

Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer

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Abstract Trastuzumab (Herceptin[®]) is currently used as a treatment for patients whose breast tumors overexpress HER2/ErbB2. Trastuzumab-DM1 (T-DM1, trastuzumab emtansine) is designed to combine the clinical benefits of trastuzumab with a potent microtubule-disrupting drug, DM1 (a maytansine derivative). Currently T-DM1 is being tested in multiple clinical trials. The mechanisms of action for trastuzumab include inhibition of PI3K/AKT signaling pathway, inhibition of HER-2 shedding and Fc γ receptor mediated engagement of immune cells, which may result in antibody-dependent cellular cytotoxicity (ADCC). Here we report that T-DM1 retains the mechanisms of action of unconjugated trastuzumab and is active against lapatinib resistant cell lines and tumors.

Keywords Breast cancer · HER2 · ErbB2 · Trastuzumab · Therapeutic antibodies · Antibody drug conjugate (ADC) · Trastuzumab-DM1 (T-DM1)

Introduction

HER2 positive breast cancer is a well-recognized subgroup of breast cancer [1]. Gene amplification of HER2 defines the class and this alteration manifests itself in the overexpression of HER2 protein on the tumor cell surface in ~20% of all primary breast cancers [2]. Indeed, HER2 positive breast cancer is known to portend a poor clinical outcome and appears to be more prevalent in younger

women [3]. Trastuzumab (Herceptin[®]) is a humanized monoclonal antibody directed against domain IV of the extracellular domain of HER2 [4, 5]. Herceptin[®] was first approved for the treatment of metastatic breast cancer and later for the adjuvant treatment of early breast cancer [6–8]. Retrospective analysis of disease-free survival of distant metastasis reveals that Herceptin[®] in combination with chemotherapy changes the natural history of the disease. Specifically, the outcome of Herceptin[®]-treated patients is similar to that observed in patients whose tumors are HER2 negative and received standard of care chemotherapy [9].

In general terms, the mechanisms of action of trastuzumab are thought to involve inhibition of constitutive HER2 signaling and the activation of immune effector cells [10]. Trastuzumab is known to disrupt ligand-independent HER2/HER3 interactions in HER2-amplified cells [11]. This dissociation leads to the uncoupling of PI3K-AKT signaling and correlates with the antiproliferative effects of trastuzumab. As a humanized IgG1, trastuzumab binds to Fc γ RIII on immune effector cells and is a potent mediator of antibody-dependent, cell-mediated cytotoxicity (ADCC) [12]. The most compelling evidence for the role of Fc-Fc γ RIII interaction is provided by Clynes et al. [13], who assessed trastuzumab response in tumor models deficient in activating Fc γ R functions.

Although the clinical data with regimens containing Herceptin[®] are impressive, some patients' tumors will still progress on these therapies. In the metastatic setting, the HER2 tyrosine kinase inhibitor, lapatinib, is a therapeutic option for these patients. When used in combination with capecitabine, lapatinib demonstrates a therapeutic advantage over capecitabine alone [14]. However, progression is frequently observed in this setting as well. Although several additional therapeutic options for these patients are currently being investigated, we have recently described

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the properties of an antibody drug conjugate [15]. Trastuzumab-DM1 (T-DM1; trastuzumab emtansine) is composed of the humanized antibody trastuzumab and DM1, a maytansinoid derivative, linked with a non-reducible thioether linker, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, designated MCC after conjugation). Maytansinoids are natural products that are potent antimetabolic agents, which like the *vinca alkaloids* prevent microtubule assembly [16, 17]. The therapeutic potential of maytansine as an anticancer agent was extensively investigated in clinical trials [18]. Ultimately the compound was abandoned because it exhibited a poor therapeutic window. Due in part to their impressive potency, maytansinoids were investigated as drugs for conjugation to monoclonal antibodies. Derivatives of maytansinoids were synthesized to allow chemical conjugation to proteins using disulfide linkers [16]. The realization that the endocytic pathway is not reducing and our empirical experimental observations led us to conclude that the more stable thioether linkage was better suited for maytansinoid coupling to trastuzumab [15, 19].

Our previous pre-clinical studies investigated the activity of T-DM1 in model systems that were refractory to trastuzumab [15]. Early promising clinical data were recently generated in patients whose tumors have progressed on HER2-directed therapies [20]. Given these results and the potential of using T-DM1 in earlier lines in breast cancer treatment, it became increasingly important to determine whether T-DM1 retains the known mechanisms of action of trastuzumab. Here we report that T-DM1 retains the mechanisms of action of unconjugated trastuzumab. We also show that T-DM1 is able to bind to Fc γ RIII and activate ADCC. We further demonstrate that T-DM1 effectively inhibits the growth of lapatinib insensitive tumors and tumors harboring PI3K pathway activating mutations.

Materials and methods

Cell lines

Breast cancer cell line BT-474-M1 is an *in vivo* passaged subclone of BT-474 (ATCC, Manassas, VA) [13]. MCF7-neo/HER2 cells were established at Genentech, Inc. KPL-4 breast cancer cells were obtained from Kurebayashi [21]. All other cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Cell lines were maintained in high-glucose DMEM:Ham's F-12 (50:50) supplemented with 10% FBS and 2 mM L-glutamine. Generation of lapatinib resistant SK-BR-3: SK-BR-3 cells

(ATCC) were cultured in the presence of increasing concentrations of lapatinib for 9 months. Cells were determined to be resistant when the doubling time in 1.5 μ M lapatinib was similar to parental cells.

