COMBINATION IMMUNOTHERAPY WITH HER-2/NEU AND VEGF PEPTIDE MIMICS IN BOTH TRANSGENIC AND TRANSPLANTABLE MOUSE MODELS OF HUMAN BREAST CANCER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2011

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ABSTRACT

Her-2/neu (ErbB2) is a member of the epidermal growth factor family of receptors and is overexpressed in about 30% of breast cancers. Targeting this receptor is a very attractive strategy for antitumor therapy using both peptides and monoclonal antibodies. Trastuzumab and Pertuzumab are humanized monoclonal antibodies that both target the extracellular domain of the Her-2 receptor. Great limitations still exists with these treatments due to their high cost and limited duration of action, thereby necessitating repeated administration of the drugs. The overexpression of Her-2 leads to overexpression of another protein known as vascular endothelial growth factor (VEGF) which turns on the angiogenic switch aimed at causing increased blood flow and oxygen to the tumors. Without an increase in blood flow, the tumors cannot increase in size, hence angiogenesis is required for tumor growth and metastasis. VEGF is known to interact with one of its receptors VEGFR-2 before it can stimulate angiogenesis. The disruption of this interaction is considered another attractive target for cancer therapy and monoclonal antibodies like Bevacizumab have been developed to prevent this interaction.

This work is mainly centered on the use of peptides for cancer treatment that are cost effective, specific and non-toxic to target both Her-2 and VEGF. Treatment with conformational B-cell epitopes affords the possibility of generating an enduring immune response and eliciting protein reactive high affinity peptide antibodies. We have designed

conformational peptides of the pertuzumab epitope and also peptides that mimic the VEGF binding sites. With the use of in vitro assays and two in vivo tumor models (transgenic and transplantable), we have shown that combination treatment aimed at targeting the HER-2/neu and VEGF signaling pathways produce additive effects with no problems of cardiotoxicity. We also showed that combining these peptide mimics with standard chemotherapy produces increase survival free rates and a decrease in tumor growth and development. Combination immunotherapy with antibodies raised against these peptide vaccines was also able to additively inhibit tumor growth in vitro as demonstrated using Her-2 signaling processes like proliferation and phosphorylation of the HER-2 receptor.

Dedication

Dedicated to my family

particularly my parents, Mr. John Chu Foy and Mrs. Florence Enam Foy, and my sister of blessed memory, Hilda Chou Foy

ACKNOWLEDGMENTS

I wish to start by thanking my advisor, Dr. Pravin Kaumaya, for giving me this unique opportunity to work in his lab. His unconditional support, enthusiasm and encouragement have been instrumental to the success of this project. I am also grateful to my committee members, Drs. Nic Moldovan, Marshall Williams and Jesse Kwiek for their time, guidance and dedication to the success of this project.

My sincere acknowledgements to Zhenzhen Lui and Megan Miller, who worked with me on several of the studies described in this project. I thank Dr. Sharad Rawale for his helpful suggestions and assistance with the synthesis of some of the peptides used in this study. My acknowledgments also go to Dr Tim Eubank and Julie Wallace of the Ostrowski lab for providing breeding pairs of the PyMT transgenic mice. I am grateful to the members of the Moldovan's lab especially Drs Mirela Aghelina and Leni Moldovan for their assistance in the angiogenesis assays described in this thesis. I am also grateful to all members of the Kaumaya lab past and present especially Dr Daniele Vicari, Nina Osafo, Aravind, Eric, Erica and Jay for their advice and help in some parts of the project. My appreciation also goes to those who helped me throughout graduate school especially Dr Steve Oghumu, Dr Theresa Rogers, Tracy, Michelle, Eusondia, Samhita and Kiley.

Finally, I would like to thank God, my family and friends. I thank my parents for their unconditional love and support. I am grateful to my twin brother and his wife, Kenneth and Pauline Foy, my cousins Anye Biame and Immaculate Foy for their support.

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FIELDS OF STUDY

Major Field: Microbiology.

