, surgical transplants were performed and drug treatment was initiated 4 wk after tumor implantation. The mice were randomized into groups of five per treatment arm. Mice were housed in standard rodent microisolator cages. For pharmacokinetic analysis of trastuzumab-maytansinoid conjugates, blood was collected from tail veins at various time points after dosing, and sera were obtained after centrifugation. Sera were stored at −80 °C prior to analysis. 

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-MCC-DM1 conjugate made with the SPPD 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, SNNPP-DM3, or SNNPP-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

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-MCC-DM1 conjugate made with the SPPD 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, SNNPP-DM3, or SNNPP-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.
reflects addition of methyl groups to the DM1 (drug) moiety (addition of one or two methyls, respectively). An additional ADC containing a thioether linker (SMCC, designated MCA after conjugation) was also constructed. *In vitro* potency assays were conducted for 3 days with the HER2-amplified breast cancer lines BT-474 and SK-BR-3 treated with various trastuzumab ADCs. This short treatment period was selected to minimize the effects of unconjugated trastuzumab on these trastuzumab-sensitive lines. The graphs in Fig. 2A show enhanced potency of trastuzumab-maytansinoid conjugates compared with trastuzumab in both cell lines, with no significant difference in activity among the various ADCs tested (IC50, 0.085–0.148 μg/mL for BT-474 and 0.007–0.018 μg/mL for SK-BR-3). Because the effect of trastuzumab is cytotatic in nature, the enhanced potency of the ADC is, thus, due to exposure of the cells to the cytotoxic maytansinoid. In addition, brief exposure of SK-BR-3 cells for 10, 30, or 60 minutes to trastuzumab-MCC-DM1, followed by a 3-day incubation in culture medium, also resulted in substantial growth inhibition (data not shown). When comparing potency as molar DM1 equivalents, DM1 conjugated to trastuzumab is 5-fold more potent than free L-DM1 on SK-BR-3 and shows equal potency to free L-DM1 on BT-474 cells (Fig. 2B). Nontargeted effects of trastuzumab-maytansinoid conjugates were assessed on breast cancer lines expressing normal levels (MCF7) or lacking expression (MDA-MB-468) of HER2 (37). All ADCs tested showed minimal antiproliferative activity in both cell lines (Fig. 2A), whereas free DM1 showed potency equal to that observed on the HER2-amplified lines (Fig. 2B). Treatment of the four cell lines with an isotype-matched control ADC, anti-IL-8-MCC-DM1, resulted in negligible growth inhibition (data not shown). Thus, like trastuzumab, maytansinoid-conjugated trastuzumab ADCs are specific for HER2-overexpressing cells. Furthermore, the different linkers behave similarly with respect to *in vitro* antiproliferative activity.

Pharmacokinetic analyses in nude mice were performed to determine the effect of different trastuzumab ADC linkers on serum concentration. In contrast to the cell culture results, we observed a clear correlation between ADC exposure and linker hindrance. The ADC with the least hindered disulfide bond (no adjacent methyl groups), trastuzumab-SPDP-DM1, showed the fastest clearance and was undetectable by day 3. The addition of methyl groups (CH3) on either side of the S-S bond (trastuzumab-SSNPP-DM3 with two CH3, one on the antibody side and two on the drug side of the S-S) resulted in increased antitumor activity for trastuzumab-SSNPP-DM3 (log-rank P values of 0.0165 and 0.0414, respectively). Further characterization of the HER2 transgenic models revealed the presence of high circulating levels of shed HER2 ECD in the serum of Fo5 tumor-bearing mice. Moreover, analysis of these tumors revealed increased expression of the transmembrane-containing fragment p95HER2, which is highly tyrosine-phosphorylated, indicating constitutive activation. In comparison, the trastuzumab-sensitive F2-1282 model showed 1,000-fold lower levels of circulating HER2 ECD, no detectable p95HER2, and significant tumor growth reduction after a single injection of different doses of trastuzumab-DM1 (data not shown). Thus, the trastuzumab-DM1 conjugate is efficacious in models, such as Fo5, which express reported markers of resistance to trastuzumab, i.e., high circulating HER2 ECD and activated p95HER2.

