Monoclonal antibody against the ectodomain of E-cadherin (DECMA-1) suppresses breast carcinogenesis: Involvement of the HER/PI3K/Akt/mTOR and IAP pathways

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Running Title: Suppression of breast carcinogenesis by DECMA-1 mAb therapy

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Translational Relevance

In the last decade, significant improvements in the prognosis of patients with HER2-positive breast cancer have been achieved using HER2-targeted therapies. However, despite encouraging results, the majority of patients develop acquired resistance due to extensive cross-talk and feedback loops associated with alternate HER family members and downstream MAPK-PI3K/Akt/mTOR pathways. Our current study demonstrates in vivo and in vitro proof-of-concept that an antibody against the ectodomain of E-cadherin (DECMA-1) selectively induces apoptosis and reduces tumor growth by down-regulating all HER family members, and components of the MAPK-PI3K/Akt/mTOR and IAP pathways, thereby successfully overcoming multiple resistance pathways. More importantly, we show that this occurs without any detectable cytotoxicity to mice, end-organs or a panel of normal human epithelial or mesenchymal cells in culture. These findings provide a rationale for the development and clinical testing of antibodies directed against the ectodomain of E-cadherin that may, in combination with Trastuzumab or other combination therapies, improve the survival of patients with HER-positive tumors.
Abstract

Purpose: Although targeted therapies against HER2 have been one of the most successful therapeutic strategies for breast cancer, patients eventually developed acquired resistance from compensatory upregulation of alternate HERs and MAPK-PI3K/Akt/mTOR signaling. As, we and others, have shown that the soluble ectodomain fragment of E-cadherin (sEcad) exerts pro-oncogenic effects via HER1/2-mediated binding and activation of downstream pro-survival pathways, we explored whether targeting this ectodomain (DECMA-1 mAb) was effective in the treatment of HER2-positive breast cancers.

Experimental Design: MMTV-PyMT transgenic mice and HER2+/E-cadherin-positive MCF-7 and BT474 Trastuzumab-resistant (TtzmR) cells were treated with the DECMA-1 mAb. Antitumor responses were assessed by BrdU incorporation, apoptosis and necrosis. The underlying intracellular pro-oncogenic pathways were explored using subcellular fractionation, immunoprecipitation, fluorescence microscopy and immunoblotting.

Results: Treatment with DECMA-1 mAb significantly delayed tumor onset and attenuated tumor burden in MMTV-PyMT mice by reducing tumor cell proliferation and inducing apoptosis without any detectable cytotoxicity to mice or end-organs. In vitro, treatment of MCF-7 and BT474 TtzmR cells reduced proliferation and induced cancer cell apoptosis. Importantly, this inhibition of breast tumorigenesis was due to concomitant down-regulation, via ubiquitin-mediated degradation through the lysosome and proteasome pathways, of all HER family members, components of downstream PI3K/Akt/mTOR pro-survival signaling and suppression of IAPs.

Conclusions: Our results establish that the E-cadherin ectodomain-specific mAb DECMA-1 inhibits Ecad+/HER2+ breast cancers by hindering tumor growth and inducing apoptosis via down-regulation of key oncogenic pathways involved in Trastuzumab resistance, thereby establishing a novel therapeutic platform for the treatment of HER2+ breast cancers.
Introduction

Breast cancer, a heterogeneous disease with multiple subgroups and molecular signatures, remains the second leading cause of cancer-related deaths in women (1). One of the most successful strategies in the treatment of breast cancers involves the administration of monoclonal antibodies directed against epitopes of the human epidermal growth factor receptor family (HER) that are abundant on tumor cells. In this regard, Trastuzumab (Herceptin®), a humanized mAb against the extracellular domain of HER2 has revolutionized the care of HER2-positive cancers, an aggressive subtype representing 20-25% of breast cancers (1). However, despite encouraging clinical trials, targeted mAb therapies for HER2 positive breast cancers have only had a modest efficacy due to the development of cancer cell resistance (1, 2).

Several key cell survival pathways have been suggested to contribute to cancer cell resistance, including signaling by other HER family members (HER1, 3, 4), hyperactivation of the PI3K (phosphatidylinositol 3-kinase)/Akt and MEK/ERK (mitogen-activated protein kinase) signaling axis and dysregulation of the inhibitor of apoptosis (IAPs) proteins (1, 2). It is well documented that Trastuzumab efficiently blocks HER2-HER2 homodimer signaling, but has little effect on HER1, HER3 or HER4 homodimers or heterodimers (1, 2). Along these lines, the TtzmR BT474 cell line exhibited higher levels of endogenous phospho-HER1 and HER1/HER2 heterodimers, the latter of which was not inhibited by Trastuzumab treatment (3). This is consistent with clinical reports, in which patients with HER2-overexpressing tumors that became resistant to Trastuzumab responded to the HER1/HER2 inhibitors Lapatinib and HKI-272 (4, 5). HER3 contains six PI3K binding sites, which make the HER2/HER3 heterodimers among the most potent activators of the PI3K-Akt pathway (6). Studies have shown that sustained MAPK and PI3K/Akt signaling is integral to Trastuzumab-induced resistance (7). Notably, constitutive PI3K/Akt signaling prevented cell-cycle arrest and apoptosis mediated by Trastuzumab, and BT474 TtzmR clones demonstrated enhanced phospho-Akt and Akt kinase activity (8, 9). Not surprisingly, preclinical studies in HER2-amplified cell lines and xenograft
models demonstrated that the bispecific mAb Pertuzumab, which blocks ligand-induced HER2/HER3 dimerization, effectively disrupted HER2-HER3 heterodimers, leading to inhibition of downstream MAPK and PI3K signaling and significant anti-tumor activity (9). Combination therapy with Trastuzumab and Pertuzumab also exhibited enhanced anti-tumor activity in models of Trastuzumab resistance, suggesting that both drugs have complimentary mechanisms of action (10). Interactions of the HER family, with other major gene families regulating cell survival, such as the inhibitor of apoptosis proteins (IAPs) also confer resistance to apoptosis in breast cancer cells (11, 12). Constitutive overexpression of survivin, the smallest IAP family member downstream of PI3K, was shown to be indispensable for survival of HER2-positive breast cancer cells that exhibited intrinsic cross-resistance to multiple HER1/2 inhibitors (13). Moreover, co-expression of HER1 and HER2 enhanced survivin levels, resulting in enhanced resistance to etoposide-induced apoptosis (11). Therefore, it is clear that HER2-positive cancers may benefit from therapeutic approaches that simultaneously block multiple HER receptor family members.