HER2 binding

Nunc break apart immunomodule plates (Nunc, Roskilde, Denmark) were coated with 20 ng/ml HER2-ECD-Ig fusion protein in 50 mM HEPES, pH 8.2, 150 mM NaCl overnight at 4°C. Non-specific binding was blocked with 2 mg/ml BSA, 25 mM Tris, pH 7.5, 150 mM NaCl for 2 h at room temperature. Wells were washed three times with assay buffer (2 mg/ml BSA, 10 mM HEPES, pH 7.2 in RPMI). The competitive binding reaction with a dilution series of non-labeled competitor antibodies and constant 125 I-trastuzumab was carried out for 2 h at room temperature. The 125 I-trastuzumab bound to HER2 was then detected by a gamma counter and the data were analyzed using the non-linear regression method of Munson and Rodbard [22]. All measurements were done in quadruplicate.

ADCC assay

In vitro ADCC assay was performed as previously described [23]. In short, PBMCs were separated from the blood of healthy volunteers using lymphocyte separation medium (MP Biomedicals, Solon, OH). Serum-free RPMI supplemented with 0.1% BSA was used as an assay buffer. Target cells (BT-474-M1, 1×10^4) were pre-incubated with antibodies for 30 min at 37°C before adding the effector PBMCs in a 25:1 E:T ratio. The cells were incubated for an additional 4 h before detecting cell death by measuring the lactate dehydrogenase activity from the media using Cytotoxicity Detection Kit (LDH; Roche, Mannheim, Germany). The percentage of cytotoxicity was calculated as follows: % cytotoxicity (Experimental lysis – spontaneous target lysis)/(maximum target lysis – spontaneous target lysis) \times 100.

pAKT

BT-474-M1 cells were lysed and 50 μ g of total protein was subjected to pAKT analysis using PathScan Phospho-Akt1 (Ser473) ELISA Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions.

HER2 shedding

For HER2 ectodomain shedding analysis, 1×10^5 BT-474-M1 cells were plated in each well of a 24-well plate. Cells were treated with antibodies for 24 h in a volume of

0.5 ml. HER2 ECD was detected from the media using ELISA [24]. Nunc immunoplates were coated with 0.8 µg/ml anti-HER2 ECD antibody (Genentech) in 50 mM HEPES, pH 8.2, 150 mM NaCl overnight at 4°C. Non-specific binding was blocked with 0.5% BSA in PBS for 1 h at room temperature. Conditioned media (25 µl) was diluted with 75 µl of assay buffer (0.35 M NaCl, 0.5% BSA, 0.05% Tween 20, 5 mM EDTA pH 8.0, 0.25% CHAPS, 0.2% Bovine gamma globulin in PBS), was applied to wells and incubated for 2 h at room temperature. After three washes (0.05% Tween 20 in PBS), HER2 ECD was detected using 0.03 µg/ml rabbit anti-HER2-Biotin antibody (Genentech, 2 h room temperature) followed by a 30 min incubation with 0.2 µg/ml ELISA grade streptavidin–horseradish peroxidase conjugate (BioSource International, Camarillo, CA) and a peroxidase substrate (Sigma Fast o-phenylenediamine dihydrochloride tablet; Sigma, St. Louis, MO).

Cell viability assay

Cell proliferation/viability was detected using CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Madison, WI). For the assay, 5×10^3 cells/well were plated in 96-well plate and incubated overnight for cell attachment before treatments. All measurements were done in triplicate.

In vivo drug efficacy

Fo5 is a mouse tumor model derived from a transgenic mouse carrying a human HER2 gene under the control of a murine mammary tumor virus (MMTV) promoter. Generation, amplification, and implantation (nude mice, Harlan) of Fo5 tumor allografts has been previously described [25]. For MCF7-neo/HER2 xenografts, NCR.nude mice (Taconic) were supplemented with subcutaneous estrogen pellets (0.36 mg, 60-day release; Innovative Research of America) 3 days prior to implanting 5 million MCF7-neo/HER2 tumor cells in 1:1 HBSS-matrigel suspension (BD matrigel; BD Biosystems, San Jose, CA) to mammary fat pads. When tumor volumes reached 130–250 mm³, mice were randomly grouped for the treatment cohorts. Dosing is described in the figure legends. Lapatinib was synthesized by the Genentech Chemistry Dept. Tumor volumes were calculated with the formula: (mm³) = (L × W2) × 0.5.

Statistical analysis

All statistical analysis was performed using JMP 7.0 software (SAS institute Inc. Cary, NC) or Prism For Windows 3.03 (GraphPad Software Inc. La Jolla, CA).

Results

Potency of maytansinoids and their derivatives on human breast cancer cell lines

Paclitaxel and doxorubicin are two established cytotoxic agents that are frequently used in the treatment of breast cancer. To demonstrate the potency of maytansinoid derivatives relative to these two drugs, we examined a panel of six breast cancer cell lines using in vitro cell viability assays. DM1 is a derivative of maytansine where a thiolpropanoyl group replaces the N-acetyl group. The presence of the free thiol was designed to facilitate conjugation to proteins. However, the thiol also introduces a complexity for assessing the potency of free DM1, since the compound can form a disulfide-mediated dimer or a mixed disulfide with other thiol-containing substituents in the cell culture media or intracellularly. To circumvent this issue, we also examined the activity of the methyl form of DM1 (DM1-CH₃; where the thiol group is methylated). As shown in Table 1, DM1-CH₃ was more potent than DM1. IC₅₀ values for DM1-CH₃ ranged from 0.02 to 0.13 nM. In contrast, IC₅₀ values for paclitaxel were 1.5–6.5 nM and for doxorubicin 9–110 nM. These data indicate that on a molar basis the maytansinoid is 24- to 270-fold more potent than paclitaxel and two to three orders of magnitude more potent than doxorubicin.

