Combination trastuzumab metronomic low-dose chemotherapy for the treatment of erbB2 overexpressing breast cancer

by

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Abstract

The purpose of these studies was to evaluate the preclinical anti-tumor and antiangiogenic effects of long-term metronomic chemotherapy in combination with trastuzumab relative to standard MTD regimens. Using an orthotopic mammary tumor model with an erbB2 overexpressing variant of MDA-MB-231, SCID mice treated with metronomic or MTD cyclophosphamide (CTX) in combination with trastuzumab showed long-term tumor growth delay compared to single agent therapy. The superior benefit of metronomic dosing was indicated by an absence of severe toxicity. Tumor perfusion and Matrigel assays indicated an additive antiangiogenic effect of metronomic CTX and trastuzumab. Acquired resistance observed after prolonged treatment with trastuzumab alone or combined with CTX was observed and led to studies of possible mechanisms of resistance. Of interest was the upregulation of VEGF and the detection of stable *in vivo/in vitro* acquired resistance to trastuzumab. Resistant tumors responded to subsequent treatment with bevacizumab, cetuximab, A12 and in some circumstances, pertuzumab.

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I would like to thank all of those who have been inspirational and influential before and during, and hopefully after my graduate studies.

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ADCC	antibody-dependent cellular cytotoxicity
ATCC	American Type Culture Collection
bFGF	basic fibroblast growth factor
CEP	circulating endothelial progenitor cell
CDK	cyclin-dependent kinase
CTX	cyclophosphamide
DMEM	Dulbecco's modified eagle's medium
EC	endothelial cell
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Grb	growth factor receptor-bound protein
HER	human EGFR-like receptor
HIF1-α	hypoxia inducible factor-1 alpha
IC_{50}	inhibitory concentration of 50% of cells
IGF-1R	insulin-like growth factor receptor-1
IgG	immunoglobulin class G
IP	intraperitoneal
KIP	kinase inhibitory protein

MAPK	mitogen-activated protein kinase
MTD	maximum tolerated dose
NF-κB	nuclear factor kappa B
PBS	phosphate buffered saline
PE	phycoerythrin
PI3K	phosphatidyl-inositol 3 kinase
PLCγ	phospholipase C γ
PTB	phosphotyrosine binding
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
SCID	severe combined immunodeficieny
SHC	SH2-containing collagen-related proteins
STAT	signal transducers and activators of transcription
TBST	tris-buffered saline with tween 20
TGFα	transforming growth factor alpha
TKI	tyrosine kinase inhibitor
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
4-HC	4-hydroperoxycyclophosphamide

1 Introduction

1.1 erbB2 and breast cancer

ErbB2 (Her2/neu) was first identified for its oncogenic potential when a mutated form of the receptor was identified in carcinogen-induced rat neuroblastomas (Schechter et al, 1984, 1985). This same receptor tyrosine kinase was identified in a number of human tumors, including breast (Slamon et al, 1987), ovarian (Slamon et al, 1989) and gastric cancers (Kury et al, 1990), yet was found to be overexpressed as a consequence of gene amplification in a non-mutated form, especially in breast cancer. Since its initial discovery considerable preclinical and clinical information has accumulated on the role of this receptor in normal and malignant cellular processes, its prognostic value, and the benefits of therapeutic intervention with erbB2-directed therapies, particularly with neutralizing antibodies.

ErbB2 is a member of the type I transmembrane tyrosine kinase family of receptors, which also contains the epidermal growth factor receptor (EGFR) also called erbB1, erbB3 and erbB4. These four receptors have a similar overall structure, with an extracellular ligand-binding domain containing two cysteine-rich regions, a transmembrane region, and an intracellular kinase domain flanked by a carboxy-terminus with tyrosine autophosphorylation sites. Three distinct classes of ligands, all containing an EGF-like domain, are responsible for binding to these receptors, and inducing the formation of 10 different dimers, 4 homodimers (3 of which are functional) and 6 heterodimers (Figure 1.1) (Jones et al, 1999). The EGF-like ligands, including EGF, transforming growth factor α (TGF-α) and amphiregulin, bind to EGFR, heregulins bind exclusively to erbB3 and erbB4, while the more promiscuous ligands heparin binding EGF, betacellulin and epiregulin bind to either EGFR, erbB3 and erbB4. A

ligand for erbB2 has yet to be identified and likely does not exist, so that it is referred to as an orphan receptor; it apparently relies on dimerization with other erbB receptors or homodimerization in situations of overexpression to initiate downstream signaling (Klapper et al, 1999). The erbB3 protein is lacking a functional tyrosine kinase domain, and therefore relies on the formation of heterodimers to activate signaling (Guy et al, 1994).

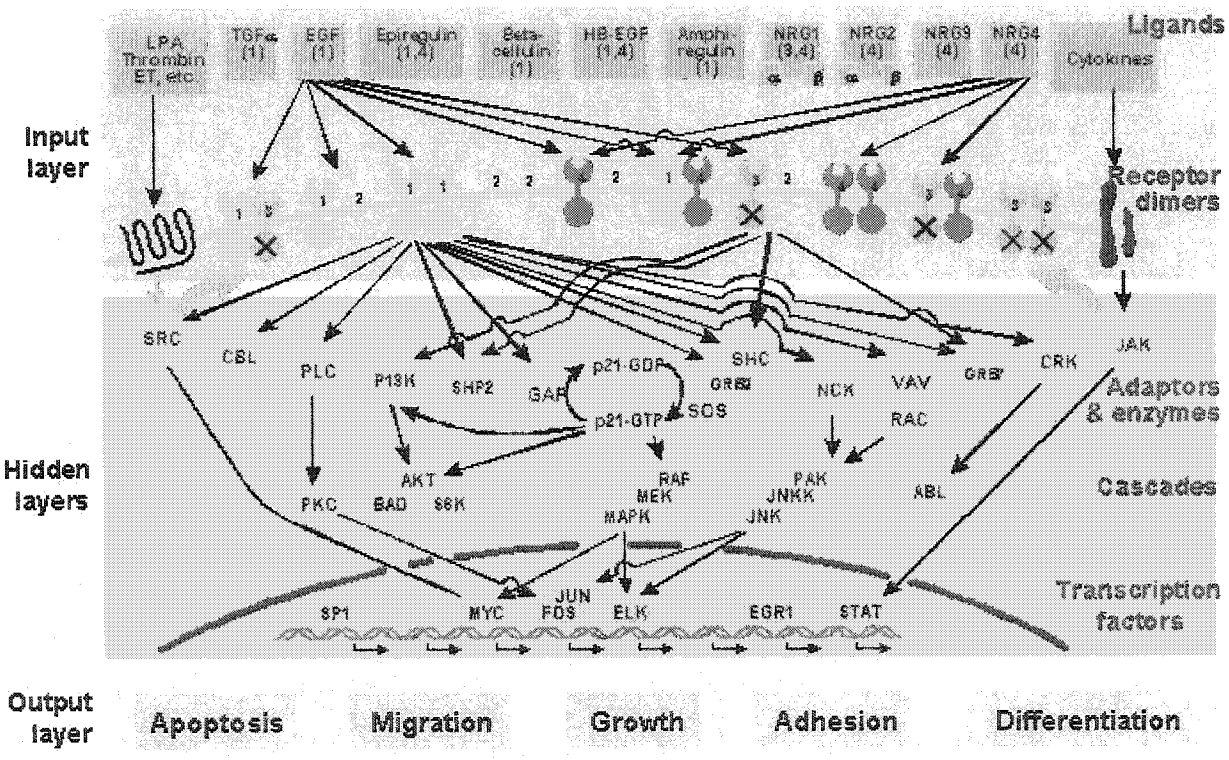

Figure 1.1: Complexity of the erbB signalling network. Input layer) Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. **Hidden layers)** Signaling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1 homodi mer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **Output layer)** The cellular processes that are controlled by transcriptional regulation of genes in the erbB network. Reproduced from Yarden & Sliwkowski, 2001.

In the case of erbB2 overexpression, the tumorigenic action was found to be associated with high levels of basal phosphorylation of the receptor (Bargmann & Weinberg, 1988). ErbB2 was determined to be capable of spontaneous, ligand-independent homodimerization when overexpressed (Weiner et al, 1989). Recent elucidation of the crystal structure of the erbB2 receptor extracellular domain has identified a long, finger-like projection that is permanently fixed in the open conformation (Cho et al, 2003; Garrett et al, 2003). In contrast, the EGFR and erbB3 receptors require ligand binding to convert the receptors from the closed to open conformation, whereby the finger-like domain folds down, which subsequently allows for dimerization of the receptors (Cho & Leahy, 2002; Garrett et al, 2002; Ogiso et al, 2002). In addition, erbB2 was found to be the preferred heterodimerization partner of both EGFR and erbB3 and when erbB2 is co-expressed with either of these two receptors, has a much more potent transforming potential (Kokai et al, 1989; Wallasch et al, 1995). To further intensify the transforming ability of erbB2, it was reported that erbB2 homodimers and erbB2-containing heterodimers had increased stability compared to other dimers, due to the enhanced affinity of ligands to erbB2 heterodimers, a decreased rate of endocytosis, an increased rate of receptor recycling to the cell membrane and a reduction in lysosomal targeting (Baulida et al, 1996; Worthylake et al, 1997; Waterman et al, 1998).

The differential activation of downstream signaling from the erbB receptors is regulated by tyrosine phosphorylation sites located on the c-terminal domain of the receptors which recruit effector molecules containing either Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Muthuswamy et al, 1999) (Figure 1.2). Which tyrosines become phosphorylated is determined by the ligand that has bound to the receptor and the dimerization partner of the receptor (Olayioye et al, 1998). The primary signaling pathway mediated by erbB2 is the mitogen-activated protein kinase (MAPK) pathway. Recruitment of adaptor proteins, such as grb2, phospholipase C γ (PLCγ) and

shc, are responsible for activation of the Ras-Raf-MAPK cascade. This eventually leads to transcriptional upregulation of the early response genes, such as c-fos, c-myc and c-jun, which are involved in promoting cellular proliferation. Dysregulation of the G1-S cell cycle checkpoint occurs due to an erbB2-mediated increase in the expression of cyclin D1 and modulation of $p27^{KIP1}$ and $p21^{Waf1}$ levels, potentiating the rate of proliferation of erbB2 overexpressing cancer cells (Lee et al, 2000). In addition, erbB2 is responsible for activation of the phosphatidylinositol 3'-kinase (PI3K) through binding of the regulatory subunit p85 to phosphorylation sites on erbB3, which plays a key role in regulating cell survival. The PI3K pathway, as well as the MAPK and nuclear factor-κB (NF-κB) pathways, all of which are in part regulated through erbB2, have been implicated in the upregulation of anti-apoptotic proteins bcl-2 and bcl-x_L, as well as the inhibitors of apoptosis which block caspase activation to prevent the induction of apoptosis of the cancer cells (Lane et al, 2000; Neve et al, 2000; Zhou et al, 2001). The NF-κB pathway has recently been implicated in both cell proliferation and apoptosis of breast cancer cells (Biswas et al, 2004), and constitutive activation of NF-κB has been correlated with expression of erbB2 in human breast tumor specimens (Hou et al, 2003).

Through the analysis of genetically modified mice, the erbB receptors have been shown to be essential for embryonic and perinatal development of a variety of organs, including the heart, nervous system, lungs, brain, skin and gastrointestinal tract (Gassman et al, 1995; Lee et al, 1995; Miettinen et al, 1995; Reithmacher et al, 1995; Sibilia & Wagner, 1995; Erickson et al, 1997; Sibilia et al, 1998; Morris et al, 1999). These receptors are also essential at times of puberty, pregnancy and parturition, when mammary development continues with ductal formation and milk production (Schroeder & Lee, 1998; Darcy et al, 2000). The importance of erbB receptors, particularly erbB2, in mammary tumor progression has also been shown using transgenic mouse models,

where the expression of oncogenic neu is sufficient to induce mammary adenocarcinomas (Muller et al, 1988; Siegel et al, 2000). The dependence of tumors on erbB2 was confirmed when studies were carried out where expression of erbB2 was controlled via a tetracycline-regulated mouse tumor model. In an absence of tetracycline, where the expression of erbB2 is switched 'off', there was an activation of apoptosis in the tumor cells and subsequent tumor remission (Schiffer et al, 2003). It has also been found that neu overexpressing transgenic mice exhibit co-overexpression of EGFR (DiGiovanna et al, 1998) and erbB3 (Siegel et al, 1999), suggesting that the oncogenic properties of erbB2 are dependent on or enhanced by the expression of other erbB receptors.

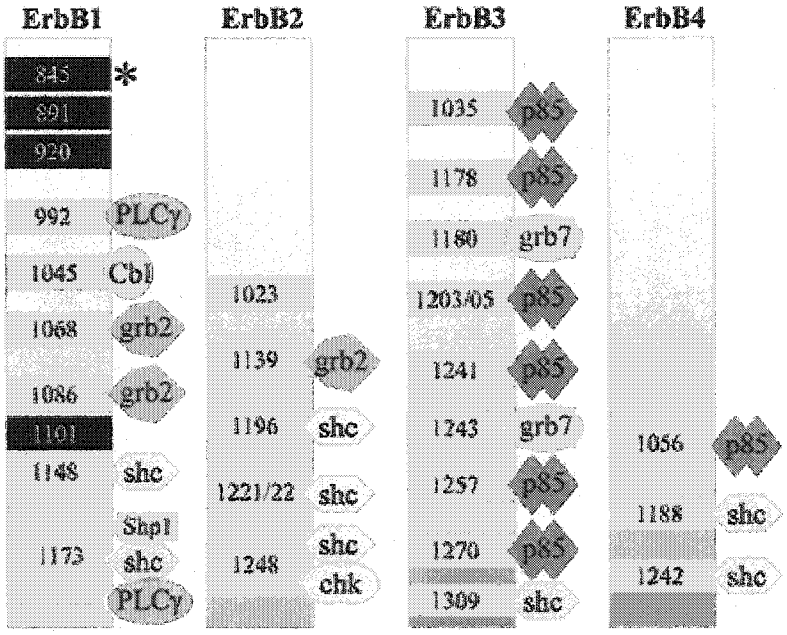

Figure 1.2: Specific phosphotyrosine residues and binding of signaling molecules to the carboxy terminus of the erbB receptors. Reproduced from Olayioye et al, 2000.

Overexpression of erbB2 can be detected in 20-30% of breast cancer patients due to genomic amplification of the erbB2 proto-oncogene, and is associated with a particularly poor clinical outcome compared to patients with erbB2 negative tumors

(Slamon et al, 1987). At least two theories have been proposed to explain why erbB2 is such a poor prognostic indicator. First, erbB2 upregulates cell signaling pathways involved in the expression and secretion of invasion-promoting matrix metalloproteases (Tan et al, 1997), expression of angiogenic factors (Viloria-Petit et al, 1997), and increased cell survival (Yu et al, 1998), all of which are essential properties for the successful formation of metastases. Indeed, erbB2 overexpression has been found to correlate with the extent of lymph node metastases found in breast cancer patients (Slamon et al, 1987; 1989). Second, extensive studies have shown that overexpression of erbB2 confers resistance to a number of therapeutic interventions used to treat breast malignancies, including some chemotherapeutic agents (particularly taxol-based therapies) (Yu et al, 1996; 1998), hormonal therapy (Leitzel et al, 1995), radiation (Burke et al, 1998; Pirollo et al, 1997), and cytokines such as tumor necrosis factor (Zhou et al, 2000). Therefore, erbB2 positive tumors have an enhanced ability to evade cell death induced by anticancer therapies and a higher potential to metastasize to distant sites (Figure 1.3).

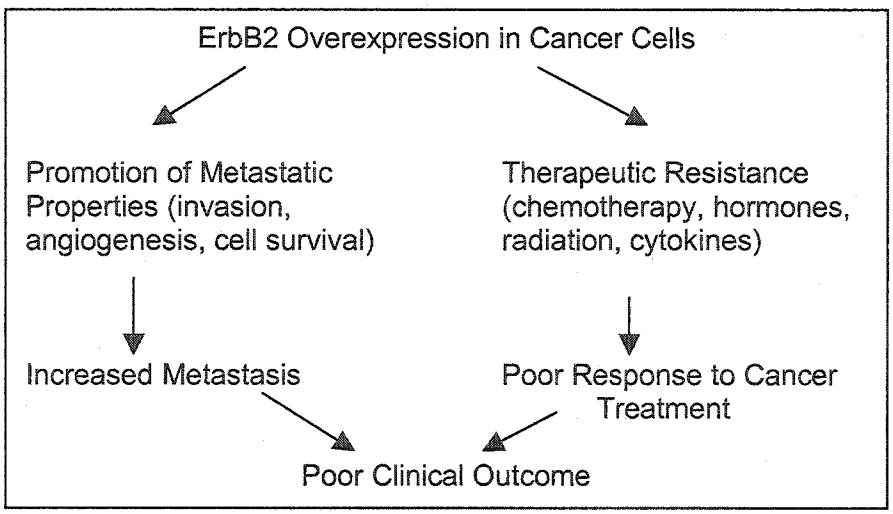

Figure 1.3: Two major detrimental consequences of erbB2 overexpression in cancer cells, which can lead to poor clinical prognosis for patients. Modified from Yu & Hung, 2000.

1.2 Mechanisms of action of trastuzumab

Shortly after the discovery of erbB2, antibodies raised against the receptor were shown to down modulate the oncoprotein, and subsequently revert the malignant cell into an untransformed, normal state as determined by anchorage-independent growth properties (Drebin et al, 1985). This work not only confirmed the oncogenic potential of erbB2 overexpression, but also identified a potentially useful, clinically relevant means to target this receptor in human tumors. The development of a murine monoclonal antibody, 4D5, directed to the extracellular domain of erbB2 (Hudziak et al, 1989), was rapidly followed by the humanization of this antibody, which is referred to as trastuzumab or by its trade name Herceptin® (Carter et al, 1992) (Genentech Inc., South San Francisco, CA). Since their development, the basis for the cytostatic effects of 4D5 and trastuzumab have been partly resolved, and a number of additional effects of targeting erbB2 with such antibody therapies have also recently been discovered.

Unlike small molecule tyrosine kinase inhibitors (TKIs), which intracellularly suppress phosphorylation and activation of receptor tyrosine kinases, trastuzumab acts at the cell surface by promoting dimerization and autophosphorylation of the erbB2 receptors. This phosphorylation triggers uptake of the receptors by endocytosis and the recruitment of the ubiquitin-ligase c-Cbl. Polyubiquitination of the erbB2 receptor induces proteosome-dependent degradation of the receptor (Klapper et al, 2000). By directing the erbB2 receptors for degradation, rather than recycling back to the cell surface, there is a downregulation in the number of erbB2 receptors and a subsequent decrease in erbB2-driven cell signaling activity.

While erbB2 is known to dysregulate the G_1-S cell cycle checkpoint, trastuzumab appears to reinstate the necessary machinery to induce growth arrest. An increase in the percentage of cells in G_0/G_1 phase and reduction of cells in S phase has been

observed in erbB2 positive breast cancer cell lines treated with trastuzumab (Sliwkowski et al, 1999). A trastuzumab-induced upregulation of p27, a cyclin-dependent kinase (cdk) inhibitory protein (kip), leads to the increased formation of $p27^{Kip1}$/cdk2 complexes, thereby preventing the cyclin-dependent activation of cdk2, a necessary event for G_1-S phase transition. By preventing progression of the erbB2 positive cancer cells through the cell cycle, trastuzumab acts as a cytostatic agent.