Prior studies have shown a clear interaction between the HER receptor family and E-cadherin, a transmembrane protein that mediates calcium-dependent homophilic cell-cell adhesions. HER1 (EGFR) activation was shown to disrupt adherens junctions and chronic HER1 stimulation enhanced the E-cadherin transcriptional repressors, TWIST and Snail (14, 15). Conversely, in lung and breast cancer cell lines, inhibition of HER1 signaling increased intact E-cadherin and restored adherens junctions (16, 17), suggesting an inverse correlation between E-cadherin and HER family members. Recent studies in our laboratory, as well as others, also demonstrate a direct interaction between the shed E-cadherin ectodomain fragment, sEcad, and the HER receptors (18, 19). sEcad is derived from the proteolytic cleavage of the extracellular domain of E-cadherin, which contains five subdomains, termed EC1-5 (20). This ectodomain shedding, mediated by metalloproteinases (MMPs) or members of the ADAMs (a disintegrin and metalloproteinases), leads to the release of an 80 kDa sEcad fragment into the extracellular space (21). sEcad is constitutively shed at low levels in normal
epithelial cells, but is significantly elevated in breast cancer patients and correlates with tumor size, clinical response to chemotherapy and predicts a shorter disease-free interval (22). In prostate, skin and ovarian cancer cells, addition of a purified 80kDa sEcad fragment or recombinant sEcad chimeric protein (rhEcad/Fc) destabilized adherens junctions, enhanced tumor cell proliferation, migration and invasion (18, 19, 21, 23, 24). This sEcad induced invasion was shown to be MMP-2, MMP-9 and/or MT1-MMP dependent in lung and skin cancer cells (18, 25). Although the mechanisms for these pro-oncogenic effects have yet to be elucidated, we recently demonstrated endogenous and exogenous sEcad binding to HER1, HER2 and the insulin-like growth factor-1 receptor (IGF-1R) in human skin squamous cell cancer specimens and skin cancer cells (18). Similarly, Najy et al (2008) demonstrated endogenous sEcad binding to HER2 and HER3 in E-cadherin-positive MCF-7 breast cancer cells, and exogenous sEcad associating with HER2 in E-cadherin-negative SKBR3 cells (19). The latter resulted in HER2-HER3 heterodimerization, HER3 phosphorylation and ERK activation. Consistent with these results, exogenous sEcad promoted cell survival and acted as a potent anti-apoptotic protein, via HER1, Akt and ERK signaling in serum starved transformed Madin-Darby canine kidney (MDCK) cells and in polarized epithelial acini (26). Furthermore, exogenous sEcad induced the phosphorylation of HER1, HER2 and IGF-1R in PAM212 and CC4A skin cancer cells, and depending on the cell-type, resulted in the activation of downstream MAPK-PI3K/Akt/mTOR signaling (18). Therefore, although an inverse relationship exists between intact E-cadherin and HERs, it is clear that the shed ectodomain sEcad fragment binds to HER family members and nurtures the growth and survival of various cancers, demonstrating a potential important role of targeting sEcad and sEcad-HER receptor interactions in breast carcinogenesis.

In this study, we set out to explore whether a commercially available monoclonal antibody (DECMA-1) that specifically targets epitopes within the extracellular domain of E-cadherin, is able to suppress breast carcinogenesis in vivo and in vitro. We show that DECMA-1 mAb treatment exhibits potent anticancer activity and effectively downregulates HER1-4 family members and multiple
downstream resistance pathways, without untoward cytotoxicity to normal cells, tissues or mice. Our results suggest that antibodies targeting specific extracellular domains of E-cadherin may provide a novel therapeutic platform for HER2+ breast cancer patients that develop de novo or acquired resistance.
Materials and Methods

Animal studies and in vivo treatments
MMTV-PyMT mouse breeders were obtained from Jackson Laboratories and mated according to the vendor's specifications. When mice reached 47 days of age, virgin female mice were randomly assigned to different groups (n=5, each group) to receive weekly intraperitoneal (i.p.) injections of 1mg/kg DECMA-1 (U5885, Sigma; custom order), rat IgG1 (0116-14, Southern Biotech; custom order), or equivalent volumes of saline (200μL). Palpable tumors were monitored twice weekly from 40 days until sacrifice at 90 days of age. At the end of the protocol, mice were given 2mg/mouse of BrdU by i.p. injection 2 h before sacrifice and organs were collected and mammary tumors were excised, weighed and either formalin fixed or flash frozen at -20°C until further use.

Patients and tissues. HER2+ human breast cancer specimens were obtained from the NCI Cooperative Human Tissue Network (CHTN) and from Proteogenex (Culver City, CA). Tumors from CHTN included 7 HER2+ infiltrating ductal carcinomas with an age range of 52y to 77 y with corresponding adjacent normal breast tissues from reduction mammoplasty tissues (n=7).

Cells and reagents
Human MCF-7, BT474-parental, MCF-10A, primary epidermal keratinocytes (PHK), and primary epidermal melanocytes (Mel) were purchased from ATCC, and cultured according to ATCC recommendations. Trastuzumab-resistant BT474 (TtzmR) cells were generously provided by Dr. Kute (Wake Forest University, Winston-Salem, NC) and grown in 10% FBS RPMI-1640 medium (ATCC). Primary antibodies used in this study were as follows: for immunoprecipitation, EGFR (Ab-15), HER2 (Ab-17) (Thermo Fisher Scientific); for Western Blot and immunostaining, EGFR (4267), HER2 (2165), HER3 (4754), HER4 (4795), pPI3K (4228), pAkt (4060), Akt (4691), pERK1/2 (4370), ERK1/2 (5695), pmTOR (5536), mTOR (2983), pRaptor (2083), p4E-BP1(2855), pp70S6K (9234), survivin (2808) (Cell Signaling); E-cadherin (H-108), β-actin (C4) and p53 (c-11 and FL-393) (Santa Cruz); G3PDH (AM4300, Ambion),ubiquitin (13-1600, Zymed); E-cadherin SHE78-7 and HECD-1.
(Calbiochem) and E-cadherin C-terminus (clone 36/E-cadherin, BD Transduction Laboratories). For in vivo studies of MCF-7 cells, DECMA-1 (mAb, U2354, Sigma) and IgG (0116-14, Southern Biotech, equivalent sodium azide) were used, respectively. For parental BT474 in vitro cultures, DECMA-1 (U5885, Sigma; custom order) and IgG (0116-14, Southern Biotech; custom order), were used.