Short-term, single-dose toxicity studies were performed in female rats comparing the effects on body weight of free or conjugated (SMCC or SPP) DM1. ADCs were given as DM1 equivalents (μg/m2). As trastuzumab does not recognize rat erbB2, this model measures antigen-independent ADC effects. Of the agents tested, trastuzumab-MCC-DM1 showed the best safety profile. Body weight gain in rats given 1,632 μg/m2 DM1 (25 mg/kg antibody) was comparable with vehicle control animals (15.9% and 16.3%, respectively; Fig. 3C). In contrast, administration of trastuzumab-SPP-DM1 at an equivalent DM1 dose resulted in considerable body weight loss (~10%) by end of study. Rats treated with 3,264 μg/m2 (50 mg/kg) trastuzumab-MCC-DM1 did not exhibit body weight loss; however, the amount of weight gain during the course of study (+6.7%) was less than in the vehicle control and the 1,632 μg/m2 trastuzumab-MCC-DM1 groups. The change in body weight with 3,264 μg/m2 trastuzumab-MCC-DM1 was similar to that observed with free DM1 at a dose of 653 μg/m2 (+6.5%). In a separate study, clinical chemistry analysis of rats treated with trastuzumab-MCC-DM1 showed transient elevation of liver enzymes (aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transpeptidase) and mild, reversible thrombocytopenia at a dose of 20 mg/kg, and no clinical signs of toxicity at 6 mg/kg (data not shown). Evidently, substituting a reducible linker (SPP) with a nonreducible linker (SMCC) results in a less toxic but still efficacious trastuzumab-DM1 conjugate. Consequently, further studies focused on defining the activity of trastuzumab-MCC-DM1.

**HER2-overexpressing, trastuzumab-resistant tumor cells respond to trastuzumab-MCC-DM1, whereas normal cell lines are unaffected.** The effects of trastuzumab-MCC-DM1 on *in vitro* proliferation were further examined in several tumor lines shown to be resistant to trastuzumab. Additional breast cancer lines, HCC1954, KPL-4, and BT-474 EEI, all with 3+ HER2 expression, were first tested. The BT-474 EEI cell line was developed by...
Figure 2. Trastuzumab-maytansinoid conjugates are selective for HER2-amplified breast cancer lines, whereas free DM1 shows equivalent potency on all cell lines tested regardless of HER2 expression. A, trastuzumab-maytansinoid conjugates show enhanced antiproliferative activity on HER2-overexpressing breast tumor cell lines compared with trastuzumab. The nature of the linker, however, does not affect in vitro activity. B, in vitro potency of Tmab-SPP-DM1 and Tmab-MCC-DM1 compared with the free drug L-DM1 after 3 d of treatment (IC_{50} values, nmol/L DM1 equivalents). HER2 expression: SK-BR-3 (HER2 3+), BT-474 (HER2 3+), MCF7 (normal HER2 expression), MDA-MB-468 (HER2-negative).

<table>
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Cells were treated with trastuzumab-DM1 conjugates or L-DM1 as free drug for 72 h; IC_{50} values were determined from four-parameter curve fitting.
subculturing BT-474 tumors grown in vivo in the absence of estrogen pellet supplementation. The cell line established in this manner, while maintaining high levels of HER2 expression, had surprisingly become resistant to trastuzumab (G.P. and L.C.) and provides a useful model for determining efficacy of trastuzumab ADCs. These three breast cancer lines showed the same degree of sensitivity to trastuzumab-MCC-DM1 as the sensitive lines SK-BR-3 and BT-474 (Fig. 4A). Moreover, tumor lines from different tissues of origin were also responsive to treatment with trastuzumab-MCC-DM1. Calu 3 lung carcinoma (3+ HER2), SK-OV-3 ovarian carcinoma (2+ HER2), and MKN7 gastric carcinoma (2+ HER2) cells (37) displayed dose-dependent inhibition of cell growth upon treatment with the ADC while showing no response to nonconjugated trastuzumab (Fig. 4B). In contrast, exposure of normal human cells, HMEC and NHEK, to trastuzumab-MCC-DM1 resulted in minimal growth inhibition, similar to that observed on HER2 nonamplified MCF-7 and MDA-MB-468 breast carcinoma cells (Fig. 4C).