The humanization of the monoclonal antibody 4D5 was carried out by one-step gene conversion mutagenesis, such that the humanized antibody trastuzumab contains human variable region and IgG_1 constant domains and only the antigen binding loops from the parental mouse 4D5 monoclonal antibody (Carter et al, 1992). Trastuzumab binding to the tumor cell surface leads to recognition by the innate immune cells in the body, due to the presence of the IgG_1 F_c domain. $F_c\gamma$ receptors, located on effector cells such as natural killer cells, macrophages and monocytes bind to the F_c portion of trastuzumab and release perforin and granzymes to kill the opsonized tumor cell (Steplewski et al, 1983). The importance of antibody-dependent cellular cytotoxicity (ADCC) for the anti-tumor activity of trastuzumab was illustrated in $F_c\gamma$ receptor deficient mice, which showed a partially impaired anti-tumor effect of trastuzumab compared to immune-competent mice (Clynes et al, 2000).

The identification of a soluble erbB2 protein in the serum of breast cancer patients has recently been studied for its clinical relevance with respect to the prediction of treatment response and prognosis (Fehm et al, 2002; Köstler et al, 2004). The consequences of soluble erbB2 are at least two-fold; the soluble form of the receptor is capable of binding to trastuzumab in the blood of patients thus reducing the concentration of free drug available for targeting tumor cells, and the truncated erbB2 receptor, referred to as p95, which remains in the cell membrane after loss of the soluble

domain, is in a constitutively active state which leads to the ligand and heterodimerization independent activation of downstream signaling cascades (Segatto et al, 1988). Metalloproteases are responsible for the cleavage of the erbB2 ectodomain (Condony-Servat et al, 1999), and trastuzumab has been found to inhibit this cleavage (Molina at el, 2001). It is believed that the binding site of trastuzumab is on or in very close proximity to the cleavage site of the metalloprotease, such that the bulky structure of the antibody bound to the erbB2 receptor prevents the metalloprotease from gaining access to the receptor to cleave it.

The co-administration of trastuzumab with various chemotherapeutic agents, *in vitro*, *in vivo*, and in the clinic, appears to revert the intrinsic resistance of erbB2 overexpressing tumor cells to chemotherapy. Several mechanisms of trastuzumab have been elucidated to explain this effect. Trastuzumab-treated breast cancer cells were found to have significantly more DNA strand breaks than untreated tumor cells, suggesting that trastuzumab inhibits normal DNA repair mechanisms (Mayfield et al, 2001; Peitras et al, 1994). There is also evidence that trastuzumab induces apoptosis of erbB2 positive tumor cells, in a p53-independent manner (Brodowicz et al, 2001), which may be secondary to the lack of functioning DNA repair mechanisms. In addition, a decrease in Akt activity is frequently observed following treatment with trastuzumab, due primarily to reduced signaling through the PI3K pathway, allowing cells to more readily undergo apoptosis when exposed to cytotoxic agents (Clark et al, 2002; Klos et al, 2003).

Additional effects of trastuzumab on erbB2 positive cancer cells continue to be uncovered. For example, trastuzumab has been found to downregulate the expression of autocrine motility factor/phosphohexose isomerase, a cytokine involved in promoting migration and angiogenesis in tumors (Talukder et al, 2002). As well, trastuzumab may affect other cells in the tumor microenvironment. At high concentrations of trastuzumab,

there is preliminary evidence to suggest that it may inhibit human stromal cells from proliferating, adhering to tumor cells, and expressing certain growth factors such as vascular endothelial growth factor (VEGF) (Corsini et al, 2003).

1.3 erbB receptors and angiogenesis

The formation of new blood vessels, primarily through the proliferation of vascular endothelial cells (ECs), is referred to as angiogenesis. In order for a tumor to grow beyond a size of 1-2 mm^3, whereby nutrients and oxygen can no longer freely perfuse to the tumor cells, there is a requirement for sustained angiogenesis to take place (Folkman, 1995). Angiogenesis is regulated through a large number of pro- and antiangiogenic factors, and the way in which these factors co-operatively act to suppress or activate angiogenesis is referred to as the 'angiogenic switch' (Hanahan & Folkman, 1996). VEGF has been identified as an important player in the angiogenic process, as it is a potent mitogen and chemoattractant for vascular ECs (Dvorak et al, 1995) and has been detected in a variety of tumor types (Ferrara et al, 2003). Like many other oncogenes, overexpression of erbB2 or EGFR in human tumor cell lines has been associated with an increased expression of VEGF and hence an enhanced capacity for these tumors to promote angiogenic processes (Viloria-Petit et al, 1997).

The importance of angiogenic processes for the progression of erbB2 positive tumors was shown using transgenic mouse models coexpressing erbB2 and VEGF in the mammary fat pads (Oshima et al, 2004). Mice coexpressing these two proteins developed tumors which were highly vascularized and appeared in a much shorter time than mice expressing only erbB2. In addition, erbB2/VEGF co-overexpressing mice were found to have increased metastases to the lung compared to erbB2 overexpressing mice. The prognostic significance of VEGF expression has been extensively studied in human breast cancer, with controversial results. The most recent,

and largest study by Konecny et al (2004) determined that a larger cohort of patients with tumors overexpressing erbB2 had elevated levels of VEGF compared to erbB2 negative tumors (77.2% versus 54.5% for the $VEGF_{121-206}$ isoforms and 87.7% versus 71% for the $VEGF_{165-206}$ isoforms). In addition, the overexpression of both erbB2 and VEGF resulted in a decreased overall survival compared to patients with erbB2 positive, VEGF negative tumors (Figure 1.4). Conversely, a study which indicated an absence of increased VEGF expression in erbB2 positive tumors found erbB2 overexpression to positively correlate with microvessel density and inversely correlate with hypoxia of the tumor (Blackwell et al, 2004), which implies that while erbB2 overexpressing tumors have an enhanced ability to induce tumor angiogenesis, it may alternatively be through a mechanism other than VEGF upregulation.

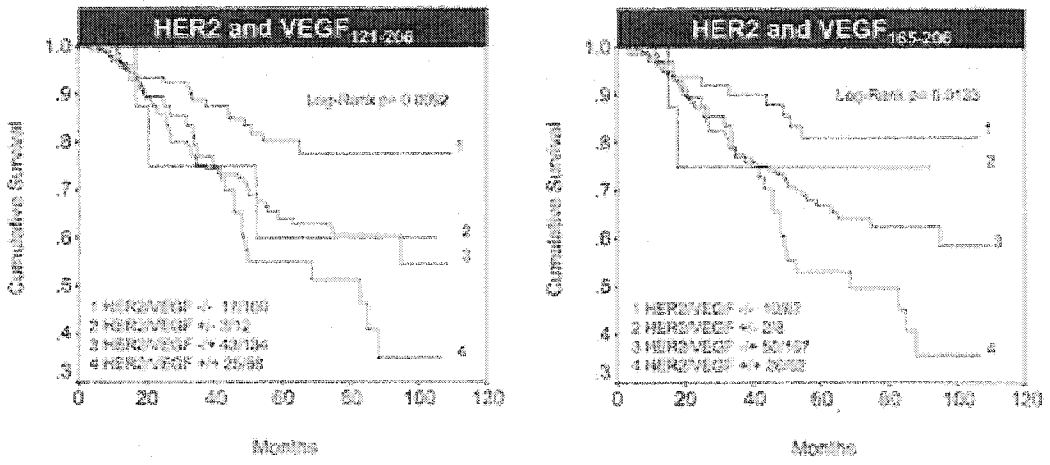

Figure 1.4: Kaplan-Meier survival plots of axillary lymph node-positive patients classified by erbB2 (HER2) and VEGF expression status (n = 304). Univariate analysis demonstrated statistically significant differences in survival between the four groups (log rank test P = 0.0092; *A*), with the poorest outcome being among patients with tumors demonstrating both erbB2 overexpression and VEGFexpression, and the most favorable outcome for patients whose tumors had both normal erbB2 expression and no detectable VEGF expression. Reproduced from Konecny et al (2004).

The mechanism by which erbB2 upregulates VEGF mRNA and protein expression is complex, due to the multiple signaling pathways that are mediated by erbB2. VEGF gene transcription is regulated in part by the transcription factor hypoxia-inducible factor (HIF-1α), which can be activated by hypoxia or genetic alterations that dysregulate or activate intracellular signal transduction pathways such as MAPK or PI3K (Ravi et al, 2000; Zundel et al, 2000; Laughner et al, 2001). Under normoxic conditions, HIF-1α is rapidly degraded via the ubiquitin pathway (Huang et al, 1998), but under hypoxic conditions, HIF-1α mRNA is stabilized such that HIF-1α protein expression is increased (Laughner et al, 2001). In contrast to hypoxic conditions, ErbB2 induces HIF-1α protein synthesis at the transcription level, primarily through activation of the PI3-kinase pathway (Laughner et al, 2001) (Figure 1.5).

The interaction of erbB receptors has been found to be of particular importance for VEGF expression. Studies by Yen et al (2002) analyzed the capacity of all homo- and heterodimeric complexes of erbB receptors to induce VEGF expression in an NIH3T3 fibroblast transduction system. EGFR/erbB2 and erbB2/erbB3 combinations were found to be the most potent activators of VEGF mRNA expression, when stimulated with EGF and heregulin respectively, and tumor xenografts of these cell lines had increased vascularity. Further studies are required to determine the role of erbB2 overexpression and erbB heterodimers in the expression of other pro- and antiangiogenic factors.

There is evidence to suggest that the 'angiogenic switch' associated with erbB2 overexpression can be attenuated by trastuzumab. Viloria-Petit et al (1997) were the first to implicate erbB2 (and trastuzumab) as regulators of tumor angiogenesis by showing a dose-dependent decrease in VEGF expression upon treatment of the erbB2 positive breast tumor cell line, SK-BR3, with the antibody in an *in vitro* system, which has

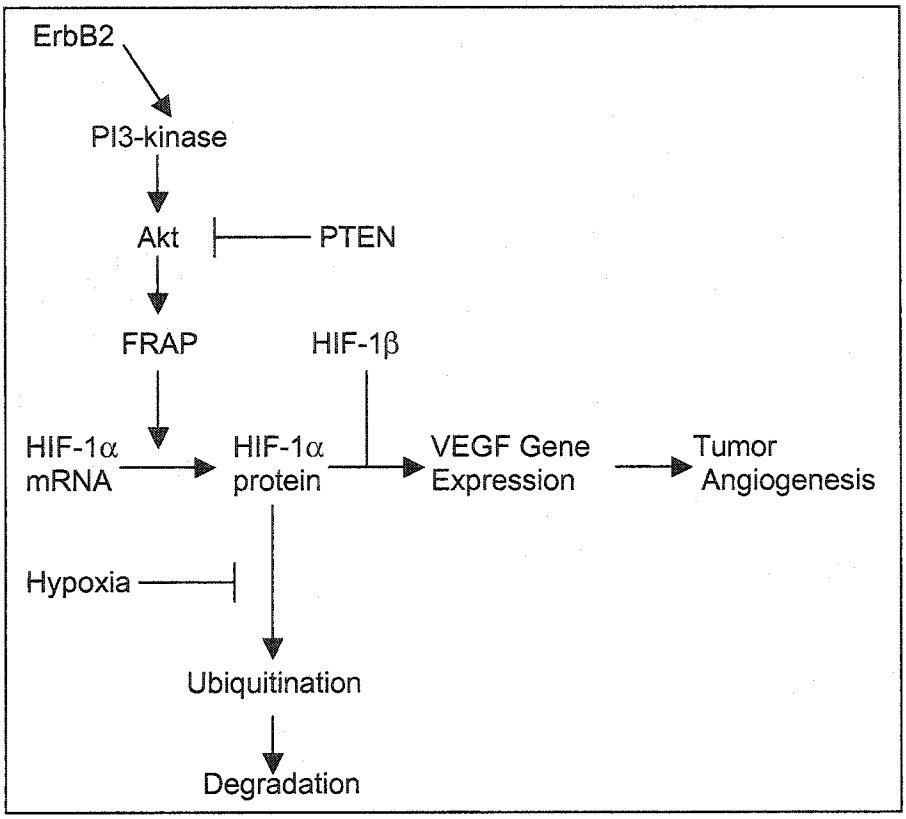

Figure 1.5: ErbB2 mediated upregulation of VEGF expression through enhancement of HIF1α translation. Modified from Laughner et al, 2001.

been confirmed by others using different breast tumor cell lines (Klos et al, 2003). This was extended to an *in vivo* context and to include other angiogenic factors by Izumi et al (2002), who found that trastuzumab treatment reduced the expression of VEGF, transforming growth factor α (TGF-α), angiopoietin I and plasminogen-activator inhibitor-1, while increasing the expression of the antiangiogenic factor, thrombospondin-1 (TSP-1) in human breast tumor xenografts. This correlated with a decrease in blood vessel diameter and volume in the tumors. It has been suggested that the antiangiogenic effect of trastuzumab therapy involves HIF1-α-dependent expression of VEGF (Koukourakis et al, 2003). Comparing two erbB2 positive breast cancer patients with tumors expressing

VEGF, it was found that trastuzumab was able to decrease blood vessel density in the tumor of one patient but not the other, as determined by staining of blood vessels in tumor biopsies acquired post-treatment. While the responding patient had HIF1-α-dependent expression of VEGF, the patient that showed no response to trastuzumab apparently expressed VEGF in a HIF1-α-independent manner as HIF-1-α could not be detected in the tumor biopsy, implying that trastuzumab may have antiangiogenic effects only in a subset of patients.

1.4 Cytotoxic drugs as antiangiogenic agents

Chemotherapeutic agents are still considered to be the gold standard for the systemic treatment of a variety of cancer types, yet in spite of numerous advances in the application and use of such cytotoxic drugs, serious limitations remain with respect to efficacy, toxicity and resistance. Chemotherapy is frequently administered at maximum tolerated doses (MTD), where patients are administered relatively high doses of the drug(s) in an attempt to maximize the number of tumor cells that are killed by the drug without causing excessive harm to the patient. The patient is then allowed a rest period, allowing time for the hematopoietic and intestinal mucosal cells to recover from the non-specific cytotoxic effects of these drugs. While chemotherapy can be effective at eradicating some rare tumor types, especially certain liquid tumors, many solid tumors typically show at most a limited response duration followed by relapse, sometimes very rapidly, due to drug resistance. Resistance to chemotherapy has been attributed to many possible mechanisms, including poor drug penetration or tumor cell alterations which increase the efflux of drug out of the tumor cells (Giai et al, 1991), changes in the expression of proteins or cofactors involved in drug detoxification (Perez et al, 1990), acquisition of mutations which prevent apoptotic machinery from inducing programmed cell death (Reed, 1995), or accelerated repopulation of more aggressive, rapidly

proliferating tumor cells (Wu and Tannock, 2003), to name some of the better known possibilities.

While the development of additional chemotherapeutic agents to overcome resistance does not appear to be a major focus of pharmaceutical companies, clinicians have extensively been studying the importance of different dosing schedules for maximum effectiveness of chemotherapy. By way of example, reports of clinical trials involving dose-dense therapy, where large, frequent doses of chemotherapy are administered in addition to granulocyte colony-stimulating factor, autologous stem cell transplants and/or antibiotic support to aid in recovery from myelosuppression, have shown only slight improvements in disease-free survival for the treatment of node-positive breast cancer patients (Citron et al, 2003). Perhaps more encouraging, the administration of chemotherapy at more regular and frequent intervals, such as weekly paclitaxel at doses less than the conventional MTD, has been shown to be more effective in the treatment of metastatic breast cancer (Perez et al, 2001) and has been increasingly adopted as a standard protocol in the clinic. Regardless of the administration protocol, chemotherapy given at any of these doses leads to some level of toxicity as a result of the non-specificity of these agents.

This non-discriminating nature of chemotherapeutic agents is responsible for such drugs being considered as 'accidental' antiangiogenic agents, a category of drug that includes all therapeutic agents that were found to have an antiangiogenic effect independent from the therapeutic effect that the drug was designed to induce (Kerbel et al, 2000; Spieth et al, 2003). The antiangiogenic potential of a wide array of chemotherapeutic drugs has been shown using various *in vitro* and *in vivo* assays (reviewed in Miller et al, 2001). It has been speculated that although MTD chemotherapy has an antiangiogenic effect, an anti-tumor response mediated by this mechanism is not observed as lost or damaged ECs are replenished and able to re-establish a vascular

network within the tumor during the rest phase between successive cycles of chemotherapy. This led to the concept of administering lower doses of chemotherapy in a chronic fashion, with few or no break periods, to maximize the antiangiogenic effects of chemotherapeutic agents (Figure 1.6). The importance of dosing schedules to maximize this antiangiogenic effect of chemotherapeutic agents was first shown by Browder et al (2000). Their study showed that the administration of chemotherapy weekly at 1/3 of the MTD, rather than in a more conventional fashion involving cycles of three doses followed by a sustained rest period of several weeks, significantly improved anti-tumor activity against both drug sensitive and drug resistant tumors *in vivo*. The increased efficacy of the chronic low-dose chemotherapy dosing regimen, now referred to as metronomic chemotherapy, was attributed in part to a sustained level of EC apoptosis within the tumor, as opposed to the conventional dosing regimen which caused significant EC apoptosis immediately after administration of chemotherapy but not during the rest phase (Browder et al, 2000).

An 'antiangiogenic window' was identified for ECs treated with chemotherapeutic agents, where low-doses of chemotherapy, including cyclophosphamide (CTX) and paclitaxel, administered in a metronomic fashion to ECs *in vitro* had a significantly lower IC_{50} value compared to tumor cells (i.e. human umbilical vein EC IC_{50} = 45 nM, MDA-MB-435 human breast tumor cell IC_{50} = 781 nM for the alkylating agent 4-hydroperoxycyclophosphamide) (Bocci et al, 2002). This study also confirmed the finding that ECs only underwent apoptosis following protracted exposure to low-doses of chemotherapy. This selective sensitivity of ECs to low concentrations of drug was not extended to normal mammary or prostate epithelial cells, fibroblasts, astrocytes or smooth muscle cells when exposed to ultra-low-doses of paclitaxel *in vitro* in the picomolar range (Wang et al, 2003).

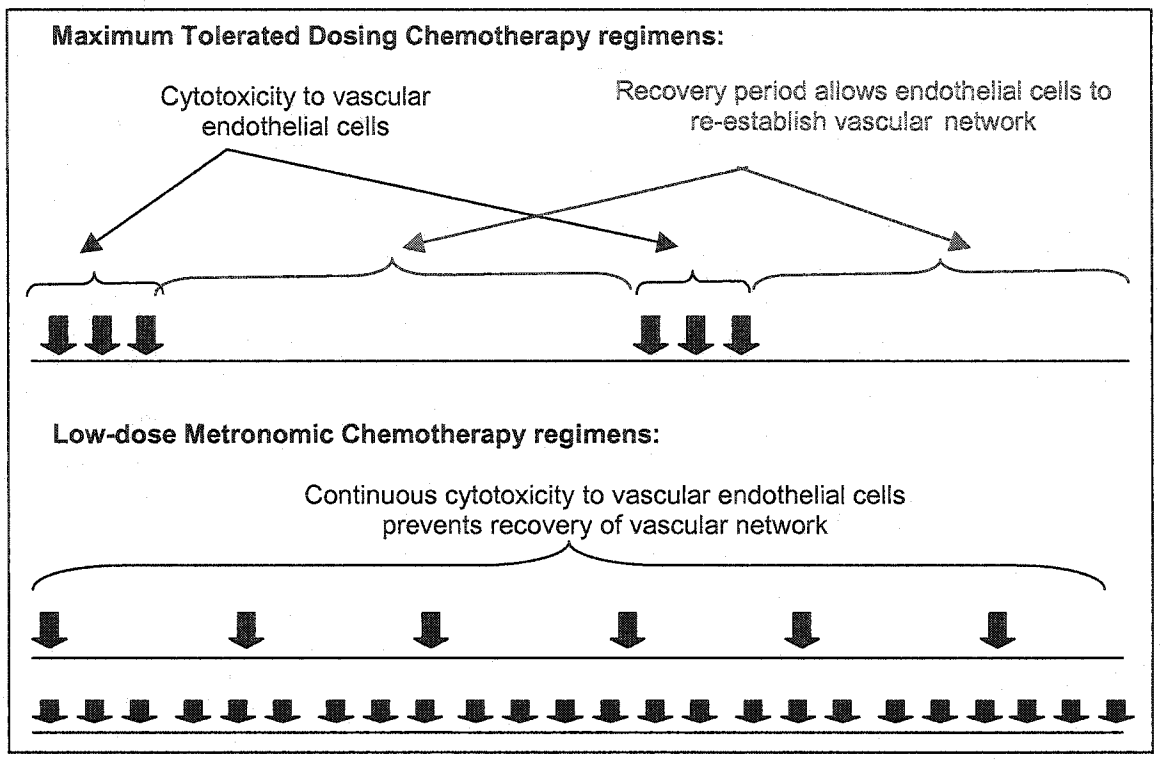

Figure 1.6: Illustration of maximum tolerated dosing and potential metronomic schedules of chemotherapy. Cytotoxic effects on vascular ECs occur during and briefly following administration of chemotherapy.