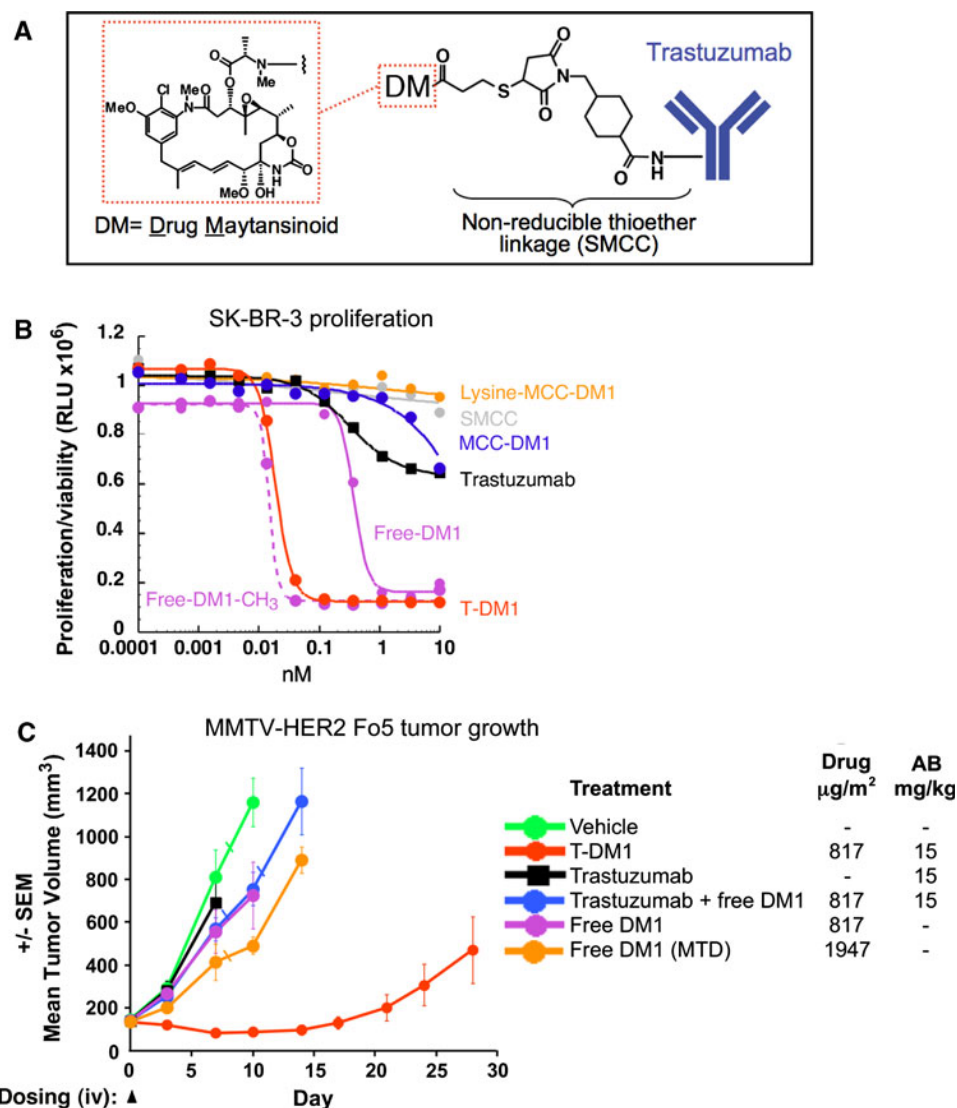
To further address the potency of the individual components of T-DM1 in an in vitro system, additional studies were conducted with the HER2 amplified breast cancer cell line, SK-BR-3. As shown in Fig. 1b, DM1-CH₃ and T-DM1 have similar potency on a molar basis. In contrast and as expected, the linker, SMCC, has no activity. Consistent with the metabolic data reported for the C242-MCC-DM1, the active metabolite of T-DM1, lysine-MCC-DM1, is also inactive due to its net positive charge which compromises its ability to cross the plasma membrane [26].

Although encouraging, the aforementioned in vitro comparison of T-DM1 and its various constituents has certain limitations. To more fully determine the antitumor

Table 1 IC₅₀ (nM) values for DM1 in comparison to paclitaxel and doxorubin in breast cancer cell lines

Cell line	DM1-CH ₃	DM1	Paclitaxel	Doxorubin
HCC1954	0.02	0.32	5.43	84.35
KPL-1	0.06	0.95	1.50	54.20
MDA-468	0.03	0.51	3.31	9.07
T47D	0.13	1.49	3.96	24.11
ZR-75-1	0.10	2.59	6.52	110.07

Fig. 1 Antiproliferative and antitumor effects of trastuzumab-DM1 components. **a** Structure of T-DM1. **b** In vitro dose response of SK-BR-3 cells to T-DM1 components. Proliferation/viability was measured after 5 days of treatment with T-DM1 (red), trastuzumab (black), free-DM1 (magenta), free-DM1-CH₃ (magenta, dashed line), SMCC (gray), MCC-DM1 (blue) and lysine-MCC-DM1 (orange). **c** Effect of T-DM1 components on the treatment of Fo5 tumor allografts. Fo5 tumor allografts were grown in the mammary fatpads of nude mice. Mice were treated with single iv dose at day 0



effects of T-DM1, we performed studies in a trastuzumab-insensitive mouse xenograft model (MMTV-HER2 Fo5). As shown in Fig 1c, this model is insensitive to trastuzumab but responds robustly to T-DM1. Very little anti-tumor activity is observed with a dose of DM1 that is equivalent to the amount of DM1 conjugated to trastuzumab. No demonstrable increase in activity is observed when free DM1 is increased to its maximally tolerated dose. Finally, to rule out possible potentiating or chemosensitization effects, a cohort of tumor-bearing mice were treated with an equivalent dose of trastuzumab combined with free DM1. Again no significant therapeutic effect was observed with this combination. We conclude in this trastuzumab-insensitive model that conjugation of DM1 to trastuzumab allows for effective delivery of the cytotoxic agent to the tumor.