### Immunoprecipitation, subcellular fractionation and western blotting

Protein extraction in cells was performed on ice using total protein extraction buffer: 20mM Tris pH7.5, 137mM NaCl, 100mM NaF, 10% glycerol, 1% NP40, 1mM PMSF and protease inhibitor cocktail (Sigma). Protein concentration was measured using a BCA Protein Assay Kit (Pierce). Protein samples (10-100µg) were denatured at 95°C (unless otherwise stated) and subsequently separated by 4-15% SDS-PAGE gel electrophoresis. After transfer to nitrocellulose membrane and blocking with 5% nonfat milk, samples were probed with primary antibodies. Western Blot images were captured using HP Scanjet G4050 and analyzed relative to G3PDH or actin using NIH Scion Image. Subcellular fractionation was performed using the BioVision FractionPREP Cell Fractionation System (BioVision Inc), as per the manufacturer’s instructions. IAP family members, in the presence or absence of DECMA-1, were analyzed using a Human Apoptosis Array kit (R&D Systems). Immunoprecipitation assays were carried out by harvesting cells with immunoprecipitation lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 100 mM NaF; 10% glycerol; 1% Nonidet P-40; 1mM PMSF and protease inhibitor cocktail) (Sigma). After brief sonication, lysates were cleared by centrifugation at 4°C. Supernatants were precleared and incubated with EGFR/HER1 (Ab-15) or HER2 (Ab-17) -specific antibodies for 4 h and protein A/G plus agarose beads (Santa Cruz, sc-2003) for 2 h at 4°C. The immunocomplexes were washed three times, boiled in sample buffer (60 mM Tris-Cl, pH 6.8; 2% SDS (vol/vol); 10% glycerol (vol/vol); 5% β-mercaptoethanol (vol/vol); and 0.01% bromophenol blue (vol/vol), and loaded on SDS-PAGE for protein analysis.

### Assessment of apoptosis, cell number and proliferation
Cellular apoptosis was quantitatively determined using the Cell Death Apoptosis Detection ELISA Plus kit (Boehringer Mannheim), which is based on the detection of DNA-histone complexes in the form of mono- and oligo-nucleosomes, according to the manufacturer’s instructions. Briefly, cells were treated in the presence or absence of various concentrations of DECMA-1 or rat IgG1 for 24 to 48 h. Cells were harvested by trypsinization, counted and lysates were subjected to the ELISA. In vitro and in vivo apoptosis was further analyzed by in situ detection of fragmented DNA, using the DeadEnd Fluorometric TUNEL System (Promega), the ApopTag Peroxidase In situ Apoptosis Detection Kit (Millipore) and the human Apoptosis Array Kit (ARY009, R & D), according to the manufacturer’s instructions. The ApopTag kit was performed on deparaffinized 5μm-thick sections treated with proteinase K (20μg/ml) at RT. For cell counting, the cells were trypsinized and counted on a haemocytometer. For tumor BrdU analyses, tissues were stained using an anti-BrdU (ab2284, abcam) antibody, as previously described (27). The proliferation of cells was monitored by a cell proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) (colorimetric) kit (Roche, Stockholm, Sweden), according to the manufacturers protocol.

**Immunofluorescence**

Cells cultured on chamber slides (no. 177437, Nalge Nunc International) were fixed with 4% formaldehyde, blocked in PBS containing 1% (wt/vol) BSA and incubated in p53 (Santa Cruz Biotechnology) or HER1-4 antibodies (4267, 2165, 4754, 4795, Cell Signaling). Formalin fixed and paraffin-embedded MMTV-PyMT mouse breast tumors and human breast tumor sections were incubated with antigen retrieval reagent (Dako), and then blocked in 1% BSA PBS and probed with DECMA-1 (U5885, Sigma), as previously described (18, 27).

**Histological analysis and immunohistochemistry**

Paraffin-embedded tumors were fixed, sectioned at 5μm, deparaffinized and stained with H&E according to standard protocols. H&E-stained slides were evaluated by a board-certified pathologist (C. Tornos). For immunohistochemical analyses, tissues were stained using anti-HER1, HER2, HER3...
HER4, p53, ERK1/2, Akt, mTOR and survivin (Cell Signaling) antibodies, as previously described (18, 27).

**Statistical Analysis**

Comparisons between groups were made using ANOVA followed by post-hoc analysis using the Student-Newman-Keuls or Dunnett's method unless otherwise stated. Statistical significance is indicated in figures as *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$.

**Study approval**

This study was conducted in accordance with NIH guidelines for the use of experimental animals. Protocols were approved by the Institutional Animal Care and Use Committee and the use of human tissues was according to the Institutional Review Board at Stony Brook University.
Results

Targeted mAb therapy against the ectodomain of E-cadherin (DECMA-1) suppresses breast tumorigenesis in MMTV-PyMT transgenic mice by inhibiting tumor growth and inducing apoptosis

The in vivo efficacy of DECMA-1 was tested in the HER2+ MMTV-PyMT transgenic mouse model of breast cancer, in which mammary targeted over-expression of the polyomavirus middle T antigen (PyMT) leads to the rapid development of palpable tumors that progress to aggressive adenocarcinomas with metastasis to the lungs that are similar to human ductal carcinomas of the breast (28). Mice were treated intraperitoneally once-weekly with saline, IgG or DECMA-1 (1mg/kg in 200μL saline) beginning at 47 days of age (just prior to the development of carcinomas in our study) until 90 days of age when the mice were sacrificed. Treatment with the DECMA-1 mAb significantly delayed tumor onset and tumor numbers compared with saline or IgG control mice (Fig. 1A-B). In these tumors that developed, pathological analysis by H&E staining of histologic sections revealed that control, IgG and mAb treated tumors were poorly differentiated (tumor grade of 3, architectural grade of 3, and nuclear grade of 3), except in one of five DECMA-1 treated mice in which the tumor was moderately differentiated (tumor grade 2, architectural grade 2, nuclear grade 1) (Fig. 1B).