The basis of this remarkable specificity may not necessarily be due to an inherent ultra-sensitivity of endothelial cells, but rather, a secondary effect due to induction of endothelial cell inhibitors such as TSP-1. TSP-1, a highly specific and potent endogenous inhibitor of angiogenesis, has been shown to be upregulated in ECs by sustained exposure to low-dose concentrations of paclitaxel or CTX (Bocci et al, 2003). The effects of low-dose chemotherapy appear not to be limited to the ECs, as changes to the tumor cell population have been reported as well. TSP-1 was found to be upregulated within the tumor microenvironment in *in vivo* studies, due to increased protein expression by both tumor cells and peri-vascular stromal cells (Hamano et al, 2004). In both of these studies, low-dose CTX was found to promote anti-proliferative and pro-apoptotic effects on vascular ECs. More impressively, chronic low-dose

metronomic administration of CTX showed an improved anti-tumor effect compared to mice treated only with MTD CTX, but this effect was not observed in TSP-1 null mice (Bocci et al, 2003; Hamano et al, 2004). Together, these studies suggest that TSP-1 plays an important role in the antiangiogenic and anti-tumor effects observed *in vivo* with low-dose metronomic CTX (Browder et al, 2000; Man et al, 2002) and possibly other chemotherapeutic agents.

Additional work is being carried out to identify what metronomic chemotherapy does to alter the tumor microenvironment and the ECs. Picomolar concentrations of vinblastine, far too low to induce cytotoxic effects on ECs, have been shown to interfere with several mechanisms thought to be essential for angiogenesis, including EC proliferation, chemotaxis, spreading on fibronectin and vascular tube formation *in vitro* (Vacca et al, 1999). The role of circulating endothelial progenitor cells (CEPs) in the vasculogenic processes of tumors has become a topic of increasing interest since implicated by Lyden et al (2001). Recent evidence suggests that low-dose metronomic CTX may effectively suppress the mobilization and viability of CEPs, thereby preventing vasculogenic processes from taking place within the tumor, while MTD CTX was found to promote mobilization of such cells during the extended break period following an acute drop in their levels (Bertolini et al, 2003). In addition to the induction of TSP-1 by tumor cells, as mentioned above, long-term exposure to nanomolar concentrations of paclitaxel has been shown to reduce the expression of two pro-angiogenic proteins, angiopoietin-1 and VEGF, in ovarian tumor cells (Hata et al, 2004). A significant reduction in serum VEGF levels has also been observed in metastatic breast cancer patients receiving metronomic methotrexate and CTX combinations (Colleoni et al, 2002). A decrease in pro-angiogenic factor expression within the tumor may act to facilitate or selectively enhance the antiangiogenic effects of low-dose chemotherapy by reducing the expression of EC survival proteins, such as VEGF (Tran et al, 2002).

While there is evidence to suggest that low-dose metronomic chemotherapy may induce EC apoptosis and alter tumor cell expression of growth factors, there is increasing evidence, both preclinical and clinical, to suggest that there is minimal chronic or acute toxicity associated with this therapy in both mice and humans. Weight fluctuations are frequently used as a measure of toxicity in experimental mouse tumor models, and both Man et al (2002) and Klement et al (2000, 2002) reported no weight fluctuations of mice receiving metronomic CTX therapy through the drinking water, or drugs such as vinblastine or taxol injected twice or three times a week, respectively. An extensive study by Emmenegger et al (2004) which evaluated the chronic effects of long-term metronomic CTX therapy of several mouse strains was recently undertaken. Two of the most sensitive tissues/cell populations normally affected by conventional doses of chemotherapy, bone marrow and gut epithelial mucosal cells were unaffected; only lymphopenia was noted. These observations are similar to the toxicities observed in two clinical trials involving low-dose CTX, which both reported only mild hematological side effects which could be reversed by dose-modification (Colleoni et al, 2002; Glode et al, 2003). The lack of severe acute toxicities associated so far with metronomic chemotherapy could be of particular importance for patient compliance, as many metronomic chemotherapy regimens are designed to be administered orally on an out-patient basis.

1.5 Metronomic chemotherapy and antiangiogenic agent combinations

In addition to the minimal or reduced toxicity associated with metronomic chemotherapy regimens, it was also thought that by targeting angiogenesis, one of the greatest drawbacks of chemotherapy - the development of drug resistance, might be avoided or delayed (Kerbel, 1997; Boehm et al, 1997). Resistance to chemotherapy is

in part due to the genetic instability and heterogeneity of the targeted tumour cells, while ECs have historically been considered to be a more genetically stable, homogenous target, although recent evidence may suggest otherwise (Streubel et al, 2004). Nevertheless, metronomic dosing has been repeatedly shown to be an effective administration protocol for chemotherapeutic agents compared to conventional dosing regimens, but the *in vivo* efficacy of these regimens is typically a limited delay in tumor growth (Klement et al, 2000; Man et al, 2002) or the development of drug resistance following a long-term absence of tumor progression (Browder et al, 2000). Blagosklonny (2004) has recently proposed that resistance to metronomic chemotherapy and other antiangiogenic therapies are likely to result from the selection of hypoxia-resistant tumor cells and/or an upregulation of HIF-1α or hypoxia-inducible growth factors such as VEGF. Indeed, evidence obtained from this laboratory by Yu et al (2002) would seem to support this possibility. It has also been suggested that the lack of efficacy of this treatment regimen may be due to angiogenesis-independent tumor cell growth, with tumors obtaining sufficient nutrients and oxygen from pre-existing blood vessels (Bassukas et al, 1990), or vessel remodeling (Huang et al, 2004).

Similar to conventional chemotherapeutic regimens in which chemotherapeutic agents can be combined or used with molecular targeted drugs in order to promote successful outcomes with metronomic chemotherapy a similar combination strategy is likely to be required. The recent development of a variety of targeted antiangiogenic agents, many of which have no or minimal toxicity, provide interesting options for combination with metronomic chemotherapy. The ability of these combinations to improve the efficacy and/or delay the onset of resistance to metronomic chemotherapy has been tested by a number of groups using a wide variety of *in vivo* tumor models. A rat monoclonal anti-mouse VEGF receptor 2 (VEGFR2) antibody, DC101, has been shown to significantly enhance the anti-tumor effects of metronomic vinblastine,

doxorubicin, or CTX relative to the chemotherapy regimen or DC101 treatment alone (Klement et al, 2000, 2002; Man et al, 2002; Zhang et al, 2002b). Additional antiangiogenic agents such as SU5416 (a small molecule VEGFR2 inhibitor), TNP-470 (a specific inhibitor of methionine aminopeptidase-2), PEX (a fragment of metalloprotease 2), or matrix metalloprotease inhibitors such as BB-94, have also shown anti-tumor benefits when used in combination with metronomic chemotherapy schedules (Browder et al, 2000; Bello et al, 2001; Bergers & Hanahan, 2002; Svensson et al, 2002; Kerbel & Kamen, 2004).

In recent years, a number of clinical trials have been initiated to test the promising preclinical results of metronomic therapy. In a phase II trial, 64 patients with advanced metastatic breast cancer received a low-dose oral methotrexate and CTX treatment regimen. An overall response rate of 19% and overall clinical benefit of 31.7% (which included stable disease) was observed using this combination of chemotherapeutic agents (Colleoni et al, 2002). Somewhat similar benefits were reported in an androgen-independent prostate cancer trial using a low-dose dexamethasone and daily oral low-dose CTX combination (Glode et al, 2003). Several trials have combined low-dose chemotherapy with 'accidental' antiangiogenic agents, such as thalidomide and celecoxib (reviewed in Kerbel & Kamen, 2004). The recent United States Food and Drug Administration (FDA) approval of bevacizumab (Avastin®), a humanized antibody directed against VEGF, has provided yet another promising option for combination with metronomic chemotherapy, and two such clinical trials are already underway, one for advanced or metastatic ovarian cancer and the other for metastatic breast cancer (Kerbel & Kamen, 2004). It is anticipated that the number of clinical trials to assess metronomic chemotherapy combinations will continue to increase as additional antiangiogenic drugs are translated into the clinic and other accidental

antiangiogenic drugs are discovered, assuming that some of the trials currently underway are sufficiently positive.

1.6 Thesis Objective

It has been shown that the antiangiogenic and anti-tumor effects of metronomic chemotherapy can be enhanced by combination with targeted EC antiangiogenic agents such as anti-VEGF receptor 2 monoclonal antibodies (Klement et al, 2000), or with 'accidental' antiangiogenic agents (Kerbel et al, 2000; Spieth et al, 2003). The antiangiogenic potential of trastuzumab, and the benefits of combining this relatively non-toxic antibody therapy with a less-toxic dosing regimen than MTD chemotherapy, led us to evaluate the possible effects of combining trastuzumab with a metronomic chemotherapy regimen. The original purpose of these studies was to assess the anti-tumor and antiangiogenic effects of metronomic CTX and trastuzumab, compared to single agent therapies and MTD dosing schedules. An unanticipated observation - the development of acquired resistance to trastuzumab, led to further studies on mechanisms of such resistance and to identify potential second-line therapies for trastuzumab-resistant tumors that might be used in the clinic.

2 Anti-tumor and antiangiogenic effects of combining metronomic chemotherapy with trastuzumab

2.1 Development of an erbB2 overexpressing breast tumor model

In order to establish a reliable erbB2 positive orthotopic breast tumor model in mice, the tumorigenic human breast tumor cell line, MDA-MB-231, which does not express erbB2, was retrovirally transduced by M.E. Mossoba and J.A. Medin (University of Toronto) to derive an erbB2 overexpressing variant referred to as 231-H2N. MDA-MB-231 was selected as an ideal breast tumor model for these studies, in part due to the reported involvement of VEGF-dependent angiogenesis in the progression of these tumors (Zhang et al, 2002a). In addition, in vivo tumor growth of MDA-MB-231 mammary tumors has been shown to be effectively delayed by treatment of mice with metronomic CTX administered through the drinking water (Man et al, 2002).

Other breast cancer cell lines were tested but not selected for these studies due to difficulties encountered with the in vivo growth of cell lines endogenously overexpressing erbB2, such as BT-474 and SK-BR3, in the mammary fat pad of SCID mice (i.e. they were non or very poorly tumorigenic). Studies which have successfully used these cell lines *in vivo* suggest that irradiation prior to tumor cell implantation (Spiridon et al, 2004), subcutaneous injection rather than orthotopic implantation of tumor cells (Spiridon et al, 2004; Moulder et al, 2001), or supplementation with subcutaneously implanted estrogen tablets (Moulder et al, 2001) are required to assist tumor take in the animals. While the insulin-like growth factor I receptor (IGF-1R) and erbB2 co-expressing cell line MCF7-HER2-18, a transfected variant of the MCF-7 human breast tumor cell line, was found to grow well in the mammary fat pad of mice and metastasize to the lung, these tumors were unresponsive to trastuzumab, as has

previously been reported by Lu et al (2001). Response to trastuzumab in this cell line requires simultaneous blockade of the IGF-1R receptor.

2.1.1 Characterization of 231-H2N cell line

Expression levels of erbB2 were determined by Western blot for 231-H2N cells relative to the parental MDA-MB-231 cell line, and the erbB2 overexpressing cell lines MDA-MB-361, SK-BR3 and BT-474. ErbB2 gene copies per cell has been determined by fluorescence *in situ* hybridization to be 11, 43, and 47 for the cell lines MDA-MB-361, SK-BR3 and BT-474, respectively (Pegram et al, 2004). While the parental cell line expresses low levels of erbB2, 231-H2N was found to express erbB2 in the range of other endogenous erbB2 positive cell lines (Figure 2.1). Also, when the blot was probed for phosphorylated erbB2, the receptor was found to be constitutively phosphorylated in 231-H2N cells, suggesting that the receptor is biologically active in our cell line.

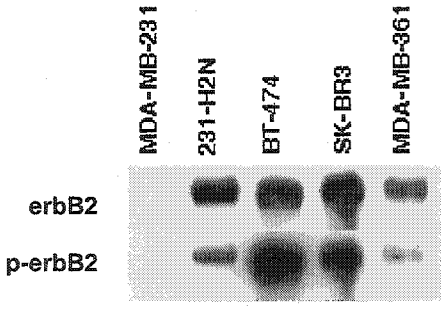

Figure 2.1: Western blot to determine levels of erbB2 and constitutively active phospho-erbB2 in whole cell lysates of the 231-H2N erbB2 transduced cells, compared to parental MDA-MB-231 and endogenous erbB2 overexpressing cell lines BT-474, SK-BR3 and MDA-MB-361.

2.1.2 Cell sensitivity to trastuzumab and cyclophosphamide

To confirm that erbB2 was functional and involved in the activation of downstream intracellular signaling pathways within the transduced cell line, *in vitro*

assessment of the cytostatic effects of erbB2-targeted therapies was carried out on the 231-H2N and MDA-MB-231 cell lines. The erbB2 targeted antibody, trastuzumab, as well as the erbB2 heterodimerization inhibitory antibody, pertuzumab (2C4), and the EGFR-targeted antibody, cetuximab, were shown to inhibit cell proliferation of 231-H2N cells by 25% at antibody concentrations as low as 1 μg/mL (Figure 2.2A). In contrast, inhibition of cell proliferation of the parental cell line was observed only at concentrations of 50 μg/mL.

The benefits of combining chemotherapeutic agents with trastuzumab have been reported both *in vitro* and *in vivo* (Pegram, 1999; Pegram, 2004). *In vitro* synergistic or additive effects were observed *in vitro* with several classes of chemotherapeutic agents, including platinum-based drugs, topoisomerase II inhibitors, alkylating agents, anthracyclines, taxanes, vinca alkaloid-based drugs, and some antimetabolites. The beneficial effects of combining these drugs with trastuzumab was confirmed *in vivo* by substantial increases in anti-tumor efficacy when compared to treatment with trastuzumab or chemotherapy alone.

To determine whether trastuzumab altered the cytotoxic effects of CTX in our *in vitro* model system using 231-H2N cells, we carried out 3 and 6-day dosing assays as previously described (Bocci et al, 2002). Chemotherapy was replaced daily for 3 or 6 days to mimic the metronomic administration of oral CTX in our *in vivo* studies. Rather than using CTX, which is a prodrug that requires activation by liver microsomal cytochrome P450 mixed function oxidases, we used a precursor, 4-hydroperoxycyclophosphamide (4-HC), to one of the active metabolites, 4-hydroxycyclophosphamide for our *in vitro* studies (Sladek, 1999; Flowers et al, 2000). The IC_{50} of 4-HC was determined to be between 30 and 40 nM following continuous exposure for 144 hours, which was consistent for both the 231-H2N and parental MDA-

MB-231 cell line (Figure 2.2B). Work is presently being carried out in our lab in collaboration with M. Colvin and S. Ludeman (Duke University, Durham, NC) to determine the plasma levels of 4-HC in mice receiving oral metronomic daily CTX. Preliminary experiments indicate that plasma levels were between 100 and 277nM when mice were given approximately 20 mg/kg/day though the drinking water, suggesting that there should be some cytotoxic effects of oral metronomic CTX on 231-H2N cells *in vivo* as this is well above the IC_{50} value determined *in vitro*.

To determine the effects of combining trastuzumab and CTX, trastuzumab was added at 10 μg/mL and dosing with 4-HC was carried out for 3 consecutive days. Trastuzumab appeared to have no additive, synergistic or antagonistic effect when combined with 4-HC in a 72 hour exposure assay at dose ranges of 0-10 μM 4-HC (Figure 2.2C).

2.1.3 *In vitro* VEGF expression and inhibition in 231-H2N cell line

Due to the finding by Viloria-Petit et al (1997) that treatment of the erbB2 overexpressing cell line SK-BR3 with trastuzumab could reduce the *in vitro* expression of VEGF, the same study was carried out using the 231-H2N cell line. 231-H2N and parental MDA-MB-231 cells, when cultured in the conditions of near confluency and serum reduction, were both found to express ~3000 pg VEGF/10^6 cells/24 hours, when conditioned media was measured by ELISA (data not shown). Treatment of 231-H2N cells with trastuzumab resulted in a dose-dependent reduction in secretion of VEGF into the culture medium, similar to that previously found in the SK-BR3 cell line (Figure 2.3). This effect was not observed in normal serum conditions, indicating the trastuzumab may elicit an antiangiogenic effect exclusively under conditions of microenvironmental stress, such as hypoxia.

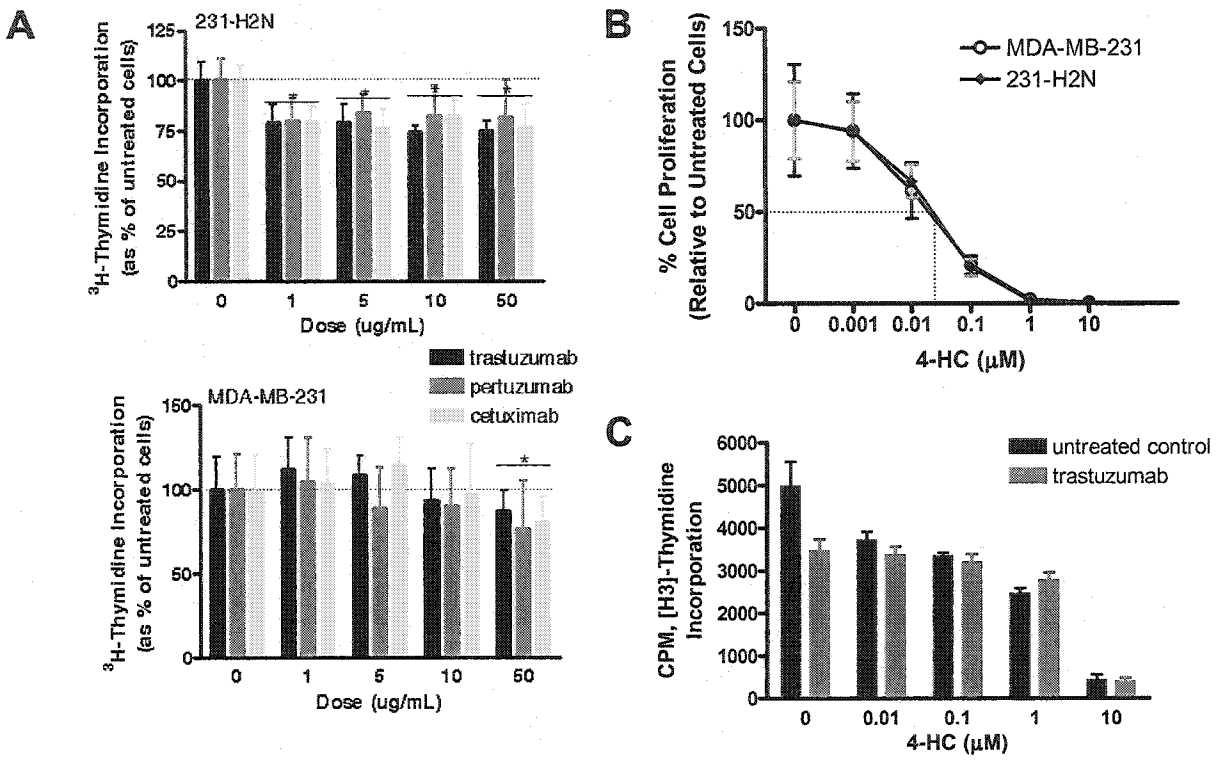

Figure 2.2: *In vitro* cytostatic and cytotoxic effects of trastuzumab and CTX. **A)** Inhibition of cell proliferation of 231-H2N and MDA-MB-231 cells after 72 hours of treatment with varying concentrations of erbB targeted antibodies trastuzumab, pertuzumab, and cetuximab, measured by [^{3}H]-thymidine incorporation and reported as % of cell proliferation relative to untreated cells. **B)** Cytotoxic effects of 4-HC on 231-H2N cells, following 6 days of consecutive treatment, measured by [^{3}H]-thymidine incorporation and reported as % of cell proliferation relative to untreated cells. **C)** Inhibitory effects of trastuzumab in combination with 4-HC, showing no additive or synergistic effects in 231-H2N cells after 3 days of treatment, measured by [^{3}H]-thymidine incorporation. All data reported as mean ± SD, n=8, and representative of triplicate experiments, * $p<0.05$.