Trastuzumab-MCC-DM1 induces cell death in HER2-overexpressing breast cancer cells. Further studies were performed to address the mechanism(s) of action of trastuzumab-MCC-DM1. Cell cycle analysis of conjugate-treated cells resulted in the expected arrest in the G2-M phase (data not shown), a known mechanism of agents that inhibit microtubule function. We examined the effects on cell death induction, as well. Trastuzumab-MCC-DM1 induced a dose-dependent increase in cell death in SK-BR-3 and BT-474 cells (Fig. 5), with EC50 values (in parentheses) similar to those observed in proliferation assays. Release of the

Figure 3. Trastuzumab conjugated to DM1 through the thioether SMCC linker displays better efficacy and pharmacokinetics and lower toxicity than conjugates with disulfide linkers. A, pharmacokinetic analysis of trastuzumab-maytansinoid conjugates shows increased serum concentrations of conjugates with more hindered disulfide linkers. Female beige nude mice were given a single i.v. injection (2 mg/kg) of different trastuzumab-maytansinoid conjugates (n = 4 mice per group). Serum samples were collected at various time points after injection for measurement of total and conjugated trastuzumab as described. B, increased in vivo linker stability results in improved efficacy in vivo. Mice bearing mammary tumor transplants from the MMTV-HER2 Fo5 line were given a single i.v. injection (10 mg/kg) of Tmab-SPP-DM1, Tmab-SSNPP-DM3, Tmab-SSNPP-DM4, Tmab-MCC-DM1, or vehicle (n = 7 mice per group), and tumor growth was monitored for 25 d. C, Tmab-MCC-DM1 displays the best safety profile, as assessed by changes in body weight, of the conjugates tested. Female Sprague-Dawley rats were given a single i.v. injection of Tmab-MCC-DM1 (1,632 or 3,264 µg/m², 25 or 50 mg/kg, respectively), Tmab-SPP-DM1 (1,632 µg/m²), or free DM1 (653 µg/m²) on day 1, and body weights were measured daily for 5 d. To give equivalent doses of DM1, animals in the Tmab-SPP-DM1 group were given 22 mg/kg due to the higher drug-antibody ratio (3.6) compared with Tmab-MCC-DM1 (3.2).
intracellular enzyme adenylate kinase into the culture medium, an indicator of cellular lysis, was observed after treatment with trastuzumab-MCC-DM1 for 3 days (Fig. 5A). Activation of caspase-3/caspase-7 also occurred in these cell lines after a 2-day treatment with trastuzumab-MCC-DM1, indicating induction of apoptotic cell death (Fig. 5B). Proteolytic cleavage of 116-kDa PARP with release of the 23-kDa fragment, a known hallmark of apoptotic cell death, was also examined. PARP cleavage was observed in SK-BR-3 cells treated for 48 hours with 100 and 300 ng/mL trastuzumab-MCC-DM1, as well as with 20 nmol/L free DM1, indicating induction of

Figure 4. Trastuzumab-MCC-DM1 induces a potent antiproliferative effect in trastuzumab-resistant tumor cells but has no effect on normal human cells. A, breast tumor lines BT-474 EEI, HCC1954, and KPL-4 (all HER2 3+). B, Calu-3 lung carcinoma (HER2 3+), SK-OV-3 ovarian carcinoma, and MKN7 gastric carcinoma (both HER2 2+). C, normal cells HMEC and NHEK were all treated with either trastuzumab or trastuzumab-MCC-DM1 for 3 d, and relative proliferation was determined using CellTiter-Glo. IC₅₀ values for each cell line are in parentheses.
Figure 5. Trastuzumab-MCC-DM1, but not trastuzumab, induces cell lysis and apoptosis in SK-BR-3 and BT-474 breast tumor cells. Cytotoxic response (A), as measured by adenylate kinase release into the culture medium, and apoptosis induction (B), measured by activation of caspase-3/caspase-7, after treatment with trastuzumab or trastuzumab-MCC-DM1 for 3 d (cytotoxicity) or 2 d (caspase-3/caspase-7 activation). EC50 values are shown in parentheses. C, trastuzumab-MCC-DM1 and free DM1 induce PARP cleavage, whereas unconjugated trastuzumab has no effect. SK-BR-3 cells were treated with 10 ng/mL trastuzumab; 30, 100, or 300 ng/mL Tmab-MCC-DM1 (0.02, 0.67, and 2.0 nmol/L, respectively) or 20 nmol/L free DM1 for 48 h. Cells were then lysed for Western blot analysis of cleaved PARP. Appearance of the 23-kDa PARP fragment indicates apoptotic activation of PARP cleavage.
apoptosis (Fig. 5C). Thus, in contrast to the cytostatic action of trastuzumab (7, 30), these data show that treatment of HER2-amplified breast tumor cells with trastuzumab-MCC-DM1 results in apoptotic cell death and cellular lysis.