Immunohistochemical analyses of these tumors were then performed, so as to determine the potential mechanisms for the decreased tumorigenesis after DECMA-1 mAb therapy. Consistent with decreased tumor burden, there was a statistically significant decrease in BrdU staining in mAb treated tumors versus controls (Fig. 1C). In contrast, apoptosis was significantly increased in tumors from mice treated with DECMA-1 mAb compared with all control groups as measured by TUNEL analyses (Fig. 1D). Taken together, these data show that targeted mAb therapy against the membrane proximal region of the ectodomain of E-cadherin significantly delayed tumor growth and tumor burden by reducing proliferation, but more importantly by inducing tumor cell death.
Anti- E-cadherin ectodomain-specific mAb inhibits cell proliferation, induces cell death and upregulates wild type p53 expression \textit{in vitro} and \textit{in vivo}

Because DECMA-1 mAb treatment suppressed breast carcinogenesis \textit{in vivo} by decreasing MMTV-PyMT tumor growth and inducing apoptosis, we next tested whether this treatment would induce similar effects on MCF-7 and the E-cadherin/HER2-positive BT474TtzmR cell lines that express varying levels of HER2 amplification. DECMA-1 treated MCF-7 and TtzmR cells exhibited a significant decrease in cell numbers (Fig. 2A) and cellular proliferation (Fig. 2B) compared to control groups. Blebbing of the cellular membrane began to appear after incubation with the mAb for 24-48 h and resulted in \textasciitilde 70\% loss of attached cells (Fig. 2C), suggesting induction of programmed cell death and/or necrosis. ELISA quantification of histone-associated DNA fragments confirmed the phase contrast images and demonstrated a significant DECMA-1 mAb-mediated induction of apoptosis 48 h following treatment (Fig. 2D), but no effects on necrosis (data not shown). As p53 activation is important in the induction of apoptosis, we next investigated whether the effects of DECMA-1 treatment involved p53. The intensity of p53 immunohistochemical staining and western blotting in tumors from DECMA-1 treated MMTV-PyMT mice was dramatically increased compared to saline or IgG treated mice (Fig 3A). Interestingly, DECMA-1 also remarkably increased p53 expression in MCF-7 cells as demonstrated by immunoblotting and immunofluorescence staining (Fig. 3B and C), whereas it decreased the mutant p53 expression in BT474 TtzmR cells (Fig. 3B). Since one potential mechanism for these DECMA-1-induced effects appeared to be through alteration of p53 expression, we next determined the effect of DECMA-1 on apoptosis in both wild-type p53 (MCF-7) and mutant p53 (parental BT474) cell lines. Our results show that DECMA-1 treatment significantly increased apoptosis, regardless of p53 mutation status (Fig. 3D).
Ectodomain-specific E-cadherin mAb down-regulates HER signaling pathways and IAP family members

Since the HER family of receptors are integral to the growth and progression of mammary cancers, we next used the MMTV-PyMT breast cancer mouse model and the E-cadherin/HER2-positive MCF-7 cell line to assess the effects of DECMA-1 on HER receptor regulation, as well as effects on other downstream cancer cell resistance pathways, including PI3K/Akt/mTOR, MEK/ERK and the inhibitor of apoptosis proteins (IAPs). Firstly, to confirm that DECMA-1 co-localizes with HER2, the preferred dimerization partner for the HER family of receptors, we performed double-label immunofluorescence staining on MMTV-DECMA-1 treated tumor specimens. Punctate HER2 immunostaining was visible in the cytoplasm of cells and co-localized with DECMA-1, suggesting interaction and internalization (Fig. 4A). Immunohistochemical and western blot analyses of HER1 and HER2 expression levels in resected tumors, exhibited decreased HER1/2 immunostaining and decreased HER1/2 levels compared to control or IgG treated mice (Fig. 4B). Moreover, in DECMA-1-treated mice, resected tumors exhibited a decrease in ERK1/2, Akt and mTOR immunostaining and a decrease in the expression levels of pMEK1/2, pPI3K and p4E-BP1, but not p70S6K (Fig. 4C and D). As HER receptor signaling involves IAP family members (11, 12), we next evaluated the expression level of the IAP family member survivin in the resected tumors by immunohistochemistry and western blotting. In saline and IgG treated mice, survivin immunostaining was predominately nuclear whereas in DECMA-1 treated mice a clear reduction in survivin immunoreactivity was noted (Fig. 4C) and subsequently confirmed by immunoblotting (Fig. 4D). Consistent with the in vivo findings, immunoblotting of membrane fractions of DECMA-1 treated MCF-7 cells revealed a marked reduction in HER1-4, pERK1/2, pAkt, pRaptor, pmTOR and its substrate p4E-BP1, while pp70S6K and pPI3K levels remained essentially unchanged (Fig. 4E). Moreover, analysis of IAP family members using a Human Apoptosis Array kit demonstrated decreased expression levels of several IAPs, including XIAP, survivin and c-IAP-1 following DECMA-1 treatment (Fig. 4F). Altogether, these data show that a
monoclonal antibody against the ectodomain of E-cadherin, effectively down-regulates key survival and cancer cell resistance pathways in cells that express E-cadherin and HER2 both in vitro and in vivo.

**Degradation of HER receptors by ectodomain-specific E-cadherin mAb DECMA-1**

As endocytic trafficking and ubiquitin-mediated degradation of cell surface receptors potentially provides an important mechanism of HER receptor regulation (29), we next assessed whether DECMA-1 decreased HER expression levels by inducing their internalization and degradation. HER1-4 receptor immunofluorescence in control MCF-7 cells demonstrated predominantly plasma membrane localization, whereas mAb treatment induced HER receptor down-regulation and aggregation into intracellular vesicular structures (Fig. 5A). In addition, using equal IP products for HER1 and HER2 (minimal protein was recovered from DECMA-1 treated cells), ubiquitination was dramatically increased in the cytosol and membrane fractions of treated cells (Fig. 5B). Subsequent experiments showed that DECMA-1 treatment induced tumor cell apoptosis by mediating HER receptor degradation. Chloroquine, a lysosomal inhibitor, prevented DECMA-1-induced apoptosis at low doses and rescued HER1-4 receptor membrane expression (Fig. 5C-D). In addition, the effect of DECMA-1 on apoptosis was inhibited in the presence of the proteasome inhibitor ALLN and was accompanied by the rescue of membrane HER1-4 expression (Fig. 5C-D). Thus, the lysosome and ubiquitin-proteasome pathways play an important role in the ectodomain-specific E-cadherin mAb–induced HER receptor degradation and apoptosis in E-cadherin/HER2-positive breast cancer cells.