It was expected that transducing the MDA-MB-231 cells with erbB2 would increase the expression of VEGF, but this was not found in the 231-H2N cells line, possibly due to the already high levels of VEGF being produced by the parental cells. It was still important to confirm that VEGF expression was regulated through the erbB2 receptor and downstream signaling pathways. To elucidate those pathways involved in the expression of VEGF in 231-H2N cells, a series of small molecule inhibitors were used; Gö6976, an inhibitor of EGF-driven NF-κB activity; PD098059, a MAPK inhibitor; LY294002, a PI3K inhibitor; CCI779, an mTOR inhibitor, and AG879, an erbB2 tyrosine kinase inhibitor (TKI). Since VEGF is regulated through a variety of signaling pathways, the noticeable inhibitory effects of each inhibitory molecule was not unexpected (Figure 2.3A). Yet the statisically significant downregulation of VEGF production by LY294002 (PI3K inhibitor) and AG879 (erbB2 TKI) together suggests that the erbB2-erbB3 heterodimer, which is the most potent dimer to activate the PI3K pathway, may be involved in the expression of VEGF in the 231-H2N cell line, although not exclusively. In contrast, the parental cell line MDA-MB-231, showed a similar profile for all inhibitory molecules, with the exception of AG879, which as expected had no effect on reducing VEGF expression due to the absence of erbB2 expression in the parental cell line (Figure 2.3B).

2.1.3 *In vivo* growth of 231-H2N cell line

Preceeding *in vivo* studies, tumor take in the mammary fat pad was determined. Tumors were palpable in all mice within 7 days of injection of 1-2 x 10^6 cells, and tumors reached 150 to 250mm^3 within 5 weeks with acceptable variation in tumor size (data not shown). Tumors reached a volume of 1700 mm^3 within 10 weeks, and remained

encapsulated within the mammary fat pad. No evidence of macroscopic metastases was observed in the lungs of these mice.

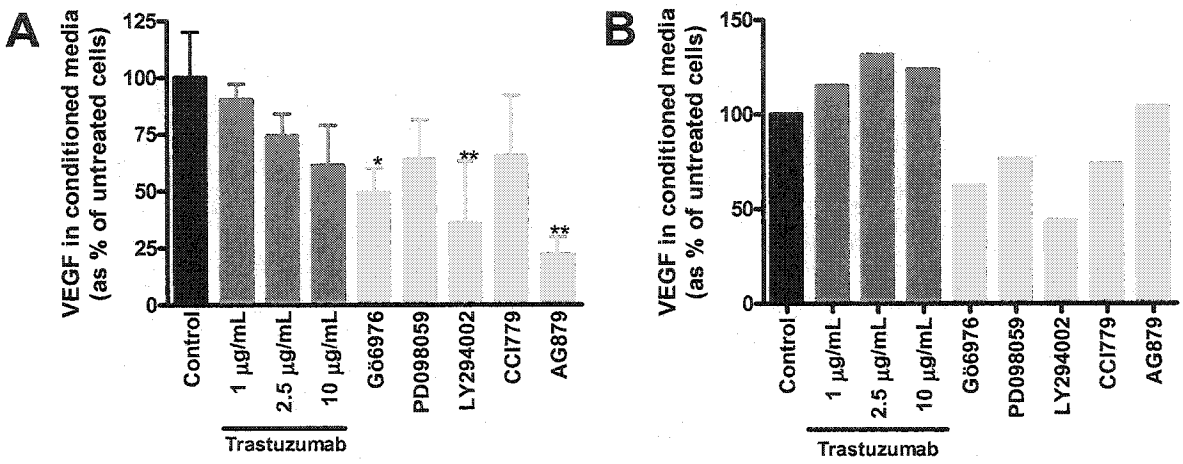

Figure 2.3: Inhibition of VEGF secretion by trastuzumab or small molecule cell signaling inhibitors in **A)** 231-H2N cells and **B)** MDA-MB-231 cells, after 48 hours of treatment. Small molecule inhibitors were used at the following concentrations: Gö 6976 (NF-κB inhibitor) 1µM; PD098059 (MAPK inhibitor) 20 µM; LY294002 (PI3K inhibitor) 20 µM); CCI779 (mTOR inhibitor) 1nM; AG879 (erbB2 receptor tyrosine kinase inhibitor) 5 µM. For A, data is reported as mean ± SD, n=4, * $p<0.05$, ** $p<0.01$, for B, n=1.

2.2 Anti-tumor effects of combination therapy

The primary purpose of these studies was to assess the anti-tumor effects of trastuzumab in combination with low-dose, chronically administered CTX in the preclinical setting, as a first step to evaluate the feasibility of translating this treatment regimen to erbB2 overexpressing breast cancer patients, especially in the adjuvant treatment setting. Two independent *in vivo* experiments were designed to evaluate the effects of metronomic CTX, trastuzumab, and combination therapy, compared to the standard MTD dosing schedule. Orthotopic 231-H2N tumors were established in the abdominal mammary fat pad of 6 week old female CB-17 SCID mice and grown to ~150 mm^3 (experiment 1, 142 ± 99 mm^3, mean ± SD, n=3 per group) and ~250 mm^3

(experiment 2, 251 ± 185 mm^3, n=7-8 per group), before initiation of treatment (day 0). Treatments were continued until tumors reached a volume of 1700 mm^3 or until mice experienced a loss of 25% of their initial body weight.

While untreated control tumors progressed rapidly, reaching a volume of 1700 mm^3 in 30-40 days, oral metronomic CTX (20 mg/kg/day) effectively delayed tumor growth to this volume for 60 days (Figure 2.4A and 2.5A), as was previously reported in parental MDA-MB-231 tumors (Man et al, 2002). A complete block of tumor growth was observed for trastuzumab-treated 231-H2N tumors (20mg/kg, twice weekly) for 4-5 weeks, after which time, the tumors rapidly began to progress, suggestive of the emergence of a variant subpopulation with acquired resistance to the drug. The initial response of the primary tumors to trastuzumab was greater than expected, as *in vitro* studies of 231-H2N cells indicated only a 25% inhibition of cell proliferation at increasing concentrations of trastuzumab. This impressive *in vivo* effect of trastuzumab is likely due to a combination of effects some of which are operative only *in vivo*, including ADCC (Sliwkowski et al, 1999) and the postulated antiangiogenic effects of this drug (Viloria-Petit et al, 1997; Izumi et al, 2002), which together with a direct inhibition of tumor cell proliferation might be responsible for this remarkable suppression of tumor growth. Both MTD CTX (100 mg/kg, days 1,3 and 5 of 21 day cycle) and metronomic CTX combined with trastuzumab delayed the apparent rapid onset of resistance by an additional 75-90 days, thus illustrating the improved efficacy of trastuzumab when it is used in combination with chemotherapeutic drugs, as reported by Pegram et al (1999).

To emphasize the advantage of the metronomic CTX and trastuzumab combination therapy compared to the standard MTD combination, a survival curve was plotted, whereby survival time was defined as a delay in time to progression of the tumor to 1500 mm^3 or loss of 25% body weight. As seen in Figure 2.5B, the combination of

MTD CTX and trastuzumab showed little benefit to survival compared to trastuzumab therapy alone due to the toxicity of the chemotherapy (as indicated by substantial weight loss), whereas combination therapy of metronomic CTX and trastuzumab provided a significant survival advantage, compared to trastuzumab therapy alone, due to both a prevention of tumor progression for 120 days and minimal chemotherapy-associated toxicity.

As indicated in Table 2.1, rates of tumor growth delay were similar for control, trastuzumab, metronomic CTX and combination therapy with trastuzumab and metronomic CTX groups in both experiments 1 and 2. Yet two differences between these experiments should be noted. First, mice receiving MTD CTX in experiment 2 showed initial response to the first two cycles of chemotherapy, then began to rapidly progress (Figure 2.5A). The complete response in experiment 1 (until day 89, at which time mice were sacrificed due to significant weight loss) and partial response in experiment 2 may be explained by the larger tumor volumes ($250mm^3$ vs $150mm^3$) at the initiation of treatment in experiment 2. Tumor growth in the MTD CTX treatment group was expected based on previous studies (Man et al, 2002). Second, in experiment 2, 5 of 7 mice receiving MTD CTX and trastuzumab were sacrificed due to large decreases in weight loss detected after the third chemotherapy cycle, which was not observed in experiment 1. The reason for this increased toxicity of CTX in the second experiment is unknown, but may be due to differences in the health status of the mice upon arrival to the animal care facility or throughout the duration of the experiment, or due to the influence of a larger tumor burden on the overall health of the animals.

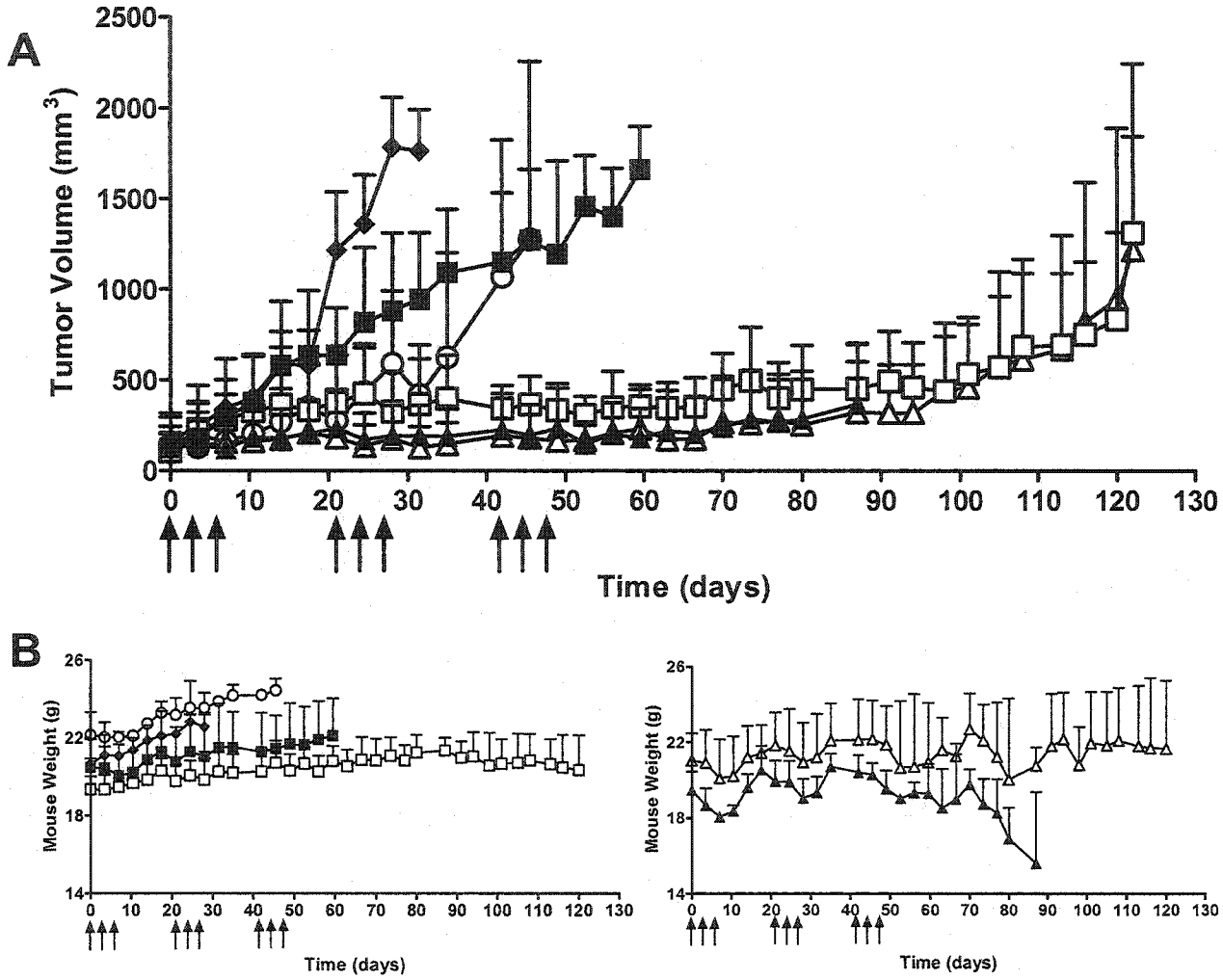

Figure 2.4: *In vivo* 231-H2N orthotopic mammary tumors in SCID mice, Experiment 1. **(A)** Anti-tumor effects of metronomic versus MTD dosing of CTX, in combination with trastuzumab. **(B)** Toxicity, as measured by fluctuations in mouse weight, of MTD CTX dosing schedules compared to less toxic metronomic regimens. (◆ control, ○ trastuzumab, ■ metronomic CTX, □ trastuzumab + metronomic CTX, ▲ MTD CTX, △ trastuzumab + MTD CTX, ↑ time of MTD dosing). Data reported as mean ± SD, n=3.

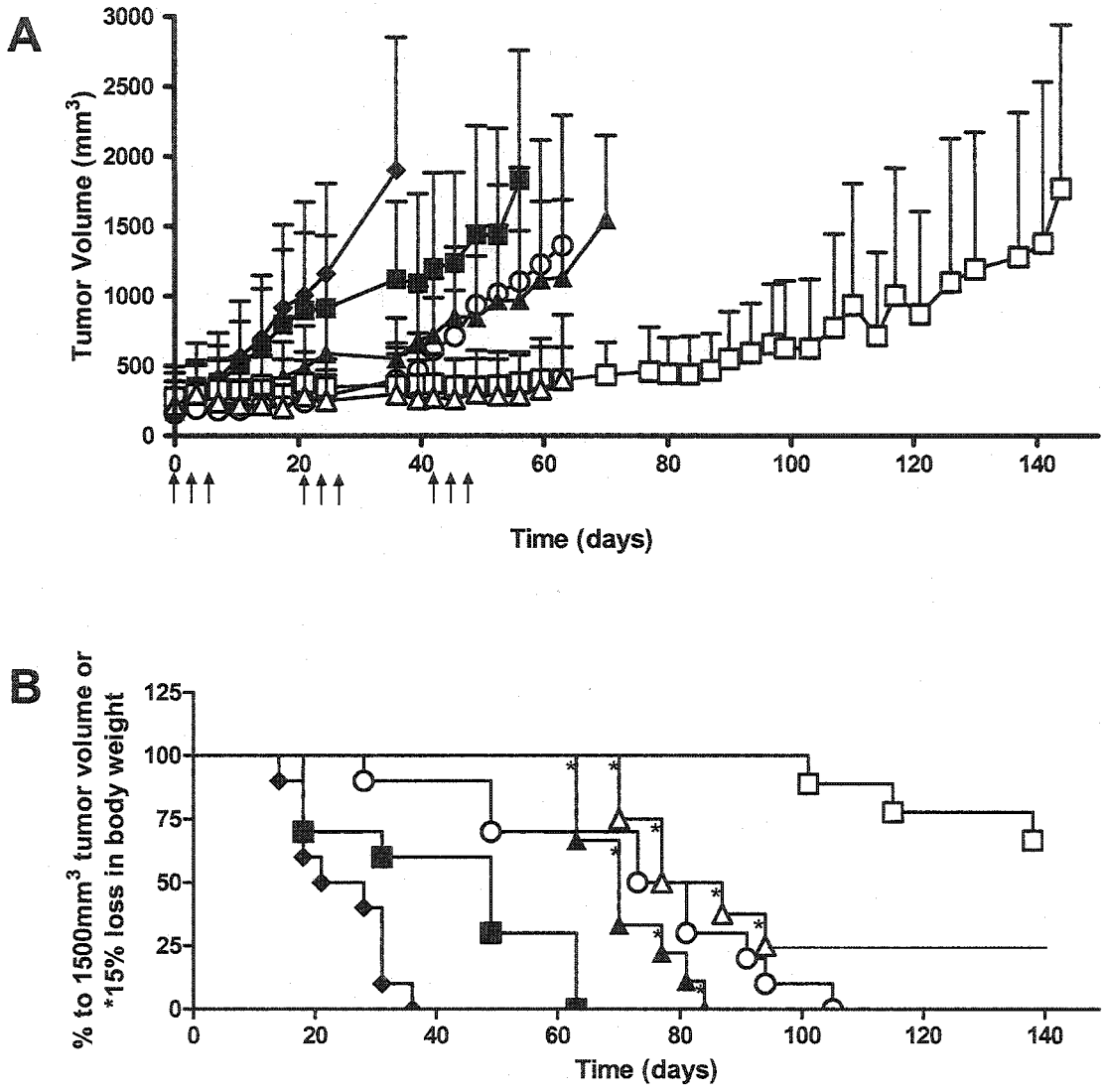

Figure 2.5: ***In vivo*** **231-H2N orthotopic mammary tumors in SCID mice, Experiment 2.** **(A)** Anti-tumor effects of metronomic versus MTD dosing of CTX, in combination with trastuzumab. **(B)** Effects of therapeutic regimens on survival, where duration of survival is defined as time to primary tumor progression to 1700mm^3 or 25% weight loss. (◆ control, ○ trastuzumab, ■ metronomic CTX, □ trastuzumab + metronomic CTX, △ MTD CTX, ▲ trastuzumab + MTD CTX, ↑ time of MTD dosing, * weight loss of >25%). Data reported as mean ± SD, n=7-8.

Table 2.1: Summary of tumor growth delay of 231-H2N orthotopic tumors to trastuzumab, CTX and combination therapies.

	Time to 1500 mm^3 Tumor Volume (days)	
Treatment Group	Experiment 1 (n=3)	Experiment 2 (n=7/8)
Control	25	21
Trastuzumab	48	73
Metronomic CTX	53	48
Trastuzumab + Metronomic CTX	122	142
MTD CTX	-	70
Trastuzumab + MTD CTX	122	77

As a measure of toxicity in these experiments, mice were monitored for changes in weight. As illustrated in Figure 2.4B, mice receiving trastuzumab, metronomic CTX or a combination of these drugs showed no significant fluctuations in weight over the duration of the experiment. In contrast, mice administered MTD CTX alone experienced weight loss following the administration of CTX. The third MTD dosing cycle was subsequently followed by an irreversible weight loss of greater than 25%, an indication of severe, cumulative toxicity from the CTX. While fluctuations in weight loss were observed in mice receiving MTD CTX with trastuzumab, in experiment 1, the combination appeared to reduce the effects of the CTX, as has been reported clinically with an improved quality of life with patients receiving chemotherapy and trastuzumab (Osoba et al, 2002). While not carried out in these experiments, a detailed analysis of the toxic effects of low-dose metronomic versus MTD CTX in mice have been reported recently in detail by our group (Emmenegger et al, 2004); with the exception of lymphopenia, toxic side effects normally associated with MTD chemotherapy such as myelosuppression were not observed.