Trastuzumab-MCC-DM1 inhibits growth and causes tumor regression in animal models of HER2-positive breast cancer. The in vivo efficacy of trastuzumab-MCC-DM1 was compared with trastuzumab in three mouse models of HER2-overexpressing breast cancer. KPL-4 human breast tumor cells were implanted into the mammary fat pads of SCID beige mice and tumors allowed to grow to an average size of 300 mm³. Mice were then treated weekly with 15 mg/kg trastuzumab i.p. or given a single i.v. injection of 15 mg/kg trastuzumab-MCC-DM1. Complete tumor regression for the duration of the study (126 days), as determined by caliper measurement, was observed in mice receiving trastuzumab-MCC-DM1, whereas unconjugated trastuzumab induced an initial decrease in tumor size, followed by regrowth upon cessation of treatment (Fig. 6A). Although the KPL4 cell line did not respond to trastuzumab in vitro, we have observed with other cell lines, such as Calu 3, response to trastuzumab in vivo, despite lack of responsiveness in vitro.

The activity of trastuzumab-MCC-DM1 was further investigated in two trastuzumab-insensitive mouse xenograft models. After transplantation of MMTV-HER2 Fo5 mammary tumor explants,
tumor-bearing nude mice were treated once every 3 weeks with 1, 3, 10, 15, or 30 mg/kg trastuzumab-MCC-DM1 (50, 150, 500, 750, and 1500 μg/m² of conjugated DM1, respectively; Fig. 6B). Doses of 1 and 3 mg/kg showed no antitumor activity, whereas administration of 10 mg/kg resulted in partial inhibition of tumor growth (this treatment group was discontinued after 30 days due to several mice having tumors in excess of 1,000 mm³). Tumors in mice treated with either 15 or 30 mg/kg trastuzumab-MCC-DM1 regressed during the entire course of treatment. Regrowth was observed ~30 days after discontinuation of treatment. Previous studies have shown that retreatment with trastuzumab-MCC-DM1 results in a further decrease in tumor size, indicating that acquisition of resistance did not occur (data not shown).

In contrast, treatment with 15 mg/kg of an irrelevant control ADC, anti-IL-8-MCC-DM1, resulted in no tumor growth inhibition (Fig. 6B, right).

The second trastuzumab-resistant model used the BT-474 EEI tumor-derived line. The data in Fig. 6C show the effects on BT-474 EEI tumor growth of different doses of trastuzumab-MCC-DM1 given once every 3 weeks for three total doses. Dose-dependent inhibition of tumor growth was observed, with 0.3, 1 and 3 mg/kg causing tumor growth delay, whereas 10 and 15 mg/kg doses resulted in tumor regression. Thus, trastuzumab-MCC-DM1 shows potent activity in trastuzumab-sensitive and trastuzumab-insensitive mouse xenograft models, with 15 mg/kg as the maximum efficacious dose.

**Discussion**

The anatomy of an antibody–cytotoxic drug conjugate can be divided into three general components: the antibody, the linker, and the cytotoxic drug. The efficacy of any such conjugate is dictated in part by the differential expression of the target antigen in tumor versus normal tissue. HER2 is a clinically validated target for the treatment of breast cancer. Trastuzumab (5, 35–41) and, more recently, lapatinib (42) are approved for clinical use in women whose breast cancer overexpresses HER2.

To be maximally effective, both trastuzumab and lapatinib require a functional or signaling HER2 receptor, which drives key biological aspects of the tumor. It is postulated that patients whose tumors progress on these therapies may acquire alterations that activate signal transduction pathways either downstream or parallel to HER2. At present, available data indicate that cancer cells do not lose HER2 expression when they become refractory to HER2-directed therapies (14, 34). For that reason, a trastuzumab conjugate, which simply uses HER2 as an address for the delivery of a potent cytotoxic agent, may offer promise as an effective therapeutic modality.