Trastuzumab-DM1 retains all the mechanisms of action of trastuzumab

To confirm that conjugating trastuzumab with DM1 (Fig. 1a) does not affect its affinity for HER2, we analyzed the binding affinities of trastuzumab and T-DM1 (Fig. 2a). In the assay, binding of radio-iodinated trastuzumab to HER2 ECD is competed with increasing concentrations of non-iodinated antibody (trastuzumab or trastuzumab DM1 conjugate). The detected antibody affinities were nearly identical ($K_D = 0.170 \pm 0.003$, and 0.140 ± 0.02 , for trastuzumab and T-DM1, respectively) indicating that conjugation does not affect HER2 binding [4].

Trastuzumab binds Fc γ R expressed by immune cells and has been shown to mediate ADCC in vitro [12]. ADCC contributes to trastuzumab activity in vivo [13]

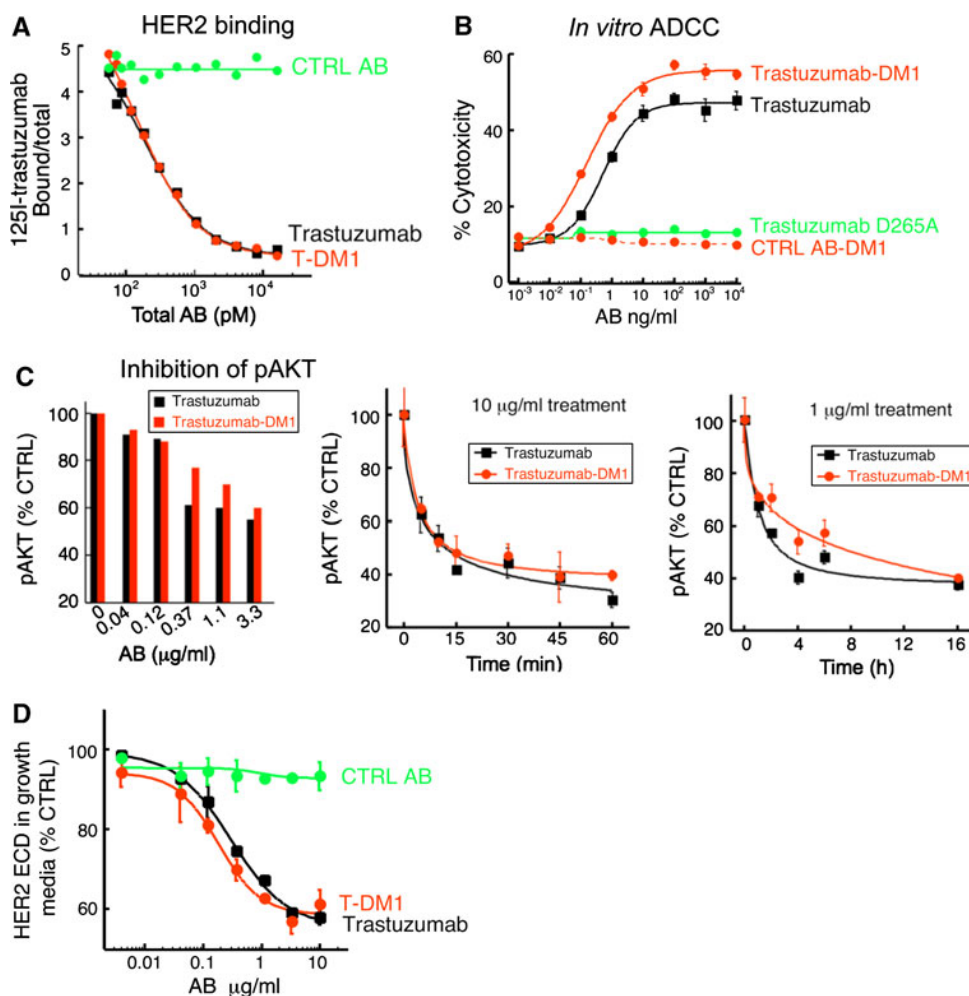


Fig. 2 Trastuzumab-DM1 retains all the mechanisms of action of trastuzumab. **a** T-DM1 binds to HER2 with affinity equal to trastuzumab. ^{125}I -trastuzumab binding to HER2 was competed with non-labeled trastuzumab (black), T-DM1 (red) or control antibody (green). **b** T-DM1 mediates ADCC. The ability of trastuzumab (black) and T-DM1 (red) to mediate in vitro ADCC by PBMCs was measured using an assay detecting LDH released from lysed BT-474-M1 cells. DM1-conjugated antibody (CTRL-AB-DM1; red, dashed line), which does not bind to BT-474-M1 cells and a trastuzumab variant, which does not bind to Fc γ R (D256A, green) were used as

controls. **c** T-DM1 inhibits pAKT. AKT phosphorylation was measured by ELISA detecting phosphorylated Ser473 from BT-474-M1 cells treated 2 h with trastuzumab (black) or T-DM1 (red; left panel). Immediate response on pAKT was investigated using 10 $\mu\text{g/ml}$ (middle panel) and long-term response by using 1 $\mu\text{g/ml}$ antibodies (right panel). **d** T-DM1 inhibits HER2 shedding. Soluble HER2 ectodomain was detected from BT-474-M1 conditioned growth media after 24 h incubation with trastuzumab (black), T-DM1 (red) or control antibody (green) using ELISA