**DECMA-1 binds to human and mouse mammary tumors but does not exert toxicity to normal tissues and cells**

Because skin toxicity is a common drug-related adverse event observed in cancer patients treated with HER-directed monoclonal antibody therapies (30), we next evaluated the binding specificity of different EC-domain-specific antibodies to intact E-cadherin or the shed E-cadherin ectodomain, on
normal human skin specimens lysed in PBS under non-reduced and reduced conditions by western blotting. In normal human skin tissues, lysed in PBS under either reduced or non-reduced conditions, DECMA-1 predominately bound to sEcad with minimal binding to FL-Ecad (Fig. 6A). In contrast, an antibody against the C-terminal region of E-cadherin mainly recognized FL-Ecad, whereas SHE78-7 and HECD-1 which were generated against EC1 and EC2, respectively, bound both FL-Ecad and sEcad by immunoblotting (Fig. 6A). To determine whether DECMA-1 binds intact E-cadherin and/or the shed soluble ~80 kDa ectodomain fragment in tumors, we next evaluated binding levels of DECMA-1 in human and mouse breast cancer specimens lysed in PBS by immunoblotting. In both human and mouse specimens, DECMA-1 bound both intact E-cadherin (120 kDa) and the soluble shed fragment (80kDa), albeit at much higher levels in the latter (Fig. 6B and C). Furthermore, immunofluorescence microscopy of Triton-X-treated tissue sections demonstrated both membrane and cytosolic staining for DECMA-1, confirming the immunoblot results (Fig. 6B and C). In our in vitro studies, TUNEL analyses in both normal human breast cells (MCF-10A) and cancer cells (MCF-7) demonstrated an increase in apoptosis in breast cancer cells, but no change of cell death in normal mammary cells (Fig. 6D). Moreover, DECMA-1 treatment of confluent human normal mammary epithelial cells (E-cadherin-positive; MCF-10A; Mam), fibroblasts (E-cadherin-negative; Fib), primary human epidermal keratinocytes (E-cadherin-positive; PHK) and melanocytes (E-cadherin-positive; Mel) also exhibited no significant induction of apoptosis compared with IgG controls as assessed by a Cell Death ELISA Assay (Fig. 6E). Because rapidly dividing cells of the intestine have been shown to be significantly impacted in conditional E-cadherin knock-out models, and gastrointestinal side effects are common with HER-targeted therapies (31, 32), we next verified that the intestinal architecture is not altered upon prolonged DECMA-1 treatment. In accordance with these in vitro findings, DECMA-1 treated MMTV-PyMT mice exhibited no overt signs of toxicity (i.e. grooming, vocalization, hunched posture, or change in appetite or body weight) and no cytotoxicity was observed in excised intestine (Fig. 6F) or in heart, liver, lung and kidney sections by H&E analysis (Data not shown). Collectively, these data show that DECMA-1 binds predominately the ~80kDa ectodomain of E-cadherin in human
and mouse tumors and normal tissues, and produces no untoward toxic effects on normal cells, tissues or mice.
Discussion

Targeted monoclonal antibodies have become attractive therapeutic drug candidates due to their potential for tumor-specific targeting and low toxicity profiles. Accordingly, Trastuzumab, a recombinant humanized monoclonal antibody against the extracellular domain of HER2, was approved by the FDA for the treatment of patients with HER2 metastatic breast cancers. However, a large percentage of patients who are initially responsive to HER-targeted therapies experience tumor recurrence and become refractory to therapy (1, 2, 3). Extensive crosstalk between multiple HER receptors and downstream key survival-signaling pathways, have been suggested to contribute to this drug resistance (1, 2, 3). Accordingly, bi-specific antibodies that simultaneously neutralize HER2 and HER3, or combinations of targeted mAb that inhibit HER1 and HER2 alone or target these receptor tyrosine kinases in combination with downstream MAPK or PI3K/Akt/mTOR inhibitors are currently being actively pursued in clinical trials (33, 34).

The tumor microenvironment is enriched with factors that nurture the growth and survival of tumors, and as such, is an indispensable player in malignant growth. Contained within this functional space are abundant proteases that facilitate the proteolytic cleavage of membrane proteins into bioactive soluble ectodomain fragments, such as sEcad. The sEcad fragment is increased in the serum of cancer patients (35), and acts in a paracrine or autocrine manner to stimulate tumor cell growth and survival (18, 26). Accordingly, levels of sEcad in breast cancer patients correlates with tumor size, response to chemotherapy and predicts a shorter disease-free interval (22). Reports further show elevated sEcad expression levels in primary gastric and prostate tumors, skin cancers and metastatic foci (18, 36, 37). This distinct pro-oncogenic property of sEcad is further supported by studies in ovarian, skin, prostate and transformed MDCK cancer cell lines, whereby recombinant sEcad disrupted E-cadherin-mediated cell-cell adhesions and enhanced migration and invasion, whereas immunodepletion of sEcad from the conditioned media reversed these effects (18, 21, 23, 24). Although the mechanisms of action of sEcad are not well understood, both HER2 overexpression and enhanced sEcad predict for a poor clinical outcome in breast cancer, making it tempting to
speculate that the two interact. Along these lines, we and others have demonstrated that sEcad binds to HER family members to increase tumor growth and cell survival (18, 26). Specifically, we have shown that endogenous and exogenous sEcad binds to HER1, HER2 and IGF-1R in human skin SCC specimens and mouse SCC cell lines (18). Similarly, Najy and colleagues (2008) demonstrated endogenous sEcad binding preferentially to HER2 and HER3, enhancing HER2-HER3 heterodimerization, HER3 phosphorylation and activating downstream ERK1/2 in MCF-7 breast cancer cells (19). In normal Madin-Darby canine kidney (MDCK) cells, exogenous sEcad promoted cell survival via activation of HER1, PI3K, Akt and ERK1/2 signaling (26). Taken together, the abundance of sEcad in tumor tissues and bodily fluids, together with its pro-oncogenic properties, HER binding and correlation with disease-free survival and therapeutic response, makes sEcad a novel candidate protein for drug targeting.