To determine whether the effects of metronomic chemotherapy and trastuzumab were extendable in scenarios involving larger, more established tumors, an experiment was carried out where treatment was commenced when tumors were ~800 mm^3 in

volume and continued for three weeks. While single agent therapy with either trastuzumab or metronomic CTX showed some delay in tumor growth, combination therapy completely blocked tumor progression during this treatment period (Figure 2.6). The erbB2 heterodimerization inhibitory antibody, pertuzumab (2C4), which was found to have cytostatic effects *in vitro*, as mentioned above, was also tested for anti-tumor efficacy as a single agent and in combination with metronomic CTX. While pertuzumab showed noticeably less tumor growth inhibition than trastuzumab, this anti-tumor effect was greatly enhanced by combination with metronomic CTX. This indicates that dimerization of erbB2 with other members of the EGFR family in our tumor cell line is important for tumor progression. In addition, MDA-MB-231 tumors were treated with trastuzumab to confirm the lack of response of the parental cell line to trastuzumab, as found above *in vitro*.

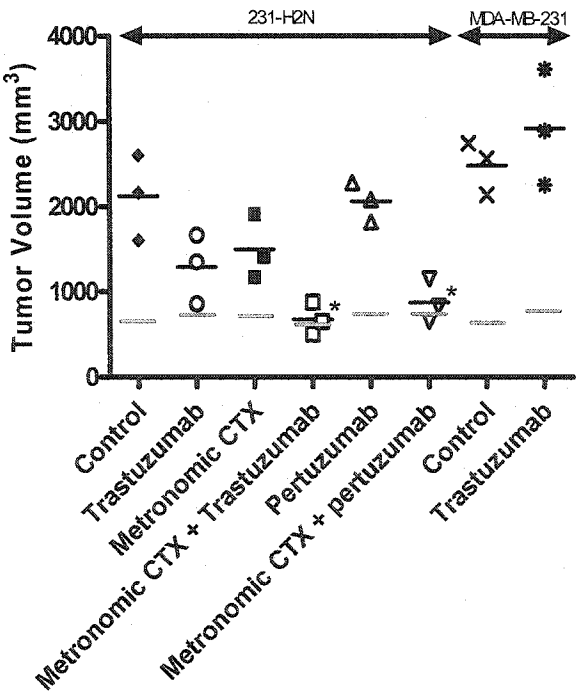

Figure 2.6: *In vivo* anti-tumor effects of CTX, trastuzumab and pertuzumab therapies on well-established (>800 mm^3) 231-H2N and MDA-MB-231 orthotopic mammary tumors in SCID mice. (grey bars = mean tumor volume at start of treatment, black bars = mean tumor volume after 3 weeks of treatment, symbols represent individual tumor volumes, * $p<0.05$).

2.3 Antiangiogenic effects of combination therapy

A strong rationale for combining metronomic chemotherapy with the erbB2 oncogene targeting drug trastuzumab comes from the reported antiangiogenic effects of both of these therapies *in vivo* (Izumi et al, 2002; Browder et al, 2000; Bocci et al, 2003). To elucidate the contribution that these therapeutic regimens may have in blocking tumor growth by acting as defacto antiangiogenic agents, several studies were designed to determine *in vivo* antiangiogenic efficacy.

2.3.1 Tumor perfusion assay

A perfusion assay was used in order to measure the extent of vascularity within the primary tumor. After one 21-day treatment cycle, tumor-bearing mice were injected intravenously with FITC-dextran. Fluorescence of tumor homogenates was taken as a measure of the volume of functional vasculature within the tumor. All single-agent therapies indicated some reduction in tumor vascular function relative to controls, ranging from 10% reduction in the trastuzumab group, and 38% and 41% reductions in the metronomic and MTD CTX groups, respectively (Figure 2.7A). Combination therapies had an additive effect, reducing tumor perfusion by 50% and 55% for metronomic and MTD CTX combination therapies, respectively.

2.3.2 Matrigel angiogenesis assay

To confirm the additive antiangiogenic effects of combination CTX and trastuzumab observed in the tumor perfusion assay, fluorescent measurements were carried out using an *in vivo* Matrigel angiogenesis assay. Typically, Matrigel containing a potent pro-angiogenic factor such as VEGF or bFGF is used, but our system is complicated by the fact that trastuzumab may act as an antiangiogenic agent by altering the expression of pro- and antiangiogenic factors by interacting with human erbB2

receptors on the tumor cells. For this reason, two Matrigel experiments were performed, one with Matrigel plugs containing the angiogenic stimulant bFGF, and the other containing viable 231-H2N cells. The purpose of the latter experiment was to determine if treatment with trastuzumab affected the ability of the tumor cells to recruit angiogenic processes. In both experiments, Matrigel plugs were established for three days prior to the commencement of treatment, to allow for the initiation of angiogenesis. Mice were treated for 7 days, after which time FITC-dextran was injected intravenously and Matrigel plugs were resected for fluorescence measurements.

As expected, trastuzumab had no antiangiogenic effect in the bFGF Matrigel plug assay due to the absence of a target of the therapy, the tumor cell (Figure 2.7B and 2.7D). Metronomic CTX alone or in combination with trastuzumab both effectively inhibited angiogenesis by 43% relative to untreated controls, while MTD CTX inhibited angiogenesis by 78% and 86% ($p<0.05$), with and without trastuzumab, respectively. DC101, the anti-VEGFR2 antibody, was used as a positive control for these experiments (Klement et al, 2000) and showed a reduction of angiogenesis of 50%. It is not surprising that this assay indicates that MTD CTX is more effective than metronomic CTX at inhibiting angiogenic processes, as measurement of total vasculature was carried out three days after the final dose of MTD chemotherapy. The underlying rationale of metronomic chemotherapy is the absence of prolonged breaks between doses that occur during MTD dosing cycles and allow for the re-establishment of angiogenic processes within the tumor microenvironment. While MTD CTX is effective at preventing angiogenesis during the dosing portion of the cycle, the remainder of the cycle when chemotherapy is not being administered allows for 'relapse' of blood vessel formation. This may occur through hematopoietic-like bursts of endothelial progenitor cells, mobilized out of the bone marrow and into the peripheral circulation, where these cells can circulate and contribute to new blood vessel formation (Bertolini et al, 2003).

Our results indicate that metronomic CTX inhibits angiogenesis to a lesser extent than MTD CTX in the short term, yet we speculate that metronomic chemotherapy may be more effective in the long term, due to the absence of break periods and sustained inhibition of CEPs (Bertolini et al, 2003).

Trastuzumab, metronomic CTX, MTD CTX and MTD CTX plus trastuzumab all noticeably reduced angiogenesis within the tumor cell Matrigel plug relative to untreated controls (Figure 2.7E), which was determined by FITC-dextran measurements to be between 25 and 32% reductions in functional blood vessel formation (Figure 2.7C). Meanwhile, the combination therapy of metronomic CTX and trastuzumab significantly reduced tumor cell-induced angiogenesis by 45% ($p<0.05$) compared to untreated controls, such that visual assessment of the Matrigel plug showed little vascularity by the naked eye. Again, DC101 was used as a positive control, indicating a reduction of 51% ($p<0.01$). These results suggest that trastuzumab alters the expression of angiogenic factors produced by the 231-H2N cells, which subsequently reduces the angiogenic capabilities of the tumor cells. Therefore, it is likely that this antiangiogenic effect of trastuzumab is translated into our *in vivo* tumor model system, where the drug can elicit similar antiangiogenic effects within the tumor microenvironment. In addition, our results indicate that by combining trastuzumab with metronomic CTX, which targets the ECs (and perhaps CEPs) migrating into the Matrigel plug, an additive antiangiogenic effect can be observed. As an additive, rather than synergistic effect was seen in this experiment, it would suggest that if the expression of pro-angiogenic factors is being reduced by trastuzumab, this reduction does not influence the susceptibility of the ECs to CTX in this experimental system, as has been suggested by Tran et al (2002). This may be due to the rich microenvironment provided by the Matrigel within the tumor cell plug.

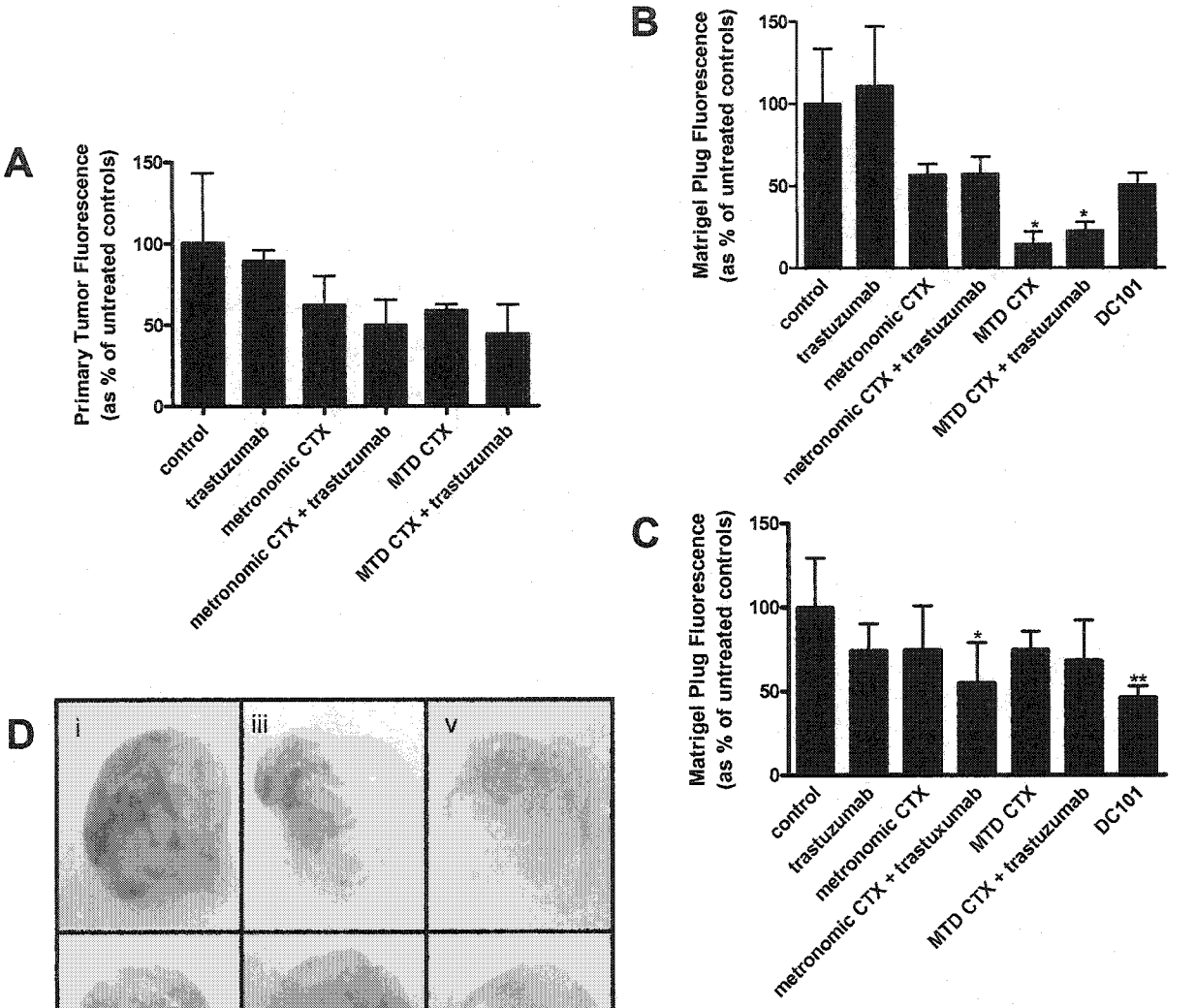

Figure 2.7: *In vivo* assessment of antiangiogenic effects of CTX and trastuzumab therapies.
(A) Tumor perfusion assay. The decrease in intravascular FITC-dextran fluorescence reflects the changes in tumor vasculature in established 231-H2N orthotopic tumors treated with various CTX and trastuzumab therapies for 3 weeks. **(B)** bFGF Matrigel assay and **(C)** tumor cell Matrigel assay. The decrease in FITC-dextran fluorescence in the Matrigel plugs indicate inhibition of angiogenic processes by therapeutic regimens administered for 8 days. All data reported as fluorescence relative to untreated controls (mean ± SD, n=5). (*$p<0.05$, **$p<0.01$) Appearance of **(D)** bFGF and **(E)** tumor cell Matrigel plugs excised from mice after 7 days of treatment. (i) Control ii) Trastuzumab iii) Metronomic CTX iv) Metronomic CTX + Trastuzumab v) MTD CTX vi) MTD CTX + Trastuzumab).

Independently, and more convincingly, together, these two experimental measures of *in vivo* angiogenesis support an additive antiangiogenic effect of trastuzumab in combination with metronomic CTX. It can be speculated that the block in tumor progression observed in the previous *in vivo* experiments (Figures 2.4A and 2.5A) may in part be due to an inhibition of blood vessel growth within the primary tumor.

3 Exploring acquired resistance to trastuzumab

While objective response rates to trastuzumab range from 12 to 34% when used as a monotherapy or in combination with chemotherapy regimens, the duration of this response is on average only 9 months (Cobleigh et al, 1999). The development of (acquired) resistance to trastuzumab is an ongoing problem observed in the clinic, and without an understanding of the underlying mechanism(s) responsible for this resistance, oncologists are left essentially guessing how to best continue treating patients with progressive, relapsed/resistant disease.

The growth profile of 231-H2N tumors treated with either trastuzumab therapy alone or trastuzumab in combination with metronomic or MTD doses of CTX, where tumors experienced rapid tumor growth after a prolonged period of tumor stabilization, was characteristic of resistance to the trastuzumab therapy. The rate of tumor growth was similar for all groups, yet those receiving trastuzumab in combination with CTX experienced tumor progression at a much later time than those receiving trastuzumab treatment alone. The similar rate of tumor progression seen in all three treatment groups led to the assumption that the tumors were becoming resistant to the trastuzumab therapy, and that this resistance also prevented any anti-tumor effects of continued administration of CTX (metronomically). The model system used in these studies, which mimics in some ways the development of resistance to trastuzumab in the clinic, provided an optimal opportunity to further assess the acquired resistance in both an *in vitro* and *in vivo* context.

3.1 Upregulation of VEGF in trastuzumab resistant tumors

Due to the finding in our lab that VEGF is significantly upregulated in human A431 squamous tumors which have become resistant to anti-EGFR antibody therapy (Viloria-Petit, 2001), we sought to determine if a similar mechanism was involved in the resistance of 231-H2N tumors to trastuzumab. Total RNA was extracted from primary tumors taken from long-term *in vivo* dosing experiments following tumor progression and was probed for VEGF by Northern blot. An increase in the expression of VEGF was observed in tumors resistant to combination therapy regimens, but not in single-agent CTX or control tumors (Figure 3.1A). Mouse plasma was tested for levels of human VEGF, but it could not be detected by ELISA in any 231-H2N tumor-bearing mice (data not shown).

The levels of VEGF in conditioned media from 231-H2N cells relative to the resistant cell lines was measured by ELISA. Unexpectedly, the increase in VEGF expression in the resistant tumors was not consistently observed in the resistant cell lines when cultured *in vitro* for 48 hours. Only two cell lines, both isolated from trastuzumab resistant 231-H2N tumors indicated a significant upregulation in VEGF secretion relative to tumor cell lines derived from untreated 231-H2N tumors ($p<0.05$) (Figure 3.1B). This suggests that the upregulation of VEGF in 231-H2N tumors resistant to trastuzumab therapy is a complicated, potentially multifaceted, *in vivo* effect, which is not conserved when cells are grown *in vitro*. For example, the increase may be regulated in part, by hypoxia, or by the stroma in the tumor.

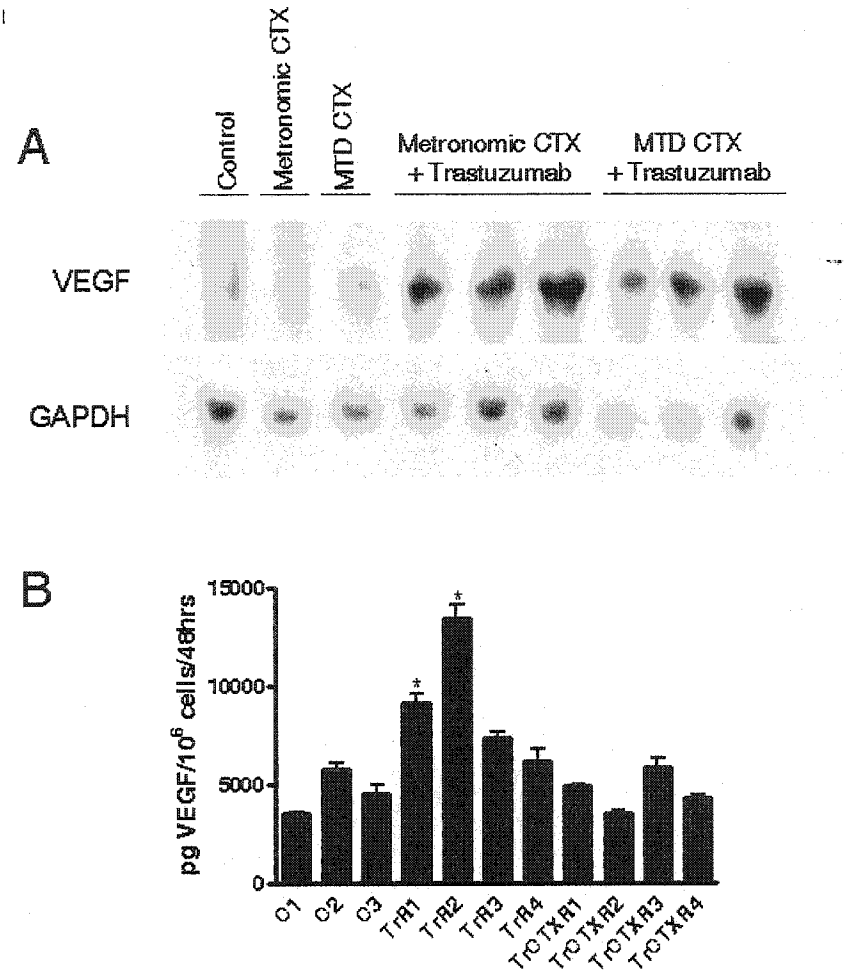

Figure 3.1: Involvement of VEGF in *in vivo* resistance to trastuzumab.
(A) Upregulation of VEGF expression observed in Northern blot of total RNA isolated from tumors progressing after long-term therapy with combination CTX and trastuzumab, compared to control, metronomic and MTD CTX treated tumors. Probing with GAPDH was used as a loading control. **(B)** VEGF levels in conditioned media of tumor-derived cell lines cultured for 48 hours in serum-reduced media, as measured by ELISA (*$p<0.05$) (C=untreated tumors, TrR=trastuzumab resistant, TrCTX=trastuzumab and CTX resistant) (mean ± SD, n=4).