and may be important for trastuzumab response in patients [27]. The ability of trastuzumab DM-1 conjugate to mediate ADCC was analyzed by measuring the lactate dehydrogenase (LDH) activity released from BT-474-M1 cells as a result of cell lysis mediated by peripheral blood mononuclear cells (PBMCs). Trastuzumab mediated in vitro ADCC efficiently (Fig. 2b), resulting in 48% maximal cytotoxicity in 4 h (EC_{50} 0.5 ng/ml). To confirm that the detected LDH leakage was a result of ADCC activity, we used trastuzumab-D265A. The D265A substitution abolishes binding of trastuzumab to Fc γ R on the immune

cells [28], thereby also preventing its ability to mediate ADCC. Trastuzumab-D265A did not induce a detectable increase in LDH release (Fig. 2b). In addition, no LDH release was detected using a control DM1 antibody conjugate, which does not bind to breast cancer cells. Long-term treatment with T-DM1 induces apoptosis of breast cancer cells in vitro [15]. To exclude the possibility that the LDH release detected in Fig. 2b would be due to apoptosis, we measured activation of caspases 3 and 7 from the cells used in ADCC assay. The 4 h treatment with T-DM1 or trastuzumab did not result in significant

caspase activity (not shown) confirming that the detected cell death is not due to apoptotic activity of the DM1, but caused by the lytic activity of immune cells.

Inhibition of HER3 phosphorylation by trastuzumab and the resulting inhibition of the PI3K pathway have been shown to be immediate and critical for the cytostatic effect of trastuzumab [11, 29]. To investigate the effect of T-DM1 on AKT phosphorylation, BT-474-M1 cells were treated for 2 h with a dilution series of the antibodies. The phosphorylation state of Ser473 of AKT1 was detected using ELISA. Maximal inhibition of pAKT was achieved using 3.3 $\mu\text{g/ml}$ concentrations for both antibodies (Fig. 2c, left panel). The immediate effect on pAKT was then investigated and rapid dephosphorylation of AKT was detectable within 5–10 min for both antibodies (Fig. 2c, middle panel). After 15 min, AKT phosphorylation had decreased by 47 and 42% in cells treated with trastuzumab and T-DM1, respectively. Similar levels of inhibition were still detected at 60 min of treatment. Prolonged treatment, up to 16 h incubation, with 1 $\mu\text{g/ml}$ antibodies resulted in 60% reduction of pAKT for both antibodies (Fig. 2c, right panel). Maximal inhibition was reached by 4 h of treatment and maintained until 16 h. These results demonstrate that treatment of breast cancer cells with T-DM1 rapidly inhibits AKT phosphorylation and that the drug conjugation does not affect this activity.

Another biological activity and a possible mechanism of action for trastuzumab is inhibition of HER2 ectodomain shedding [30]. The ability of the T-DM1 conjugate to inhibit HER2 shedding was analyzed by detecting the amount of soluble HER2 ectodomain from BT-474-M1 conditioned growth media following a 24-h incubation with the antibodies (Fig. 2d). The maximal inhibition of HER2 shedding was 42% with trastuzumab treatment and 43% with T-DM1 treatment. The EC_{50} values for the antibodies were 0.30 ± 0.05 and 0.18 ± 0.04 $\mu\text{g/ml}$, respectively. To exclude the possibility that the reduction of HER2 ectodomain in the media is due to decreased cell numbers in the treated wells, the number of viable cells was analyzed parallel to media collection. The treatments did not affect cell number at 24 h (not shown), confirming that the reduction of HER2 ectodomain in the media is due to inhibition of HER2 shedding. Taken together, these results demonstrate that T-DM1 binds HER2 with an affinity equal to that of trastuzumab and that T-DM1 retains all the mechanisms of action of trastuzumab.

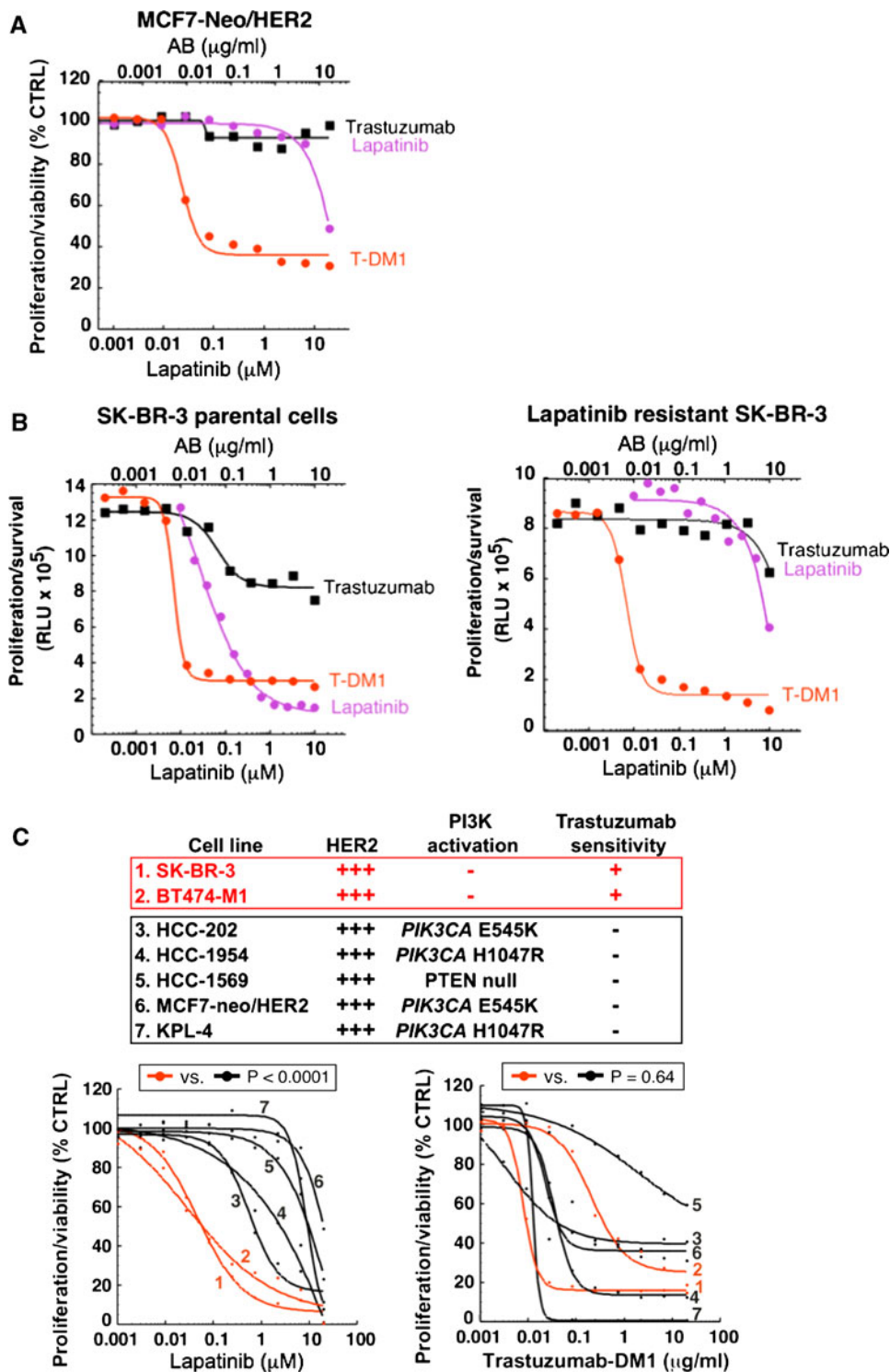
Trastuzumab-DM1 inhibits the growth of lapatinib resistant breast cancer cells with activated PI3K pathway