In this study, we evaluated the feasibility and efficacy of targeting sEcad in HER2-positive breast cancers by using a commercially available monoclonal antibody against the ectodomain of E-cadherin, DECMA-1, which may equally bind cell surface bound intact E-cadherin, sEcad or potentially sEcad-HER interactions. Here, we show that DECMA-1 mAb therapy, significantly delayed tumor onset and tumor burden in MMTV-PyMT mice by retarding cancer cell growth and inducing apoptosis selectively in cancer cells without untoward cytotoxicity toward normal cells and tissues. These results were validated in cell culture studies, wherein DECMA-1 treatment in E-cadherin-HER2 positive MCF-7 and BT474 TtzmR cells inhibited breast cancer proliferation and induced apoptosis. These results are in line with prior publications showing that DECMA-1 significantly reduced cell numbers, inhibited proliferation and induced apoptosis in PAM212 SCCs (38, 39). Moreover, DECMA-1 was shown to increase apoptotic cell death in MCF-7 cells and decrease Bcl-2 gene transcription (40). Others reported a significant inhibition of oral SCC multicellular aggregate proliferation, induction of apoptosis and decreased Bcl-2 expression after administration of SHE78-7, a commercially available antibody against the EC1 domain of E-cadherin (41). Potentiation of TPA-induced apoptosis
by SHE78-7 was further documented in LNCaP prostate and SUM185 breast cancer cells (42) and intraperitoneal administration of SHE78-8 was shown to significantly prolong survival and prevent peritoneal bleeding (indicative of advanced disease) compared to controls in an HT29 colorectal xenograft model (43). Lastly, Tomlinson et al (2001) demonstrated that 5 day injections of HECD-1 (EC2 domain monoclonal antibody) resulted in a decrease in both the size and number of pulmonary lymphovascular emboli using a MARY-X human inflammatory breast carcinoma xenograft model (44).

Because the tumor suppressor p53 is well known to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance, we next sought to determine if these DECMA-1-induced effects were p53 mediated. Interestingly, in MMTV-PyMT tumors and HER2-positive MCF-7 cells (wild-type p53), DECMA-1 treatment significantly increased wild type p53 expression, but in BT474 Tzmr cells decreased mutant p53 levels. Importantly, one of the gain-of-function effects of mutant p53 is apoptotic resistance to pro-apoptotic stimuli, including growth factors deprivation, γ-irradiation and anticancer agents (45). Accordingly, Mirzayans et al (2012) demonstrated that siRNA-mediated knockdown of mutant p53 in BT474 cells significantly increased cell death via apoptosis (46), which is consistent with our findings. Taken together, alteration of p53 expression is likely involved in DECMA-1-induced apoptosis, yet whether the latter is p53-dependent needs further study.

To gain a better understanding of the putative mechanisms by which DECMA-1 administration suppressed the development of mammary carcinomas, we next examined whether DECMA-1 modulated the HER family of receptors. Importantly, the most intriguing and clinically relevant finding of this study is the demonstration that DECMA-1 co-localized with HER2 in MMTV-PyMT mAb-treated tumors and down-regulated many of the compensatory pathways that Herceptin-treated cells eventually develop so as to sustain cell proliferation and enhance survival. That is, one of the most important strategies to improve the efficacy of HER-targeted therapies is to inhibit multiple HER receptors, thereby interfering with the horizontal cooperatively that exists between these receptors.
that currently limit the success of agents that target individual receptors. Specifically, simultaneous use of trastuzumab, pertuzumab and the HER tyrosine kinase inhibitor gefitinib inhibited HER2-overexpressing xenografts more effectively than any of these drugs used as a single agent or in dual combinations (47). Here, our mechanistic studies reveal a previously undiscovered pathway, wherein DECMA-1 mAb treatment down-regulated all HER1-4 family members, with the HER1 and HER2 family members being endocytosed and degraded by the ubiquitin-proteasome and lysosome pathways. However, downregulation of all HER family members may not be sufficient, since acquired drug resistance can still occur through reactivation of downstream pro-survival signaling pathways.

The PI3K/Akt/mTOR and MAPK pathways, along with IAP family members, are highly dysregulated in breast cancer (2), making them well-validated targets for cancer treatment. However, with extensive cross-talk and many feedback loops, results of single agent inhibitors have only had modest effects. This was demonstrated by Serra et al (2011) who showed that sole PI3K targeting in HER2-overexpressing breast cancers activated alternate compensatory pathways resulting in ERK dependency (48). Moreover, Carracedo et al (2008), demonstrated that inhibition of mTORC1, using rapamycin, resulted in MAPK activation through a PI3K feedback loop in prostate cancer (49). Here, we show that DECMA-1 mAb treatment suppressed many of the complex and redundant downstream pathways involved in Trastuzumab resistance. Specifically, membrane fractionation of mAb treated MCF-7 cells resulted in the downregulation of ERK1/2 as well as Akt, mTOR, the mTOR-binding protein Raptor and the mTOR substrate 4E-BP1. Consistent with the in vitro findings, PI3K/Akt/mTOR signaling was also significantly reduced in tumors from mAb treated MMTV-PyMT mice. Recent studies also demonstrate that IAPs are indispensable for survival of HER2-positive breast cancer cells. Xia et al (2006) showed that the HER1/HER2 inhibitor, lapatinib, markedly reduced survivin expression and induced apoptosis through its inhibition of PI3K signaling in HER2 overexpressing BT474 cells (12). Similarly, HER2-positive tumors from patients treated with lapatinib exhibited a marked inhibition of survivin expression levels (11, 12). In contrast, Trastuzumab had little effect on
steady-state survivin levels in Trastuzumab-sensitive BT474 cells, but in the HER2-positive
Trastuzumab-resistant SUM190PT cell line, it induced the upregulation of both survivin and XIAP (12,
50). In this study, we further show that DECMA-1 therapy down-regulated the inhibitor of apoptosis
protein family members survivin, XIAP and c-IAP-1, many of which are essential for tumor cell survival
and are also upregulated in Trastuzumab-resistant breast tumors (12).