3.1 Characterization of trastuzumab resistant cell lines

Since the mechanisms of resistance to trastuzumab have rarely been studied in an *in vivo* context, we decided to further explore the underlying causes of the development of resistance to trastuzumab following long-term drug administration. Initially, *in vivo* clonal selection for cells expressing lower levels of the erbB2 target was suspected as the cause for loss of response to trastuzumab, as the cell line 231-H2N was derived from a polyclonal population of erbB2-transformed cells. Cell lines were therefore derived from untreated primary tumors and tumors that had become unresponsive to trastuzumab therapy alone or to combination with chemotherapy, and were analyzed for retention of erbB2 expression. Analysis of whole cell lysates for total erbB2 and EGFR protein by Western blot indicated that all cell lines maintained expression of EGFR, and 5 of 8 of these established resistant tumor cell lines still expressed high levels of the erbB2 protein, as has been shown by others (Spiridon et al, 2002; Kute et al, 2004). In these 5 cell lines, erbB2 maintains the extracellular trastuzumab-binding domain, as determined by the C-terminal recognition site of the antibody used to detect the protein (Figure 3.2). While the remaining 3 cell lines express lower levels of the full length erbB2 protein, it remains to be determined if these three erbB2 negative cell lines express a truncated or mutant form of the erbB2 receptor that is not recognized by the antibody used to probe the membranes. In addition, blots were probed for total protein levels of Akt and p44/42 MAPK, members of two pathways known to be regulated through the erbB receptors (Yarden & Sliwkowski, 2001). No change in the expression levels of these two proteins was detected.

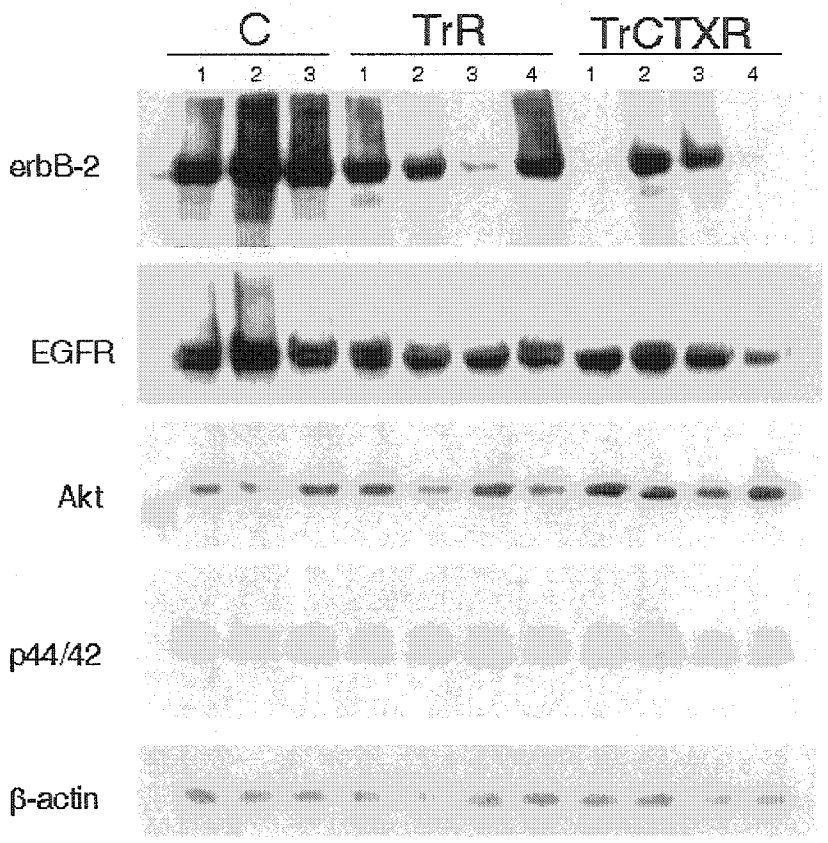

Figure 3.2: Western blot to determine expression of EGFR, erbB2, Akt and p44/42 MAPK in cell lines isolated from trastuzumab-resistant 231-H2N tumors. (C = untreated, TrR = after development of resistance to trastuzumab therapy and TrCTXR = after development of resistance to trastuzumab and CTX therapy). β-actin was used to confirm equal loading.

To determine if the acquired drug resistance was due to host-related mechanisms (i.e. increased drug clearance, poor drug penetration, or loss of ADCC function) versus genetic or epigenetic alterations in the tumor cell population, cell lines isolated from trastuzumab resistant tumors were assessed for *in vitro* and *in vivo* sensitivity to trastuzumab. Two cell lines from trastuzumab-resistant tumors were selected for further investigation, one maintaining high erbB2 expression levels (TrR2) and one with lower levels of erbB2 expression (TrR3), and were compared to a cell line

derived from an untreated 231-H2N tumor (C1). The cytostatic effect of trastuzumab on the resistant cell lines was determined by ^{3}H-thymidine incorporation as a measure of cell proliferation. Cells were exposed to varying concentrations of trastuzumab for 72 hours. The anti-proliferative effects of trastuzumab initially observed for the 231-H2N cell line and in the C1 cell line was abolished in the two resistant cell lines, suggesting that the mechanism of resistance is not a transient or host-mediated effect (Figure 3.3A).

To further strengthen this conclusion, tumor cells established from untreated tumors and from tumors resistant to trastuzumab were injected into the mammary fat pads of female SCID mice and tumor growth was monitored. Treatment with trastuzumab was commenced when tumors reached volumes of 200-450 mm^3. While trastuzumab was able to inhibit the growth of tumors established from previously untreated 231-H2N primary tumors (C1), the two trastuzumab-resistant tumor derived cell lines tested *in vivo* showed only a slight, statistically insignificant, response to drug treatment (TrR3) and a complete lack of efficacy (TrR2), similar to that observed for the parental untransduced cell line MDA-MB-231 (Figure 3.3B). The characterization of these two cell lines, for erbB2 expression (Figure 3.2) as well as VEGF secretion (Figure 3.1) indicated that while TrR2 maintained expression of erbB2 and had significantly greater *in vitro* VEGF expression compared to control cell lines, TrR3 had comparatively low erbB2 expression and only a slight increase in VEGF expression. Since these two cell lines have very different characteristics yet they both retain acquired resistance to trastuzumab *in vivo*, this suggests that mechanisms of resistance may not involve erbB2 signaling or upregulation of VEGF, at least not exclusively.

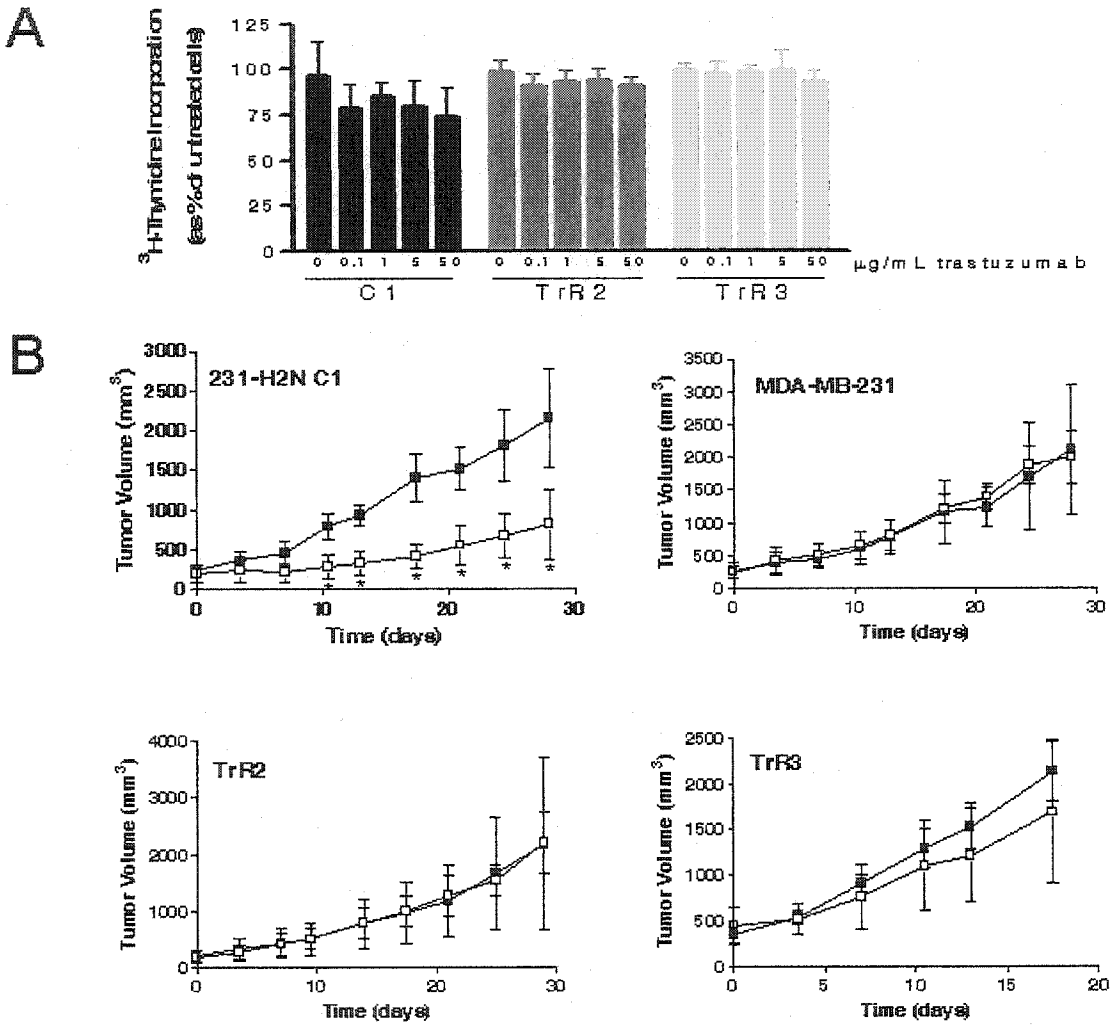

Figure 3.3: Resistance to trastuzumab is sustained *in vitro* and *in vivo* in cell lines derived from 231-H2N tumors unresponsive to trastuzumab therapy (TrR2 and TrR3), but cell lines derived from untreated tumors (C1) are still sensitive to trastuzumab. **(A)** *In vitro* inhibition of cell proliferation of 231-H2N variants after 72 hours of treatment with trastuzumab, measured by [^{3}H]-thymidine incorporation and reported as % of cell proliferation relative to untreated cells (mean ± SD, n=8). **(B)** Loss of trastuzumab induced inhibition of *in vivo* growth of orthotopic tumors from tumor-derived cell lines (■ control, □ trastuzumab) (mean ± SD, n=4).

3.3 De facto reversal of trastuzumab resistance by treatment with targeted therapeutics

Resistance to trastuzumab is a clinical reality, yet it has been given little experimental attention, leaving oncologists with little information about possible alternative (salvage) lines of follow-up therapy. The observed increase in VEGF in tumors that rapidly progressed following CTX therapy combined with trastuzumab led to further studies to identify potential second-line therapies to block tumors with an acquired resistance to trastuzumab.

As preliminary studies have yet to identify the mechanism(s) by which VEGF is upregulated in our trastuzumab resistant tumors, a logical second line therapy would be the addition of a VEGF-directed therapy such as the anti-VEGF antibody, bevacizumab. Since bevacizumab shows specificity to human and not mouse VEGF (Lin et al, 1999), the efficacy of this drug in our model system will be an indication of the importance of tumor cell-derived VEGF rather than host cell-derived VEGF for tumor growth in our model system. Determining the role of VEGF is also of importance since VEGF was the only angiogenic factor that was measured in these studies, and is certainly not the only one that may be playing a role in progression of tumors after long-term therapy.

One proposed mechanism of resistance to trastuzumab is the overexpression of EGFR ligands (Ritter et al, 2003; Valabrega et al, 2003). MDA-MB-231 cells have been shown to have autocrine activation of the EGFR/transforming growth factor-α (TGF-α) signaling cascade, which is one of the many pathways involved in regulating the expression of VEGF (Masuda et al, 2002). If the VEGF increase is indeed due to the autocrine expression of TGF-α, targeting the EGFR with the antibody cetuximab might also be effective at delaying tumor growth in trastuzumab-resistant tumors through

inhibition of EGFR signaling. Several groups have shown that the small molecule EGFR tyrosine kinase inhibitor, ZD1839, effectively inhibited cell proliferation and tumor growth in EGFR/erbB2 co-expressing cells, and noted an additive effect when this drug was combined with trastuzumab (Moulder et al, 2001; Warburton et al, 2004; Normanno et al, 2002). Cetuximab has only been combined with trastuzumab *in vitro*, but showed co-operative induction of antiproliferation of human ovarian tumor cells (Ye et al, 1999). The recent development of a dual-erbB2/EGFR small molecule tyrosine kinase inhibitor, GW572016, is also showing promise in preclinical studies (Zhou et al, 2004; Xia et al, 2002, 2004).

To determine if TGF-α was upregulated in the cell lines derived from tumors resistant to long-term trastuzumab or trastuzumab and metronomic CTX therapy, levels of TGF-α secreted into the conditioned media was measured by ELISA. While the control (C) cell lines, derived from untreated 231-H2N cells expressed on average 6 pg TGF-α/mL media/48 hours, 3 of the 4 cell lines derived from trastuzumab resistant tumors (TrR) expressed elevated levels of 15-34 pg TGF-α/mL media/48 hours (Figure 3.4). This elevation was not observed in the cell lines derived from trastuzumab and CTX tumors (TrCTXR), suggesting that these cell lines may not become resistant to trastuzumab through the same mechanism. This would also predict that trastuzumab and EGFR-targeted therapies would be more effective in trastuzumab-resistant tumors than trastuzumab and CTX resistant tumors.

The erbB2 heterodimerization inhibitor pertuzumab, has also been shown to be effective at inhibiting tumor growth rates in our 231-H2N model system both *in vitro* and *in vivo* and has been reported to act synergistically in combination with trastuzumab (Nahta et al, 2004). An improved anti-tumor efficacy of combining erbB2-directed antibodies recognizing multiple epitopes on the extracellular domain of erbB2 was also

shown by Spiridon et al (2002) when compared to the same antibodies used individually. Using pertuzumab for trastuzumab resistant tumors may be a potential second-line therapy as it inhibits the dimerization of erbB2 with EGFR and erbB3, both of which are potent heterodimers involved in the expression of VEGF (Yen et al, 2002). Pertuzumab is currently in Phase II clinical trials for a variety of cancer types, including erbB2 negative breast cancer, as this drug has shown *in vitro* and *in vivo* efficacy in cell lines that are not considered to be overexpressors of erbB2 (Agus et al, 2002). This efficacy in erbB2 negative cancers is important as clonal selection of cells expressing lower levels of erbB2 may be involved in some circumstances of trastuzumab resistance in those studies presented here, and potentially in the clinic.

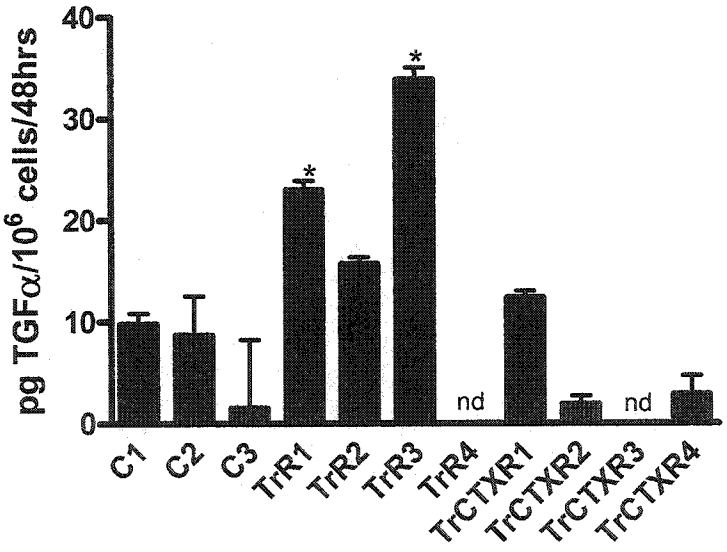

Figure 3.4: *In vitro* TGFα expression in cell lines derived from 231-H2N tumors. (C = untreated, TrR = after development of resistance to trastuzumab therapy and TrCTXR = after development of resistance to trastuzumab and CTX therapy). Data reported as mean ± SD, n=3.

A relatively new target, at least in terms of drug development in the area of cancer therapeutics is IGF-1R, which is overexpressed in a variety of cancer types, including breast cancer, and is involved in regulating a number of cellular processes that potentiate malignancy. Of particular interest to this study is the reported resistance of breast cancer cell lines co-expressing IGF-1R and erbB2 to trastuzumab (Lu et al, 2001), and the reversal of this resistance when IGF-1R signaling is inhibited (Camirand et al, 2002). Mechanistic studies suggest that an increase in $p27^{kip1}$ degradation as a result of IGF-1R signaling prevents the upregulation of $p27^{kip1}$ normally induced by trastuzumab through downregulation of erbB2 signaling, thereby antagonizing the ability of trastuzumab to induce cell cycle arrest (Lu et al, 2004). A further rationale for testing the importance of this receptor in our model system was a recent report that erbB2 interacts with IGF-1R receptors to form a heteromeric complex, and IGF-1R is capable of phosphorylation of the erbB2 receptor (Balaňá et al, 2001). A fully humanized monoclonal antibody to the IGF-1R, A12, which has been found to block ligand-dependent signaling of the receptor (Burtrum et al, 2003), was therefore evaluated as a second-line therapy in our trastuzumab-resistant tumors, as the 231-H2N cell line was found to express IGF-1R (data not shown).

The effects of these second-line therapies were assessed in two experiments, where tumors had become resistant to trastuzumab therapy alone and trastuzumab in combination with low-dose CTX. Our rationale for carrying out both of these experiments was the observation that while 75% of the cell lines derived from tumors resistant to trastuzumab therapy alone maintained high levels of expression of erbB2, only 50% of the cell lines derived from tumors resistant to combination therapy still expressed erbB2, leading us to believe that a reduction or loss of erbB2 expression may occur after more prolonged trastuzumab therapy, which is made possible by the

increased efficacy of adding metronomic CTX to trastuzumab. It was therefore important to determine if these second-line therapies would be effective in both scenarios of trastuzumab resistance, as trastuzumab is used both as a single agent therapy and in combination with chemotherapeutic agents in the clinical setting.

Mice with 231-H2N primary tumors were treated with trastuzumab alone or trastuzumab and metronomic CTX until tumors began to progress (28 and 98 days, respectively). Mice were maintained on this therapy, and treatment with bevacizumab, cetuximab, pertuzumab or A12 was commenced. There is ongoing debate amongst clinicians as to whether or not to continue the administration of trastuzumab when treating metastatic breast cancer patients with progressive disease. A recent study indicated that the continuation of trastuzumab posed no increased risk to patient safety and that there was a modest response to continuing trastuzumab with alternative chemotherapeutic agents (Gelmon et al 2004; Tripathy et al, 2004). Trastuzumab therapy was continued in the studies presented here as there was concern that the drug was still having some anti-tumor effects, and that by removing trastuzumab the tumors would rapidly progress, thereby concealing any efficacy of the additional therapies tested.

In the trastuzumab-resistance study, bevacizumab was more effective at blocking and delaying tumor growth than any of the other therapeutic strategies, but all therapies showed some delay in tumor growth compared to the group maintained on trastuzumab alone. Cetuximab, pertuzumab and A12 all slowed the rate of tumor growth, without a complete delay in tumor growth (Figure 3.5A). The anti-tumor effect of these three therapies was similar for approximately 14 days, at which time the tumor growth rate in mice treated with pertuzumab or cetuximab began to increase. A12 maintained a consistent anti-tumor effect, allowing a continuous, yet gradual increase in tumor growth

over the 34 days of treatment. Our studies indicate that bevacizumab induces a more effective inhibition of tumor growth compared to the other therapeutics, but this effect is limited as with time the tumors again begin to slowly progress.

For mice bearing tumors resistant to trastuzumab and metronomic CTX, monitoring of tumor growth illustrated that bevacizumab was the most effective therapy at delaying tumor growth for a period of time after initiation of second-line treatment (Figure 3.5B). In contrast, the addition of pertuzumab provided no benefit compared to those mice maintained only on trastuzumab and metronomic CTX. Cetuximab appeared to delay the tumor growth rate for a few weeks prior to the onset of rapid tumor progression. The A12 antibody indicated an impressive delay in tumor growth similar to bevacizumab, but mice had indications of extensive metastases throughout the peritoneal cavity. Mice from all treatment groups were sacrificed at treatment day 144 due to poor general health, which could be associated with long-term administration of CTX, systemic response to the multiple antibody-based therapies used, or a consequence of bearing tumors for greater than 180 days. The health status of the mice in the trastuzumab-only experiment did not decline, but these mice were younger, had tumors for much shorter periods of time, and were not given CTX.