In our previous report, we demonstrated that T-DM1 efficiently inhibits proliferation of trastuzumab resistant,

HER2 amplified breast cancer cells [15]. The small molecule tyrosine kinase inhibitor, lapatinib, is used for treatment of trastuzumab refractory breast cancer. We next investigated the effect of T-DM1 for treating HER2 amplified cells that fail to respond to lapatinib. Activation of the PI3K pathway either by acquiring *PIKCA* mutations or loss of PTEN causes resistance against both trastuzumab and lapatinib [11, 31, 32]. The dose responses of lapatinib and T-DM1 treatment for MCF7-neo/HER2 were determined by measuring proliferation/viability using Cell-Titer plus reagent following 6 days of treatment. Trastuzumab did not inhibit cell proliferation and cells were also resistant to lapatinib (Fig. 3a). Proliferation was affected only by the highest lapatinib concentration (20 μM). However, the cells were sensitive to low doses of T-DM1 (IC_{50} 0.024 $\mu\text{g/ml}$ (0.16 nM)). Next we subjected SK-BR-3 cells for 9 months to increasing concentrations of lapatinib, which resulted in a lapatinib resistant SK-BR-3 cell line. Of note, the parental SK-BR-3 cells are readily sensitive to both lapatinib (IC_{50} 0.027 $\mu\text{g/ml}$) and trastuzumab (IC_{50} 0.068 $\mu\text{g/ml}$ (0.45 nM); Fig. 3b, left panel). However, the lapatinib resistant SK-BR-3 cell line demonstrated resistance to high, micromolar concentrations of lapatinib and up to 10 $\mu\text{g/ml}$ trastuzumab (Fig. 3b, right panel). Importantly, the acquired lapatinib resistance did not have any effect on sensitivity to T-DM1 (Fig. 3b, right panel). IC_{50} for the lapatinib conditioned SK-BR-3 cells was identical to the parental SK-BR-3 (0.007 μM for both cell lines).

The E545K mutation in the *PIK3CA* gene (p110 α subunit of PI3K) is a likely cause for the trastuzumab and lapatinib resistance in MCF7-neo/HER2 cells. Next, we investigated a panel of cell lines that harbor PI3K pathway activating genetic modifications in addition to HER2 overexpression. KPL-4, HCC1954, MCF7-neo/HER2, and HCC202 cells all have activating *PIK3CA* “hotspot” mutations (H1047R, H1047R, E545K, and E545K, respectively) and HCC-1569 is PTEN null. We determined dose responses of lapatinib and T-DM1 in these cells and in SK-BR-3 and BT-474-M1 cells, which harbor HER2 amplification, but no additional PI3K-activating modifications. As predicted, PI3K pathway activation determined the ability of cells to respond to lapatinib (Fig. 3c, left panel). Cells with activating *PIK3CA* mutations or PTEN loss were significantly less sensitive to lapatinib ($P < 0.0001$). In contrast, PI3K activation did not predict sensitivity to T-DM1 ($P = 0.64$; Fig. 3c, right panel). In conclusion, these results demonstrate that T-DM1 is highly potent in inhibiting growth of lapatinib resistant cells and cells that have PI3K pathway activating alterations. Our results suggest that in contrast to lapatinib and trastuzumab, aberrant PI3K pathway activation is not a major determinant for T-DM1 sensitivity.