Because targeted monoclonal antibody based therapies, are not without untoward side effects and
E-cadherin forms homotypic cell-cell adhesions in normal epithelial cells, we next evaluated DECMA-
1 binding in human and mouse breast tumor specimens versus potential off-target binding in normal
tissues. Importantly, Tsuiji et al (2007) demonstrated that the EC3-EC5 domains of E-cadherin are
much less accessible to monoclonal antibody binding than EC1-EC2, and suggest that this masking
may be due to the conformational state of cadherins or by other moieties (51). In the current study, we
show that DECMA-1 binds predominately to the ~80kDa ectodomain fragment in human breast tumor
specimens and both intact E-cadherin and the 80kDa protein in mouse MMTV-PyMT tumors.
However, in non-reduced and reduced normal human skin tissue lysates (homogenized in PBS),
DECMA-1 predominately bound the ectodomain fragment. This contrasted to the EC1 and EC2
antibodies, SHE78-7 and HECD-1, which bound both intact E-cadherin and the ectodomain fragment
versus the cytosolic E-cadherin antibody which only bound the former. It would be tempting to
speculate that the monomeric conformation of E-cadherin in tumor cells, versus the homodimeric
conformation of E-cadherin in normal cells, may potentially unmask the epitopes necessary for this
tumor-specific DECMA-1 binding. This lack of DECMA-1-induced cytotoxicity was further confirmed in
an array of normal human cells. Moreover, in MMTV-PyMT mice there were no apparent signs of
distress and histopathologic analysis of end-organs, including heart, liver, kidney and intestine
showed no morphologic differences between treated mice and healthy controls.

Breast cancer is a heterogeneous disease, wherein multiple resistance mechanisms may coexist
in each individual patient with late-stage metastatic disease. Therefore, we propose that in addition to
targeting each of the HER family members and/or MAPK, PI3K/Akt/mTOR or IAP pathway alterations
individually or in combination, researchers may effectively overcome resistance by further administration of a targeted monoclonal antibody against the ectodomain of E-cadherin. Our findings suggest that this monoclonal antibody regime, together with existing therapies, may have a more significant impact in delaying or preventing further progression, resistance and impending metastatic progression for patients with E-cadherin-positive yet invasive HER2-positive breast cancers without untoward cytotoxicity to normal tissues and cells.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial or other interests.

Acknowledgements

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References


Figure Legends

**Figure 1. Inhibition of tumor growth and induction of apoptosis in DECMA-1 treated MMTV-PyMT mice.** (A), Female MMTV-PyMT mice were treated weekly with saline (Sal), IgG or DECMA-1 (mAb) (1mg/kg) intraperitoneally (i.p.), starting at 47 days of age until sacrifice at 90 days. On the indicated day after treatment, tumor multiplicity (the number of mammary glands bearing palpable tumors) was calculated and analyzed by ANOVA with post-hoc analysis using Bonferroni t-test (**p<0.01, ***p<0.001, n=5). Insert: Photographs of saline, IgG and DECMA-1 (mAb) treated mice. (B), Tumor weights and histopathology of H&E stained paraffin sections in saline, IgG and mAb treated mice at 90 days of age (n=5 per group). (C), BrdU quantification and immunohistochemical staining in control and treated MMTV-PyMT tumors. (n=3). (D), Quantification and immunohistochemical images of TUNEL-positive cells (brown) from resected mammary gland tumor sections of saline, IgG versus DECMA-1 (mAb) treated mice (n=3). Data are presented as Mean +/- SEM. **p<0.01, ***p<0.001 vs. Sal, IgG from triplicate cultures. Scale Bar = 250μm.

**Figure 2. Inhibition of proliferation and activation of apoptosis in DECMA-1 treated Trastuzumab-sensitive and -resistant cells.** (A), MCF-7 or BT474-TtzmR cells were treated with or without 20 or 400 μg/mL of IgG or DECMA-1 (mAb), and 48 h later cells were harvested and viable cells counted and analyzed using a haemocytometer. (B), BrdU incorporation in MCF-7 and BT474-TtzmR cells 48h after treatment with or without 20 or 400μg/mL of mAb, as assessed by the cell proliferation ELISA BrdU (colorimetric) assay. (C), Phase contrast photomicrographs of MCF-7 and BT474-TtzmR cells in the presence or absence of DECMA-1 (mAb) or IgG isotype control. (D), Apoptosis and Necrosis-specific ELISA of MCF-7 and BT474-TtzmR cells cultured in the presence or absence of 20 or 400μg/mL of DECMA-1 (mAb) or IgG for 48 h. Data are presented as Mean +/- SEM. ***p<0.001 vs. Sal, IgG from triplicate cultures. Scale Bar = 200μm.

**Figure 3. Upregulation of wild type p53 and downregulation of mutant p53 after DECMA-1 treatment.** (A), Immunohistochemical staining and western blot analysis of p53 expression levels in
tumors from saline, IgG or DECMA-1 (mAb) treated MMTV-PyMT mice. (B), Western blot analyses of p53 expression in MCF-7 and BT474-TtzmR cells treated with or without 20 or 400 μg/mL of IgG or DECMA-1 (mAb) for 48 h. Protein levels were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer’s instructions. A hundred micrograms of protein were loaded on each lane. G3PDH or β-actin was used to confirm equal loading. (C), Immunofluorescence staining of p53 in MCF-7 cells treated with or without 20μg/mL IgG or DECMA-1 (mAb) for 48 h. (D), Apoptosis specific ELISA of wild type p53 (MCF-7) and mutant p53 (BT474 parental) cells treated with or without 20 or 400 μg/mL of DECMA-1 (mAb) or IgG for 48 h. Data are presented as Mean +/- SEM. ***p<0.001 vs. C, or IgG from triplicate cultures. Scale Bar = 200μm.