Together, these results suggest that in our breast carcinoma model system, VEGF is actively involved in the progression of tumors resistant to trastuzumab, and implies that bevacizumab may be an effective therapy to consider as a second-line agent for the treatment of breast cancer patients no longer responding to trastuzumab due to VEGF upregulation. In addition, EGFR targeted drugs, such as cetuximab, may be effective agents in breast cancer patients with both erbB2 amplification and expression of EGFR, as suggested by the delay in tumor progression in these studies when cetuximab was administered to trastuzumab-resistant tumors which express both

erbB2 and EGFR. While pertuzumab was effective at delaying tumor growth in trastuzumab-resistant tumors, this effect was not observed in those mice also receiving metronomic CTX. This may be an indication that while erbB signaling is still playing a key role in cell proliferation in trastuzumab-resistant tumors, other signaling pathways are overriding the erbB network in tumors that also received metronomic CTX. Further work is necessary to confirm this result and determine why such differences were observed in this study. The efficacy of A12 indicates that IGF-1R signaling is important for tumor growth in our model system, and shows promise as a targeted therapy for breast cancers that are resistant to molecular targeting agents such as trastuzumab. However, the metastases observed in the trastuzumab/CTX/A12 combination group is of obvious concern and must be further addressed.

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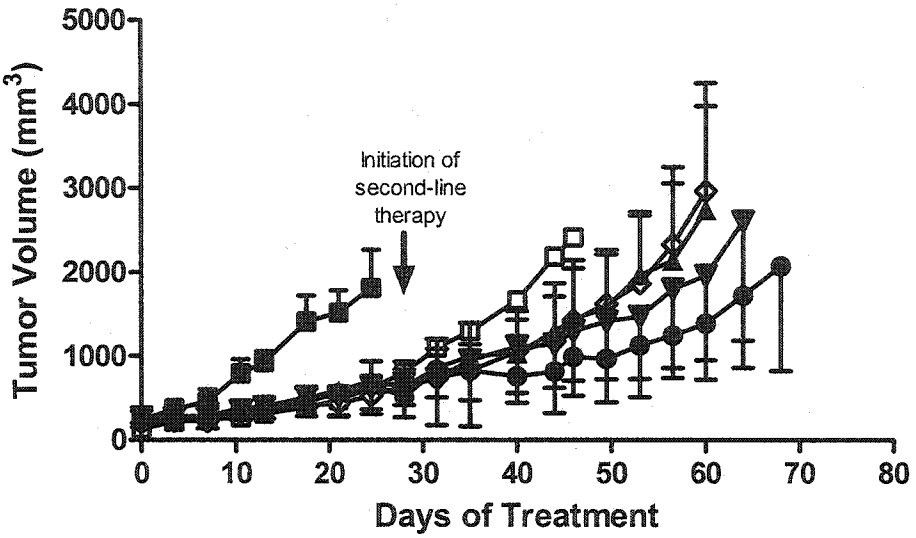

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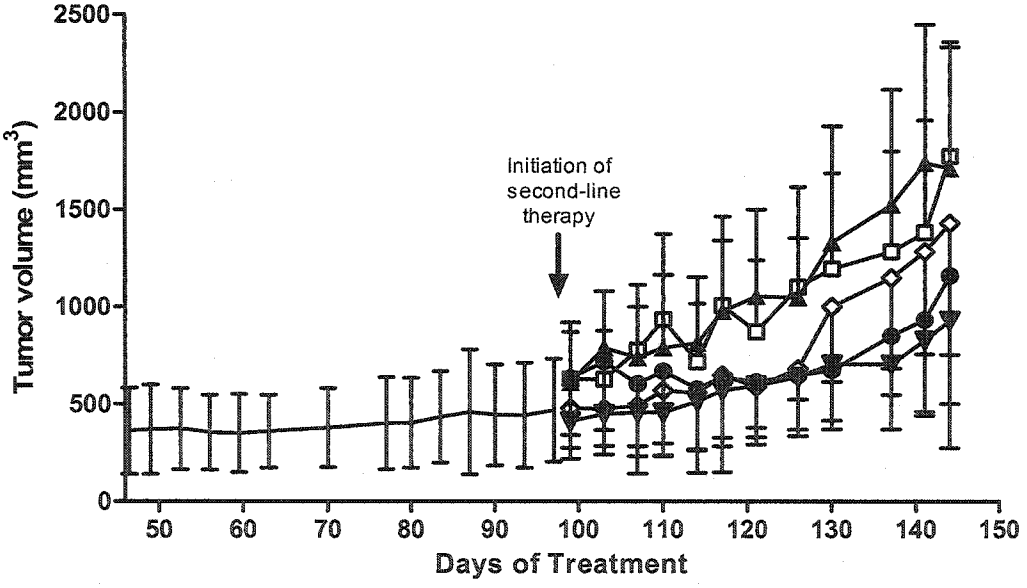

Figure 3.5: Abrogating drug resistance with second-line therapies. A) Addition of second line therapies to block rapid tumor growth after development of resistance of 231-H2N tumor bearing SCID mice to trastuzumab. Mice were maintained on trastuzumab (n=6-7). B) Addition of second line therapies to block rapid tumor growth after long-term treatment with trastuzumab and metronomic CTX. Mice were maintained on trastuzumab and oral metronomic CTX (n=4). (■ untreated controls, □ trastuzumab ▲ pertuzumab, ◇ cetuximab, ● bevacizumab, ▼A12). All data reported as mean ± SD.

4 Discussion and clinical relevance

4.1 Anti-tumor and antiangiogenic effects of trastuzumab and metronomic chemotherapy

Overexpression of erbB2/Her-2/neu, a member of the epidermal growth factor receptor family, is detected in the tumor cells of 25-30% of breast cancer patients (Slamon et al, 1987), who are therefore candidates for therapy with the humanized monoclonal anti-erbB2 antibody, trastuzumab, or other erbB2 targeting agents. While trastuzumab has been approved as a single-agent therapy for advanced metastatic disease (Arteaga, 2003), this drug is more commonly combined with paclitaxel or occasionally a combination chemotherapy regimen containing CTX, methotrexate and 5-fluorouracil. The combination of trastuzumab with high dose MTD chemotherapy eliminates or minimizes the potential benefits of using a relatively non-toxic drug such as trastuzumab. Furthermore, as the use of trastuzumab is extended into the adjuvant setting where longer term therapy regimens are employed, there is a need to assess whether this drug can be combined with an alternative to highly toxic, maximum tolerated doses of chemotherapy that are nevertheless effective. By way of example, some chemotherapy drugs such as UFT, a 5-FU prodrug comprised of uracil and tegafur, can be taken orally by early stage, resected lung cancer patients at low-dose every day for 2 years as adjuvant therapy (Kato et al, 2004)

Our studies indicate that low-dose, metronomic CTX in combination with trastuzumab was as effective as MTD CTX combination therapy at blocking tumor growth for extended periods of time as well as delaying the development of resistance to trastuzumab. Yet unlike MTD CTX combination therapy, which showed evidence of

toxicity through weight fluctuations and severe weight loss, the chronic administration of oral metronomic CTX on a daily basis had no indications of serious long-term toxicity, with the exception of slight weight loss over time, even for treatment periods extending for up to 140 days. As a consequence of the results obtained from tumor perfusion and Matrigel plug angiogenesis assays, we suggest that the prolonged anti-tumor efficacy of metronomic combination therapy may in part be due to the additive antiangiogenic effect of these two drugs. It has been reported by others that trastuzumab, when combined with weekly low-dose paclitaxel, more effectively suppresses tumor growth, metastasis and erbB2-mediated angiogenesis, than either therapy alone, in an orthotopic breast tumor model (Klos et al, 2003). Our results confirm and extend this observation with an additional low-dose metronomic chemotherapy regimen, CTX through the drinking water, which mimics the long-term treatment of metastatic breast cancer patients with daily low-dose CTX with no breaks (Colleoni et al, 2002), and supports the additive antiangiogenic effects of combination therapy.

The rationale for the decision to use CTX for our studies may be questioned in view of the fact that trastuzumab is often combined with taxane-based drugs in the clinic. There are several reasons for our choice of chronic administration of CTX. First, we have accumulated considerable information about metronomic CTX regimens including the use of a human regimen of delivering the drug orally on a daily basis, in our studies through the drinking water. This type of regimen is convenient for the type of prolonged therapy studies that we have undertaken. Second, we have determined the approximate optimal daily dose for this metronomic regimen, which is 20-25 mg/kg/d. We do not yet have such information on taxane- or platinum-based drugs. Third, our results could lay the groundwork for future clinical trials involving daily low-dose CTX and trastuzumab, in the metastatic setting, and perhaps in the adjuvant therapy setting as well. For example,

based on preclinical results and phase II clinical trial studies of daily low-dose oral CTX (50 mg) in breast cancer (Colleoni et al, 2002), trials have been initiated in advanced breast or ovarian cancer using daily low-dose CTX and bevacizumab, administered every two weeks (Kerbel & Kamen, 2004). Lastly, it has been shown, in both *in vivo* and *in vitro* (Pegram et al, 1999, 2004) contexts, that CTX in combination with trastuzumab has synergistic effects, and is therefore a rational combination for treatment of erbB2 positive breast cancer patients in the clinic, emphasizing the importance of carrying out preclinical studies, such as that presented here, to determine optimal chemotherapy dosing regimens. In this regard, a major priority in the Kerbel laboratory at the present time is to develop surrogate markers (eg circulating endothelial progenitor cells) that can be used to determine the optimal biological dose for antiangiogenic drugs or treatments, including low-dose metronomic chemotherapy (Shaked et al, 2004).

4.2 Acquired resistance to trastuzumab

The prolonged delay in tumor growth, followed by a fairly rapid progression in those treatment groups receiving trastuzumab therapy with or without CTX, was not entirely unexpected due to the frequent response, and then relapse, of patients treated with trastuzumab for metastatic erbB2 positive breast cancer (Cobleigh et al, 1999). Nevertheless, to our knowledge, our study is the first to report a preclinical example of such a relapse *in vivo* following long-term treatment, as a review of the literature revealed that there is an absence of long-term studies using trastuzumab therapy. Our studies have shown how chronic therapy experiments can be particularly useful for the evaluation of the ability of combination therapies to prevent or delay the onset of such resistance to trastuzumab. Considering the clinical importance of elucidating the potential mechanisms involved in the acquired resistance to trastuzumab, it is surprising that this area is only now just starting to be explored in more detail. Our results suggest

that this resistance to trastuzumab is likely the consequence of a genetic alteration in the cells, and not due to host-mediated effects, since resistance was maintained both *in vitro* and *in vivo* when re-injected into mice.

While clonal selection may play a role in the development of resistance in some circumstances, the majority of cell lines obtained from resistant tumors in this study maintained overexpression of the erbB2 gene, suggesting that other tumor cell alterations are required to lead to the observed resistant phenotype. It is interesting to note that while 25% of tumors resistant to trastuzumab did not retain expression of erbB2, 50% of tumors resistant to trastuzumab and CTX, which were treated for much longer periods with trastuzumab, appeared to have lost erbB2 expression. This may indicate that prolonging the duration of trastuzumab therapy by combining it with chemotherapy may potentiate the selection of cells expressing lower levels of erbB2, which emphasizes the importance of finding alternative therapies not targeted to erbB2 for treatment of tumors no longer responding to trastuzumab treatment.

While an upregulation of VEGF in tumors resistant to trastuzumab therapy was observed in our studies, further work is required to confirm this observation and identify the mechanism(s) by which this occurs. We could suggest a number of mechanisms that could potentially be involved in an upregulation of VEGF, e.g. upregulation of IGF-1R signaling (Oh et al, 2002; Fukuda et al, 2002; Stoeltzing et al, 2003) or that of other tyrosine kinase receptors, acquisition of PTEN or p53 mutations leading to loss of function (Bose et al, 2002; Zundel et al, 2000; Ravi et al, 2000), constitutive activation of PI-3 kinase (Viloria-Petit et al, 2004) or nuclear factor-κB (Shibata et al, 2002), the development of hypoxia-inducible factor-α (HIF1-α) independent VEGF expression (Koukourakis et al, 2003) or changes in expression or degradation of HIF1-α (Laughner et al, 2001). It has also not been ruled out that other angiogenic factors, in addition to

VEGF, may also be involved in this resistance. A delicate equilibrium between anti- and pro-angiogenic factors is required to prevent angiogenic processes, and it is possible that an upregulation of only one potent pro-angiogenic factor, such as VEGF, would be all that is required to promote angiogenesis, and subsequent rapid tumor growth, as we have observed in our studies. It is important to note that this VEGF increase was not consistently translated to resistant cell lines when cultured *in vitro*, which implies that this may also be a feature of the tumor microenvironment, which cannot be reproduced in conventional monolayer *in vitro* culture systems.

Regardless of the mechanism by which tumors become resistant to trastuzumab, addition of an anti-VEGF directed regimen, such as the antibody bevacizumab, to the trastuzumab therapy can be rationalized by both the upregulation of VEGF through the erbB2 oncogene (Viloria-Petit et al, 1997, Laughner et al, 2001) and our present finding that VEGF is upregulated within the tumor microenvironment upon acquisition of a resistant phenotype. The preliminary studies presented here, using our orthotopic tumor model, indicate that resumed tumor growth due to resistance to trastuzumab temporarily can be blunted by the addition of bevacizumab, the anti-VEGF monoclonal antibody, to the drug cocktail. It is important to note that bevacizumab targets only the tumor-derived human VEGF, and not the circulating mouse VEGF, thereby emphasizing the important role that this tumor-derived VEGF is playing in the progression of tumors resistant to combination therapy in our model system. It would be interesting to determine whether or not the administration of these two drugs prior to the development of resistance to trastuzumab therapy, as suggested by Pegram & Reese (2002) and Konecny et al (2004), could delay tumor regrowth due to the development of drug resistance. The derivation of cell lines unresponsive to treatment with trastuzumab, as carried out in these studies, will provide the opportunity to further study the mechanisms involved in

resistance to trastuzumab, and to continue the search for additional therapies that may be beneficial to patients no longer responding to trastuzumab. In any case, our results do provide a new rationale for undertaking clinical trials using a combination of two drugs such as bevacizumab and trastuzumab.

The administration of the anti-EGFR antibody, cetuximab, to trastuzumab-resistant tumors was also effective at delaying tumor progression compared to those mice maintained only on metronomic CTX with trastuzumab. While both EGFR and erbB2 are potent homodimers to induce the expression of VEGF, two heterodimers, erbB2/erbB3, and even more potently, EGFR/erbB2, induce VEGF expression and enhance tumor angiogenesis (Yen et al, 2002). Therefore, by simultaneously blocking EGFR and erbB2 signaling with cetuximab and trastuzumab respectively, the formation of the most potent heterodimer to regulate VEGF may be prevented, while also more efficiently inhibiting tumor cell proliferation/survival mediated by EGFR signaling. Whereas 25-30% of breast cancer patients overexpress erbB2, only 5.4% overexpress both EGFR and erbB2 (Witton et al, 2003), which leads to a particularly aggressive tumor with significantly worse disease-free survival than overexpression of either receptor alone (Torregrosa et al, 1997). Therefore, unless EGFR is upregulated through the development of resistance to trastuzumab, this may not be as appropriate a second line drug therapy as bevacizumab or other direct antiangiogenic inhibitors, since so few breast cancer patients would fit the criteria for cetuximab therapy. As a result, it will be of considerable interest to determine if resistance to trastuzumab in the clinic is accompanied by elevated expression or function of normal (wildtype) or mutated EGF receptors.

Pertuzumab, the erbB2 heterodimerization inhibitory antibody also showed efficacy in tumors resistant to trastuzumab therapy, but this effect was absent in tumors

resistant to trastuzumab and low-dose metronomic CTX. This lack of effect may be due to the loss of erbB2 overexpression observed with a greater frequency in cell lines derived from tumors treated with combination therapy than with trastuzumab alone. Since pertuzumab binds to the erbB2 receptor and blocks the ability of erbB2 to interact with the erbB3 and EGFR receptors, a loss of the erbB2 target may result in a lack of therapeutic efficacy of this therapy.

The anti-IGF-1R antibody, A12, shows potential at blocking tumor progression in trastuzumab-resistant tumors, yet as was observed with bevacizumab and cetuximab, the effect was not sustainable for extended periods of time. The metastases observed only in mice receiving trastuzumab, A12 and oral CTX is of significant concern and must be further explored. A recent report by Pennisi et al (2002) suggests that targeting of the IGF-1R may lead to a more metastatic phenotype. *In vitro* experiments using breast cancer cells indicated that reduction of IGF-1R expression by antisense technology increased cell motility, while decreasing both attachment and aggregation of the cells. It is plausible that targeting of the IGF-1R, which has previously been shown to block ligand-induced signaling from the receptor (Burtrum et al, 2003), could lead to the development of this same metastatic phenotype.

4.3 Clinical relevance

Although trastuzumab was clinically approved by the United States FDA in 1998, questions still remain as to how this drug is best administered in the clinical setting, including which chemotherapy drugs to combine with trastuzumab and what dosing schedules to use for both trastuzumab and the chemotherapeutic agents it is combined with. As the administration of trastuzumab moves into the adjuvent setting, the need for non-toxic dosing schedules is becoming of significant importance. Colleoni et al (2002) have already shown that chronic, continuous low-dose scheduling of chemotherapy may

be an effective and non-toxic treatment either in the metastatic or adjuvant treatment setting. Should the results from our study be confirmed in additional orthotopic xenograft tumor models, there would be a strong rationale for commencing clinical trials to assess the quality of life and survival benefits of low-dose, metronomic chemotherapy in combination with trastuzumab compared to the standard dosing schedules used.

While our studies do not elucidate the mechanisms involved in resistance to trastuzumab, the improved anti-tumor effects of combining therapeutic regimens with independent targets (such as VEGF and erbB2, or IGF-1R and erbB2) raises the question of whether such combination therapies would be effective in our *in vivo* model in delaying the onset of trastuzumab resistance by simultaneously inhibiting multiple intracellular signaling pathways. While these combination therapies may prove to be effective preclinically, the financial burden associated with combining multiple targeted therapies may prevent their translation to the clinic, as the tremendous cost of targeted therapies is something neither public or private health care systems would be prepared to cover (Herper, 2004).

4.4 Future directions and considerations

One obvious limitation of these studies is the cell line which was selected for use in our *in vitro* and *in vivo* studies. Since a clonal population of transduced cells was not used to create the 231-H2N cell line, the expression of erbB2 in this cell line is somewhat variable over the cell population. While erbB2 protein expression was confirmed by western blot after long-term culturing of the cell line *in vitro* and from cell lines isolated from tumors grown in SCID mice, this method of detection does not allow for the detection of slight changes in protein expression levels, and more importantly, changes in the functionality of erbB2-directed signaling in the cell line. And while erbB2 was detected in cell lines isolated from trastuzumab-resistant 231-H2N tumors, this is

not sufficient evidence to confirm that erbB2 remains active and functional in tumors treated with trastuzumab for long periods of time (ie resistance to trastuzumab could be related to the growth of tumor cells with a lack of signaling through erbB2). For this reason, the efficacy of metronomic chemotherapy and trastuzumab combination therapy should be confirmed in a model system using a cell line endogenously and constitutively overexpressing erbB2, such as BT-474 or SK-BR3, in spite of the reported difficulties of growing this tumors in the mammary fat pad of mice. Of particular interest would be the pattern of development of resistance in tumors grown from these cell lines.