Fig. 3 Trastuzumab-DM1 inhibits the growth of lapatinib resistant breast cancer cells with activated PI3K pathway. **a**, **b** T-DM1 and lapatinib dose response of MCF7-neo/HER2 cells (**a**) SK-BR-3 cells (**b, left panel**) and lapatinib resistant SK-BR-3 cells (**b, right panel**). Proliferation/viability of cells was detected using CellTiter-Glo® Luminescent Cell Viability Assay after 6 days of treatment with T-DM1 (red), lapatinib (purple), or trastuzumab (black). **c** T-DM1 and trastuzumab dose response of HER2 overexpressing breast cancer cells with (black) or without (red) PI3K pathway activating mutations or PTEN loss. Statistical significance was determined using *t*-test on 0.25 µg/ml or 0.25 µM values



Trastuzumab-DM1 inhibits the growth of lapatinib insensitive tumors and tumors harboring PI3K pathway activating mutations

Next, we explored whether T-DM1 is able to inhibit the growth of lapatinib insensitive tumors in vivo. For this

purpose, we cultured Fo5 tumor allografts [25] that express human HER2 from MMTV promoter in mammary fat pads of nude mice. After tumor establishment, the mice were treated either with 15 mg/kg T-DM1 (once every 3 weeks for two doses), 100 mg/kg lapatinib (daily dosing for 3 weeks), or vehicle. Treatment of mice with a daily high

dose of lapatinib was not sufficient to stop tumor growth (Fig. 4a) and no complete or partial responses were detected. In contrast, trastuzumab-DM1 efficiently prevented the growth of the tumors (Fig. 3a) and resulted in complete responses (CR; no detectable tumor) in 3/10 and partial responses (PR; tumor volume is 50% or less of its Day 0 volume) in 6/10 mice.

Although, the mechanism for trastuzumab resistance in Fo5 tumors is unknown, MCF7-neo/HER2 cells are resistant to lapatinib and trastuzumab likely due to PI3K pathway activation. MCF7-neo/HER2 cells harbor the E545K *PIK3CA* mutation and express similar levels of HER2 protein as trastuzumab sensitive SK-BR-3 cells. To investigate the ability of T-DM1 to inhibit the growth of MCF7-neo/HER2 tumors, cells were grown in mammary fat pads of NCR.nude mice. After tumor establishment, mice were treated with trastuzumab, T-DM1 or vehicle. Six weekly 30 mg/kg doses of trastuzumab did not inhibit the growth of MCF7-neo/HER2 tumors (Fig. 4b). However, treatment

with T-DM1 dosed 10 mg/kg once every 3 weeks for two doses completely prevented tumor growth, and again resulted in significant tumor regression (CR = 6/10, PR = 4/10). Taken together, these results demonstrate that T-DM1 efficiently prevents growth of lapatinib resistant tumors and tumors where PI3K pathway activating mutations cause resistance to currently approved therapeutic anti-HER2 compounds.

Discussion

It is well established that alterations in specific genes initiate and promote neoplastic growth [33]. As a consequence, organ specific classifications of cancer are now giving way to molecularly defined subtypes. HER2 gene-amplified tumors are now recognized as distinct subgroups within breast and gastric cancers. In both of these diseases, Herceptin[®] is generally used in combination with chemotherapy as standard of care [34]. Unfortunately, some patients with metastatic breast cancer are observed to progress on Herceptin[®]-containing regimens. Progression also occurs when these patients are treated with second line HER2-directed therapies such as lapatinib (Tykerb). To address this unmet clinical need, we designed an antibody drug conjugate that is composed of trastuzumab and the maytansinoid, DM1.

Chemical conjugation of DM1 to trastuzumab is accomplished with the heterobifunctional agent *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). The *N*-hydroxy-succinimide moiety allows for nucleophilic attack by primary amino groups contained within the antibody. As a result, derivatization of trastuzumab occurs predominantly on the epsilon amino groups of lysines, which are abundant and distributed throughout the sequence of the antibody. Thus, T-DM1 is heterogeneous with respect to these covalent modifications. In the present report, we address whether chemical modification of trastuzumab affects its known mechanisms of action. The anti-signaling properties of trastuzumab are primarily the consequence of antibody recognition of HER2 on the surface of breast cancer cells. Our studies confirm that T-DM1 binding to HER2 is not affected by the derivatization with MCC-DM1. As expected, once bound to HER2, T-DM1 blocks HER2 shedding. Perhaps more importantly, T-DM1 also downregulates PI3K-AKT signaling due to the fact that, like trastuzumab [11], it effectively disrupts the constitutive HER2-HER3 complex. Activation of immune effector function requires binding of the antibody's Fc region to Fc γ receptors. Antibody-dependent cytotoxicity assays (ADCC) performed with peripheral blood mononuclear cells obtained from health volunteers verified that T-DM1 maintains full trastuzumab activity.