We observed that anti-HER2 ADCs display impressive potency in vitro and in vivo and are active in trastuzumab-refractory models of HER2-amplified breast cancer. However, the tolerability of these conjugates varies considerably and is intrinsically dependent upon the chemical nature of the linker and the cytotoxic agent. In this report, we describe our characterization of several trastuzumab-maytansinoid conjugates with a special emphasis on linker chemistry. The disulfide-linked DM1-containing ADCs were designed to allow for endosomal reduction of the disulfide to result in intracellular release of the cytotoxic agent. Moreover, in antigen-expressing tumor cells, DM1 derived from ADCs designed with a disulfide linker can be released by the tumor cells and initiate a "bystander" killing effect (34). However, in vivo, the liberated DM1 is also rapidly cleared and, thus, may not be available for prolonged antitumor effects (43). Our results with the thioether (MCC) linker contradicted the hypothesis that selective release of DM1 by intracellular reduction of the disulfide bond in a trastuzumab-disulfide-DM1 conjugate was necessary for efficacy. Similar to huC242-MCC-DM1, a thioether-containing ADC that targets the CanAg antigen (34, 44), trastuzumab-MCC-DM1 is internalized upon binding to HER2-overexpressing tumor cells and is postulated to undergo intracellular proteolytic degradation to release active maytansinoid. As expected, the activity of trastuzumab-MCC-DM1 is blocked in the presence of protease inhibitors in cell culture experiments (data not shown). Subsequently, it has been shown that HER2 itinerary allows for lysosomal degradation of HER2-directed ADCs (45). Thus, it is likely that the trastuzumab antibody component of the trastuzumab-MCC-DM1 conjugate undergoes proteolytic degradation in the lysosome and that lysine-MCC-DM1 is a major active metabolite, as has been shown for huC242-MCC-DM1 (44). In fact, preliminary data from studies investigating the metabolism of [3H]trastuzumab-MCC-DM1 in BT-474 EEI cells show lysine-N'-MCC-DM1 as the predominant metabolite (data not shown). The primary active metabolite, lysine-MCC-DM1, does not readily cross the plasma membranes of neighboring cells and, therefore, does not elicit a bystander effect (34, 44).

Non–disulfide-containing ADCs show increased pharmacokinetic exposure in both rats and cynomolgus monkeys (data not shown). This increase in conjugate exposure did not lead to an increase in either target-dependent or target-independent toxicity. As expected, trastuzumab conjugated to DM1 through the thioether MCC linker was better tolerated compared with the disulfide (SPP) linked to DM1, with regard to toxicity signals. Despite the improved pharmacokinetic profile of DM3 and DM4-containing trastuzumab conjugates, the disulfide linkage would likely render these conjugates less tolerable than the thioether-linked trastuzumab conjugate.

In summary, trastuzumab-MCC-DM1 is shown to be efficacious in trastuzumab-sensitive and trastuzumab-insensitive models of HER2-overexpressing cancer. Of equal importance, trastuzumab-MCC-DM1 has a favorable pharmacokinetic and safety profile compared with disulfide-linked trastuzumab conjugates and represents an important new approach for treating HER2-amplified human cancers. Given these promising results, early clinical studies are currently under way to assess the safety and efficacy of trastuzumab-MCC-DM1 in patients whose disease has progressed through HER2-directed therapies.

**Disclosure of Potential Conflicts of Interest**


**Acknowledgments**

Received 5/19/2008; revised 7/23/2008; accepted 9/8/2008.

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This article is dedicated to our dear colleague, Dr. Ralph Schwall, who passed away on August 26, 2005.

We thank Drs. Vishva Dixit, Pal probe, Richard Scheller, and Robert Cohen for their support, interest, and valuable discussions; Allison Bruce for graphics support; and Dr. J. Korenbaum for providing the KPL4 cell line.

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References

2. Yarden Y. The EGFR family and its ligands in human cancer. Signaling mechanisms and therapeutic oppor-
24. Char IV, Martell BA, Gross JL, et al. Immunoconju-
gates containing novel maytansinoid promising anti-
27. Tijink BM, Buter J, de Bree R, et al. A phase I dose escalation study with anti-CD44v6 bivatuzumab mer-
mab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I pharmaco-
29. Baslega J, Norton L, Albullan J, Kim Y-M, Mendlersohn J. Recombinant humanized anti-HER2 antibody (Her-
32. Burstein BH, Keshaviah A, Baron AD, et al. Trastuzu-
mab plus vinorelbine or taxane chemotherapy for HER2-overexpressing metastatic breast cancer: the trastuzu-
34. Kortvyn YV, Auette CA, Ye Y, et al. Antibody-drug conjugates designed to eradicate tumors with homoge-
36. Austin CD, Wen X, Gazzard L, Nelson C, Scheller RH, Scales SJ. Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-
39. Cobleigh MA, Vogel CL, Tripathy D, et al. Multina-
43. Erickson HK, Park PU, Widlison WC, et al. Antibody-
maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-
44. Xie H, Auette C, Hoffe M, Lambert JM, Blätterl WA. Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (Hu2C24-1), and its two components in mice. J Pharmacol Exp Ther 2004;308:1073–82.