Some concern has been raised with respect to the toxicity associated with the MTD dose of 100 mg/kg CTX selected for the anti-tumor studies presented here. This dose was selected from previous work carried out in the Kerbel laboratory (Man et al, 2002), which found 120 mg/kg, but not 100 mg/kg to be lethal to SCID mice. It is postulated that the duration of our *in vivo* studies presented CTX-related toxicity that was not previously observed in prior more short-term experiments. It is advisable that the MTD CTX dose used for such *in vivo* experiments as those presented here be re-established, and tumor response experiments be carried out at several doses below the 100 mg/kg used in these studies to find an optimal MTD dose with manageable toxicity and reasonable tumor response. Ideally, in future studies, MTD CTX should be administered over the same duration as metronomic CTX, with no lethal side effects, such that a better comparison between metronomic and MTD CTX dosing regimens can be made.

Our results suggest that VEGF may be contributing to the development of resistance to long-term treatment with trastuzumab. To confirm this, it is recommended that 231-H2N be transfected with a vector designed to endogenously overexpress VEGF, and tumors grown from these cell lines be treated with trastuzumab and trastuzumab in combination with metronomic CTX. It is anticipated that if VEGF is indeed involved in the development of resistance to this therapy, there will be a reduced

effect of these therapies compared to the parental 231-H2N tumors. This same experiment could be repeated with TGFα overexpressing cell lines to determine if the paracrine EGFR-TGFα loop is also involved in the development of resistance to trastuzumab.

Finally, as suggested by Pegram and Reese (2002), there is a rationale for combining bevacizumab with trastazumab for the treatment of erbB2 overexpressing tumors, and indeed, a trial testing this combination has already been initiated in the United States. The rationale is that erbB2 overexpressing breast cancers show an upregulation of VEGF, which appears to be due to activation of erbB2 (Viloria-Petit, 1997; Konecny et al, 2004). Moreover, our laboratory and others have shown that blocking erbB2 can result in partial, but not full, blockade of VEGF expression (Viloria-Petit, 1997). In addition, Jain's laboratory has found that trastuzumab treatment of erbB2 positive expressing tumors *in vivo*, while leading to a reduction in tumor cell VEGF, is accompanied by an increase of VEGF expression in the tumor stroma (Izumi et al, 2002). These investigators believe that trastazumab acts as an antiangiogenic drug by increasing expression of TSP-1 in the tumor cell population, rather than by blocking tumor cell VEGF - hence, the rationale for combining two drugs such as trastuzumab and bevacizumab. Our results provide a new and additional rationale, namely, that development of resistance to trastuzumab may be mediated in part by the upregulation of VEGF. While our studies suggest that bevacizumab is an effective therapy with which to delay the growth of trastuzumab-resistant tumors, the next question to answer is whether treating 231-H2N tumors with a combination of bevacizumab and trastuzumab from the initiation of treatment (ie when tumors reach 250 mm^3) would delay or perhaps even prevent the onset of resistance. While resistance to this therapeutic combination

would likely occur at some point, it would also be of interest to determine if an alternative mechanism(s) to VEGF led to the development of resistance.

With respect to combining either bevacizumab or trastazumab (or both) with metronomic chemotherapy regimens, an additional new point to consider about testing such regimens in the clinic derives from the enormous cost of targeted genetically engineered drugs such as bevacizumab, cetuximab (C225/Erbitux), or trastuzumab. These drugs can cost anywhere from between US$4,000 to US$10,000 a month. Therefore the combination of such targeted agents would pose an unsustainable economic burden on either private or government funded health care systems (Herper, 2004). The huge cost of targeted therapies highlight the urgent need of combining such targeted agents with other drugs/regimens that are far less expensive such as daily oral low-dose metronomic CTX - provided that there is a strong scientific rationale backed up by equally strong preclinical science for the testing of such drug combinations. We hope that the results presented in this thesis represent a first, but important, step for the rationale of testing targeted oncogene directed therapies such as trastazumab in combination with metronomic chemotherapy regimens, such as daily low-dose cyclophosphamide which costs about $15 - $20 a month.

4.5 Conclusion

In conclusion, our studies indicate that metronomic chemotherapy may be an acceptable, if not preferable, alternative to MTD chemotherapy when combined with trastuzumab for the treatment of erbB2 overexpressing breast cancer, and perhaps more importantly, may be a more cost effective, potentially self-administered therapy that has efficacy, but with reduced toxicity compared to conventional MTD chemotherapy. Our studies also indicate possible strategies to deal with breast cancers which respond, and then acquire resistance to trastuzumab, provided our results can be confirmed in additional preclinical models and in clinical samples.

5 Materials and methods

5.1 Cell lines and culture conditions

Human breast tumor cell lines, MDA-MB-361, BT-474 and SK-BR3, were purchased from American Type Culture Collection (ATCC; Rockville, MA) and maintained as monolayers in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA). The human breast tumor cell line, MDA-MB-231, was kindly provided by Dr. Janet E. Price (MD Anderson Cancer Centre, University of Texas, USA). MDA-MB-231 cells were transduced to stably overexpress erbB2, as described in detail below, to generate the cell line 231-H2N. Both MDA-MB-231 and 231-H2N were maintained as monolayers in DMEM with 5% FBS. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

5.1.1 Generation of 231-H2N cell line

Full-length human Her2/neu cDNA was inserted into the pSV/IRES/enYFP shuttle vector using Nco1/Cla1 cut sites. A Nco1/Not1 fragment containing the her2/neu/IRES/enYFP was then transferred into the retroviral plasmid pUMFG. Sequencing was carried out to confirm fidelity of the Her2/neu DNA.

Amphotropic GP+envAM12 packaging cells were grown in DMEM containing 10% FBS, 2mM L-glutamine, 100units/mL Penicillin, and 10ug/mL Streptomycin (Bio Whittaker Molecular Applications, Inc., Rockland, ME, USA), to 90% confluency. pUMFG/her2/neu/IRES/enYFP (7mg) and the plasmid pGT-N28 (0.7mg, New England Biolabs), which contains the neo^R gene, were cotransfected into chloroquine-treated packaging cells using $CaCl_2$. Transfection was allowed to take place for 10 hours, prior

to commencement of G418 selection at 800mg/mL (Life Technologies, Gaithersburg, MD).

MDA-MB-231 cells were transduced for 12 consecutive days, replacing media daily with fresh conditioned media from the AM12/her2/neu/enYFP producer cells, filtered through a 0.45μm filter. Selection of transduced MDA-MB-231 cells was carried out by fluorescence-activated cell sorting (FACS; BD Biosciences, Mississauga, ON). Briefly, cells were stained with PE-conjugated anti-Her2 antibody (BD Biosciences) as directed by the manufacturer and those cells expressing the highest erbB2 and enYFP levels (top 10%) were isolated by FACS. These cells established the cell line 231-H2N.

5.1.2 Protein Isolation and Western Blots

Cells were grown to ~80% confluency in normal culture conditions. Plates were placed on ice, washed with ice-cold PBS, and cold lysis buffer (20mM Tris pH 7.5 containing 137mM NaCl, 100mM NaF, 10% glycerol, 1% NP40, 2mM Na_3VO_4, 1mM PMSF, 10μg/mL aprotinin and 10μg/mL leupeptin) was added directly to plates. Cells were scraped from plates, incubated on ice for 40 minutes and centrifuged at 10,000 rpm for 15 minutes. Protein concentrations of the supernatant was determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA). 50 μg/lane of total proteins were resolved by 8% SDS-PAGE, transferred onto Immobilon-P membrane (Millipore (Canada) Ltd., Etobicoke, ON), blocked with 10% milk in tris-buffered saline with tween (TBST), and probed with primary antibody. Anti-neu (sc-284), anti-EGFR (sc-03) (Santa Cruz Biotechnology Inc, Santa Cruz, CA), and anti-β-actin (A-5441) (Sigma-Aldrich Canada Ltd., Oakville, ON) were incubated for 1 hour at room temperature, while Akt (#9272), p44/42 MAP kinase (#9102), and phospho-erbB2 Tyr1248 (#2244) (all from Cell Signaling Technologies, Beverly, MA) were incubated overnight at 4°C. All primary antibodies were diluted 1:1000 in 5% milk in TBST,

detected with anti-rabbit IgG^{HRP} or anti-mouse IgG^{HRP} (Promega, Madison, WI) diluted 1:5000, and detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

5.1.3 *In vitro* determination of drug sensitivity

To assess the cytostatic effects of trastuzumab, two thousand cells were plated in 96 well flat bottom tissue culture plates in a volume of 100 μL media per well, and incubated overnight at 37°C and 5% CO_2. Drug treatments were added to wells in a volume of 100 μL media, and were carried out for 72 hours. Cells were then pulsed with 2 μCi/well of methyl-[^{3}H] thymidine (Amersham Biosciences) for 6 hours, followed by rapid freezing of plates at -20°C. DNA was harvested from thawed plates onto Unifilter filtermat plates (Perkin Elmer Life Sciences, Woodbridge, ON) using a Titertek Cell Harvester (Flow Laboratories Ltd., Irvine, Ayr, UK). Incorporated radioactivity was measured by scintillation counting using a Packard Topcount NXT microplate counter (Perkin Elmer Life Sciences).

To determine cell line sensitivity to CTX 500 cells were plated as mentioned above and incubated for 24 hours. 4-hydroperoxycyclophosphamide (4-HC), a precursor to the active metabolite of CTX, was solubilized in sterile phosphate buffered saline (PBS) immediately prior to each drug administration, as the half life for 4-HC is only 90 minutes. 4-HC was added to each well at various concentrations, in a volume of 100 μL of media. For 6 consecutive days, 100 μL of media was removed, and replaced with fresh media containing 4-HC. Cells were pulsed with methyl-[^{3}H] thymidine as mentioned above. To determine if trastuzumab in combination with CTX had an additive or synergistic effect, two thousand cells were plated and allowed to adhere for 24 hours. 4-HC was added to each well at various concentrations and trastuzumab was added to

each well at 20 μg/mL (10 μg/mL final concentration). Every 24 hours for a total of 72 hours, 100 μL of media was removed from each well and replaced with fresh 4-HC and 10 μg/mL of trastuzumab. Again, cells were pulsed with methyl-[^{3}H] thymidine as mentioned above.

5.2 Orthotopic murine breast tumor model

Four week old female C.B-17/IcrCrl-scidBR severe combined immunodeficient (SCID) mice were purchased from Charles River Canada (Saint-Constant, QC). Mice were allowed to acclimatize for two weeks prior to implantation of tumor cells. To prepare cells for injection, confluent plates were harvested with 1% trypsin-EDTA (Gibco BRL), cells were then washed with phosphate buffered saline, pH 7.4, and resuspended in DMEM at $4x10^7$ cells/mL. A volume of 50 μL cell suspension ($2x10^6$ cells) was injected into the abdominal mammary fat pad of isoflurane-anaesthetized mice. Wounds were secured with surgical staples.

Palpable tumors were noticeable within 7-10 days of injection, and tumors reached a volume of 250 mm^3 (measured by veniper calipers and calculated by (length x width^2)/2) within 5 weeks, at which time treatments were commenced. Mice were monitored twice weekly for tumor growth and fluctuations in body weight. When mice lost 25% of their body weight (~5 g) or tumors exceeded 1700 mm^3, mice were sacrificed by cervical dislocation following inhaled anaesthesia. Plasma was collected by cardiac puncture into heparinized syringes and plasma collection tubes, and stored at -70°C until analysis. Tumors were removed from mice, divided into three sections, and placed into RNAlater (Ambion, Inc., Austin, TX), Prefer fixative (Anatech, Battle Creek, MI) or frozen section medium (Stephens Scientific, Riverdale, NJ).

5.2.1 Drug treatments

Metronomic dosing of mice with CTX was carried out as previously described (Man et al, 2002). Cyclophosphamide (CTX) was purchased from Sunnybrook and Womens' College Health Sciences Centre pharmacy as Procytox (Baxter Corporation, Toronto, ON) or Cytoxan (Bristol Laboratories of Canada, Montreal, PQ) and prepared at 20mg/mL with 0.2μm filtered ddH_2O. Metronomic CTX was administered through the drinking water, as CTX has been determined to maintain activity in water for several days. Drinking water was prepared twice weekly from freshly reconstituted cyclophosphamide at 125μg/mL and daily consumption of CTX per mouse is estimated at 20mg/kg/day assuming mice drink approximately 1.5mL per 10g body weight. Mice administered the MTD schedule were given 100μg per day, which is a 100μL intraperitoneal (ip) injection of the 20mg/mL stock CTX solution. MTD CTX was given on days 1,3 and 5 of a 21 day cycle.

Trastuzumab (Herceptin®) was generously provided by Genentech (South San Francisco, CA). A 20 mg/mL stock solution was prepared by adding sterile PBS to the lyophilized antibody. Immediately prior to ip injection, the trastuzumab stock solution was diluted 1:5 with sterile saline, and was administered at 20 mg/kg twice weekly (100μL). Additional in vivo dosing studies were carried out with the erbB2 heterodimerization inhibitory antibody pertuzumab (Omnitarg/2C4) and the humanized anti-VEGF antibody bevacizumab (Avastin®) (both provided by Genentech), as well as the anti-EGFR antibody cetuximab (Erbitux®/C225), provided by Imclone Systems Inc., New York, NY. All antibody reagents were diluted in sterile saline, and administered ip, twice weekly, together with trastuzumab. Drugs were administered at the following doses; 400 μg pertuzumab (Agus et al, 2002); 200 μg bevacizumab (Gerber et al, 2000);

500 μg cetuximab (Ciardiallo et al, 2000; Prewett et al, 2002); 500 μg A12 (Burtrum et al, 2003) per mouse.

5.3 *In vivo* angiogenesis assessment

5.3.1 Tumor perfusion assay

231-H2N tumors were established as indicated above and treatment was commenced when tumors reached a volume of 413 mm^3 ± 266 mm^3 (mean ± SD). Mice were then treated for 21 days for the tumor perfusion assay. A 25 mg/mL solution of FITC-dextran (Sigma-Aldrich) was prepared in PBS and filter-sterilized to 0.22 μm. Mice were warmed with a heat lamp and 0.2 mL of the FITC-dextran solution was injected into the lateral tail vein. FITC-dextran was allowed to circulate for 20-25 minutes, at which time mice were anaesthetized and plasma was collected by cardiac puncture. Tumors were carefully excised, placed in 1:10 dispase, and incubated at 37°C overnight on a shaker. Tumors were then homogenized and centrifuged to remove particulate material. Fluorescence of tumor homogenates and plasma was measured on a FL-600 microplate fluorescence reader (Bio-tek Instruments Inc., Winooski, VT) at excitation wavelength of 485 nm, emission wavelength of 530 nm. Fluorescence as a measure of blood vessel density was calculated as tumor versus plasma fluorescence, and reported as percent of untreated control tumors.

5.3.2 Matrigel plug assay

Two Matrigel preparations were injected subcutaneously in 8 week old female C.B-17/IcrCrl-scidBR mice, the first containing the angiogenic growth factor basic fibroblast growth factor (bFGF), and the second containing 231-H2N tumor cells. BD Matrigel matrix (BD Biosciences, Bedford, MA), stored at -20°C, was thawed at 4°C overnight. To prepare the bFGF-Matrigel plugs, bFGF (R&D Systems, Minneapolis, MN)

was added to thawed Matrigel to a final concentration of 500 ng/mL. The solution was vortexed vigorously and allowed to degas for atleast 2 hours at 4°C. To prepare the tumor cell-Matrigel plugs, Matrigel was mixed with viable 231-H2N cells at a cell count of $3x10^6$ cells/mL. The solution was thoroughly mixed by gentle pipetting, so as to not disrupt the tumor cells.

A 0.5 mL volume of bFGF-Matrigel or tumor cell-Matrigel was injected subcutaneously into anaesthetized mice. Matrigel plugs were allowed to establish for 3 days prior to commencement of treatments. Mice were treated for 7 days as mentioned previously. On day 8, FITC-dextran was injected as described above. Matrigel plugs were carefully excised, photographed, and placed in 1:10 dispase (BD Biosciences) in PBS. Plugs were incubated at 37°C overnight on a shaker, followed by homogenization and centrifugation to remove particulates. Fluorescence of Matrigel plug homogenates and plasma was measured on a FL-600 microplate fluorescence reader (Bio-tek Instruments Inc) at excitation wavelength of 485 nm, emission wavelength of 530 nm. Fluorescence as a measure of blood vessel density was calculated as Matrigel versus plasma fluorescence. For the bFGF experiment, Matrigel plugs without bFGF were used to determine background and were subtracted from each measure. All data was reported as percent of untreated control plugs.

5.4 Exploring acquired resistance to trastuzumab

5.4.1 Northern Blot of total RNA

Tumor tissues were stored at -70°C in RNA Later. Trizol extraction (Life Technologies Inc., Gaithersburg, MD) was carried out according to manufacturer's instruction. VEGF Northern blots were performed as previously described (Francia et al, 1996). The VEGF probe spanning the 200-bp sequence common for all VEGF isoforms

was a generous gift of Dr. Brygida Berse and Dr. Harold Dvorak (Beth Israel Hospital, Boston, MA). GAPDH was probed as a loading control.

5.4.2 Measurement of VEGF and TGFα by ELISA

Cells from parental and resistant variants of the 231-H2N cell line were plated at $0.1x10^6$ cells in 24-well plates and grown for 24 hours. Media was replaced with DMEM containing 1% FBS, and incubated for an additional 48 hours. Small molecule inhibitors, Gö 6976, PD098059, LY294002, AG879 were purchased from Calbiochem Inc. (La Jolla, CA) and reconstituted in DMSO, CCI779 was generously provided by Drs. J Gibbons and P Frost (Wyeth-Ayerst Laboratories, Philadelphia, PA) and reconstituted in DMSO. Inhibitors or trastuzumab were added to cells at time of media change. Conditioned media was collected and assayed on the human VEGF ELISA kit and human TGFα ELISA kit as directed in the manufacturer's protocol (R&D Systems). Growth factor levels detected in conditioned media were corrected for final cell number per well, as counted by a particle counter (Coulter Electronics Ltd, Luton, UK).

Plasma from tumor-bearing mice was also collected. Briefly, blood was collected using heparinized syringes, transferred into plasma microtubes, centrifuged for 15 minutes at 6000rpm, then stored in eppendorf tubes at -70°C. Plasma was evaluated for content of human VEGF and mouse VEGF using VEGF ELISA kits (R&D Systems), as directed in the manufacturer's instructions.

5.4.3 Derivation of trastuzumab resistant cell lines

231-H2N orthotopic mammary tumors were grown to ~250 mm^3 prior to initiation of treatment with trastuzumab alone or in combination with metronomic CTX. In all mice, tumor growth was blocked by the treatment regimens (~ 30 days for trastuzumab, ~110 days for trastuzumab and CTX), after which time tumors rapidly progressed. Once

tumors reached a volume of 1700 mm^3, mice were sacrificed and tumor tissue removed under sterile conditions. To isolate cells from the tumor tissue, the tumor was minced into small ~2 mm^3 pieces and placed into serum free DMEM containing 2 mg/mL Collagenase 3 (Worthington Biochemical Corporation, Lakewood, NJ), 1 mg/mL Hyaluronidase (Sigma-Aldrich), and 1 mg/mL Collagenase IV (Sigma-Aldrich). The tumor-enzyme mixture was incubated on ice for 30 minutes, followed by a 30 minute incubation at 37°C with shaking. DMEM containing 5% FCS was added to the cocktail and cells were filtered through a Falcon 70 μm cell strainer (VWR International, Mississauga, ON). After centrifugation, the cell pellet was plated in DMEM containing 5% FCS, Fungizone Amphotericin B (Gibco BRL) and Penicillin-Streptomycin (Gibco BRL). Cells were grown to confluency and passaged 3 times prior to further study of the cell lines. Cells were maintained in DMEM with 5% FCS.

5.5 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows, (GraphPad Software, San Diego CA). For all statistics, one-way ANOVA with Dunnett's post test was performed.

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