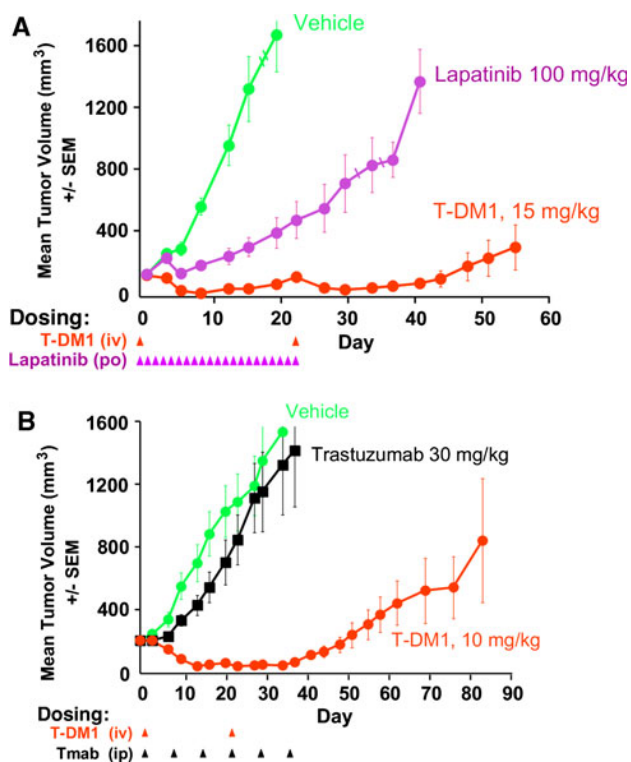


Fig. 4 Trastuzumab-DM1 inhibits the growth of lapatinib insensitive tumors and tumors harboring PI3K pathway activating mutations. **a** In vivo efficacy of T-DM1 in the treatment of Fo5 tumor allografts. Fo5 tumor allografts were grown in the mammary fatpads of nude mice. Mice were treated with 100 mg/kg lapatinib (po; bid \times 21), 15 mg/kg T-DM1 (iv; q3wk \times 2) or with vehicle (po; bid \times 21); $n = 10$ /treatment group. **b** MCF7-neo/HER2 xenografts were grown in the mammary fatpads of NCR.nude mice. Mice were treated with 10 mg/kg T-DM1 (iv; q3wk \times 2), 30 mg/kg trastuzumab (ip; q1wk \times 6), or vehicle (ip; q1wk \times 6); $n = 10$ /treatment group

Most novel anticancer agents are initially tested in model systems that are refractory to standard of care therapies. To date our preclinical focus with T-DM1 has examined tumor models that are refractory to trastuzumab. Our clinical studies have also selected patients whose tumors have progressed through Herceptin® and in many cases Tykerb® (lapatinib). In the current report we demonstrate that T-DM1 efficiently inhibits growth of cells and tumors that are insensitive to lapatinib and have hyperactivated PI3K signaling due to activating mutations in p110 α . Because the T-DM1 preclinical and clinical data are promising, we are now designing and executing clinical trials that will examine T-DM1's activity in patients who may not have received previous HER2 directed therapies. The data presented in this report suggest that T-DM1 maintains all of the known mechanisms of action of trastuzumab. As a result of these findings, we are optimistic that T-DM1 can be moved into earlier lines of therapy for the treatment of HER2 positive cancer.

Conflict of interest All authors are employees of Genentech, Inc.

References

- Perou CM, Sorlie T, Eisen MB et al (2000) Molecular portraits of human breast tumours. *Nature* 406:747–752
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177–182
- Slamon DJ, Godolphin W, Jones LA et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244:707–712
- Carter P, Presta L, Gorman CM et al (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 89:4285–4289
- Cho HS, Mason K, Ramyar KX et al (2003) Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 421:756–760
- Piccart-Gebhart MJ, Procter M, Leyland-Jones B et al (2005) Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 353:1659–1672
- Romond EH, Perez EA, Bryant J et al (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 353:1673–1684
- Slamon DJ, Leyland-Jones B, Shak S et al (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344:783–792
- Joensuu H, Kellokumpu-Lehtinen PL, Bono P et al (2006) Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med* 354:809–820
- Slivkowski MX, Lofgren J, Lewis GD, Hotaling TE, Fendly BM, Fox JA (1999) Nonclinical studies addressing the mechanism of action of Herceptin® (Trastuzumab). *Semin Oncol* 26(Suppl 12):60–70
- Junttila TT, Akita RW, Parsons K et al (2009) Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell* 15:429–440
- Lewis GD, Figari I, Fendly B et al (1993) Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother* 37:255–263
- Clynes RA, Towers TL, Presta LG, Ravetch JV (2000) Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med* 6:443–446
- Geyer CE, Forster J, Lindquist D et al (2006) Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med* 355:2733–2743
- Lewis Phillips GD, Li G, Dugger DL et al (2008) Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res* 68:9280–9290
- Chari RV, Martell BA, Gross JL et al (1992) Immunoconjugates containing novel maytansinoids: promising anticancer drugs. *Cancer Res* 52:127–131
- Remillard S, Rebhun LI, Howie GA, Kupchan SM (1975) Antimitotic activity of the potent tumor inhibitor maytansine. *Science* 189:1002–1005
- Issell BF, Croke ST (1978) Maytansine. *Cancer Treat Rev* 5:199–207
- Austin CD, Wen X, Gazzard L, Nelson C, Scheller RH, Scales SJ (2005) Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody-drug conjugates. *Proc Natl Acad Sci USA* 102:17987–17992
- Krop IE, Beeram M, Modi S et al (2010) Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol* 28:2698–2704
- Kurebayashi J, Otsuki T, Tang CK et al (1999) Isolation and characterization of a new human breast cancer cell line, KPL-4, expressing the Erb B family receptors and interleukin-6. *Br J Cancer* 79:707–717
- Munson PJ, Rodbard D (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 107:220–239
- Idusogie EE, Presta LG, Gazzano-Santoro H et al (2000) Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164:4178–4184
- Sias PE, Kotts CE, Vetterlein D, Shepard M, Wong WL (1990) ELISA for quantitation of the extracellular domain of p185HER2 in biological fluids. *J Immunol Methods* 132:73–80
- Finkle D, Quan ZR, Asghari V et al (2004) HER2-targeted therapy reduces incidence and progression of midlife mammary tumors in female murine mammary tumor virus huHER2-transgenic mice. *Clin Cancer Res* 10:2499–2511
- Erickson HK, Park PU, Widdison WC et al (2006) Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res* 66:4426–4433
- Musolino A, Naldi N, Bortesi B et al (2008) Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* 26:1789–1796
- Shields RL, Namenuk AK, Hong K et al (2001) High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem* 276:6591–6604
- Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL (2002) Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* 62:4132–4141

30. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J (2001) Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* 61:4744–4749
31. Eichhorn PJ, Gili M, Scaltriti M et al (2008) Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. *Cancer Res* 68:9221–9230
32. Nagata Y, Lan KH, Zhou X et al (2004) PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 6:117–127
33. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70
34. Smith I, Procter M, Gelber RD et al (2007) 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* 369:29–36