**Figure 4.** DECMA-1 down-regulates the HER signaling axis and IAP family members. (A), Immunofluorescent staining of DECMA-1 (green) and HER2 (red) showing co-localization in tumors from DECMA-1 (mAb) treated MMTV-PyMT mice. (B), Immunohistochemical staining and western blot analyses of HER1 and HER2 expression levels in tumors from control, IgG and DECMA-1 (mAb) treated MMTV-PyMT mice. HER expression levels normalized to β-actin were analyzed by NIH Scion Image. n=3. Data are presented as Mean +/- SEM. *p<0.05 vs. corresponding saline or IgG control. (C), Immunohistochemical staining of ERK1/2, Akt, mTOR and survivin in tumors from control, IgG and DECMA-1 (mAb) treated MMTV-PyMT mice. (D), Immunoblotting demonstrating activation of MEK1/2, PI3K, p70S6K, 4E-BP1 and survivin in tumor lysates from control, IgG and DECMA-1 (mAb) treated MMTV-PyMT mice. (E), Control, IgG and DECMA-1 (mAb) treated MCF-7 cells were subjected to subcellular fractionation followed by immunoblotting with HER1-4 and phospho- MAPK and PI3K/Akt/mTOR specific antibodies. Protein levels (100μg/lane) were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer’s instructions. Na+/K+ ATPase and β-actin were used to confirm equal loading in membrane fractions and total lysates, respectively. (F), Apoptosis array demonstrating expression of XIAP, survivin and c-IAP-1 in control and DECMA-1 (mAb) treated MCF-7 cells. Scale bar = 200μm.
Figure 5. HER degradation induced by DECMA-1 treatment is dependent on the proteasome, lysosome and ubiquitin pathways. (A), Immunofluorescence images showing HER1-4 staining in MCF-7 cells in the presence or absence of DECMA-1 (mAb). The arrows indicate staining of HER1-4 in vesicular structures. (B), Membrane and cytosolic HER1 and HER2 fractions in the presence or absence of DECMA-1 (mAb) or IgG (20μg/mL) were IP and then subjected to IB with anti-ubiquitin or HER1, 2 specific antibodies. Since HER1 and HER2 were significantly degraded at 48h after mAb treatment, double amounts of IP products from DECMA-1 mAb treated membrane or cytosolic samples were applied onto the SDS-PAGE in order to visualize the IP bands. (C), Analysis of apoptosis in MCF-7 cells treated with DECMA-1 (mAb) (20μg/mL) in the presence or absence of 10µM chloroquine (CQ) or 2.5µM of acetyl-leu-leu-norleu-al (ALLN). ***P<0.001 vs no mAb, CQ, or ALLN group. ##P<0.01 vs. mAb alone group, n=3. (D) 10µM chloroquine (CQ) or 2.5µM ALLN rescue HER1-4 expression levels following mAb treatment. Scale bar = 200μm.

Figure 6. DECMA-1 exhibits no cytotoxic effects on normal cells or tissues. (A), Western blot analysis demonstrating binding of SHE78-7 (anti-EC1 mAb), HECD-1 (anti-EC2 mAb), c-ter (anti-cytoplasmic domain mAb) and DECMA-1 (anti-membrane proximal region of the E-cadherin ectodomain) to normal human skin tissues (homogenized in PBS) under reduced or non-reduced conditions. (B), Western blotting and immunofluorescence staining demonstrating binding of DECMA-1 in human breast tumor specimens. Scale bar= 100µm. (C), Western blotting and immunofluorescence staining of DECMA-1 binding in MMTV-PyMT mouse tumor tissues. Scale bar= 100µm. Tissues from human and MMTV-PyMT mouse breast tumors were homogenized in PBS and protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer’s instructions. Ten micrograms of non-reduced protein per lane were loaded onto a SDS-PAGE and blots were probed with 5 µg/mL of DECMA-1 (Sigma). (D), Representative images of TUNEL (green) staining of normal human mammary MCF-10A and human breast cancer MCF-7 cells treated with control, IgG, or DECMA-1(mAb) (20μg/mL) for 48h. Nuclear
were labeled with Hoechst (Blue) counterstain. Scale bar= 250μm. (E), Cell Death Detection ELISA assay in MCF-10A (Mam), human WI-38 fibroblasts (Fib) primary human keratinocytes (PHK) and primary human melanocytes (Mel) treated with 20μg/mL of DECMA-1 (mAb) or control IgG. (F), Representative H&E stained intestine sections from 90 day old DECMA-1 mAb treated MMTV-PyMT mice. Scale bar = 500μm.
Figure 1

A. Number of tumors per mouse over age (days) for Sal, IgG, and mAb treatments.

B. Tumor weight comparison between Sal, IgG, and mAb treatments.

C. BrdU positive area comparison between Sal, IgG, and mAb treatments.

D. Apoptag positive area comparison between Sal, IgG, and mAb treatments.
**A**

**MMTV-PyMT MOUSE TUMORS**

![Images showing Sal, IgG, and mAb treatments for MMTV-PyMT mouse tumors.](image)

**B**

**HUMAN BREAST CANCER CELLS**

![Images showing Wt p53 and Mutant p53 in MCF-7 cells with IgG and mAb treatments.](image)

**C**

**MCF-7**

![Images showing Control, IgG, and mAb treatments for MCF-7 cells.](image)

**D**

![Bar chart showing Apoptosis fold in Wt p53 and p53 mutant with IgG and mAb treatments.](image)
Figure 4

A. DECMA-1 and HER2 images.

B. Control, HER1, HER2, and β-actin images.

C. Sal, IgG, and mAb images for ERK1/2, Akt, mTOR, and survivin.

D. Sal, IgG, and mAb images for pMEK1/2, pPI3K, pp70S6K, p4E-BP1, survivin, and β-actin.

E. C, IgG, and mAb images for HER1, HER2, HER3, HER4, Na⁺/K⁺ ATPase, pERK1/2, pPI3K, pAkt, pmTOR, pRaptor, p4E-BP1, pp70S6K, Na⁺/K⁺ ATPase, XIAP, survivin, and c-IAP-1.
Figure 5
Figure 6

A. Normal human skin epidermis

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FL Ecad (120 kDa) and sEcad (~80 kDa)

B. Human breast tumors

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sEcad (~80 kDa)     |        |

C. Mouse breast tumors

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sEcad (~80 kDa)     |        |

D. Human mammary cells

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D. Human breast CA cells

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E. Human cells

![Graph showing apoptosis fold change between IgG and mAb](image)

F. Intestine: mAb

![Intestine section with mAb](image)
Monoclonal antibody against the ectodomain of E-cadherin (DECMA-1) suppresses breast carcinogenesis: Involvement of the HER/PI3K/Akt/mTOR and IAP pathways

Sabine M. Brouxhon, Stephanos Kyrkanides, Xiaofei Teng, et al.

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