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ABBREVIATIONS

Ab	antibody
ABTS	2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
APC	antigen-presenting cell
ATP	adenosine triphosphate
Bu^t	tert-Butyl
BSA	bovine serum albumin
CD	circular dichroism
CDC	complement-dependent cytotoxicity
CDR	complementarity determining region
CTL	cytotoxic T lymphocyte
CYC	cyclized
DTT	dithiothreitol
ECD	extracellular domain

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ESI-MS	electrospray ionization mass spectrometry
Fab	antigen-binding fragment
FISH	fluorescent *in situ* hybridization
HER	human epidermal growth factor receptor
HLA	human histocompatibility leukocyte Ag
IFN	interferon (e.g., IFN-γ)
IGF-1R	insulin-like growth factor receptor-1
IHC	immunohistochemistry
IL	interleukin (e.g., IL-2)
KLH	keyhole limpet hemocyanin
mAb	monoclonal Ab
MALDI	matrix-assisted laser desorption/ionization
MAP	multiple antigenic peptide

MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus
MTT	C,N-diphenyl-N'-4,5-dimethyl thiazol-2-yl tetrazolium bromide
MVF	measles virus fusion protein amino acids 288-302
NC	non-cyclized
NK	natural killer
NSCLC	non-small cell lung cancer
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinase
TAA	tumor-associated ag
TCR	T cell receptor for Ag
TGF	transforming growth factor
Th cell	T helper cell
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor

TM	transmembrane
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth receptor 1
VEGFR-2	vascular endothelial growth receptor 2

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer Epidemiology

Breast cancer ranks second as a cause of cancer death in women (after lung cancer) and according to the American Cancer Society (http:www.cancer.org) an estimated 192,370 new cases of invasive breast cancer were expected to occur among women in the US in 2009 with an estimated 40,610 breast cancer deaths. The probability of a woman developing invasive breast cancer once in her life time is one in eight and the chance of dying from the disease is about one in thirty three. Breast cancer is no longer a disease of the old as children as young as 9 are now being diagnosed with this disease. The main question now is if this group of patients can withstand the current treatment options available. All of this makes the disease condition a great priority for many researchers. The search for other methods of treatment that are cheaper, less toxic are therefore warranted. A great breakthrough will be the development of a vaccine that can train the human immune system to take care of its own problems.

1.2 HER-2 and VEGF

Normal functions of HER-2

HER-2 is expressed in many tissues including neuronal, epithelial and mesenchymal tissues [1]. Knockout mice that are deficient in the HER-2 gene cannot complete gestation due to inadequate formation of the heart muscles [2] and when this defect is rescued, the mice still have very poor neuronal development [3]. HER-2 is also expressed in almost all postnatal developmental stages of the mammary gland [4]. Also, mice that have HER-2 mutants show serious defects in milk secretion [5].This therefore shows that HER-2 plays a very crucial role in the development of embryonic and adult tissues.

HER-2 transformation

Overexpression of HER-2 in mice has been shown to lead to its transformation and increase in protein expression thereby causing malignancy which is similar to that seen in human breast cancer [6]. Transgenic mice that were injected with the rat neu were shown to develop mammary adenocarcinomas at about 6 to 10 months of age [7-10]. All these experiments clearly demonstrate the role of HER-2 in cellular transformation. HER-2 upregulation leads to increased levels of autophosphorylation and activation of the intracellular tyrosine kinase domain [11, 12]. HER-2 receptors have been shown to downregulate receptor internalization and degradation and this is also common to all members of the HER family of receptors [13]. High expression of HER-2 makes it readily available for dimerization with other HER family receptors like HER-1 and HER-3 and this dimerization increases the transformation potential of the receptor as observed

in many types of cancers [14-17]. This is mainly because HER-2 dimers are more stable and hence less degradation of the receptors [12] and the HER-2 receptor is also able to inhibit ubiquitilation by many receptor blocking ligase [18]. All these characteristics give HER-2 the ability to cause cancer by altering the functions of normal cells in various organs of the body. Also, HER-2 is always in an open conformation which makes it a preferred dimerization partner with other HER receptors [19] and signaling by HER-2 heterodimers have been shown to be stronger than homodimers due to little or no degradation and endocytosis [20, 21] with increased activation of the intracellular kinase domain [22]. Most biological mechanisms such as phosphorylation, proliferation and cellular migration are greatly enhanced in cells that have high levels of HER-2 expression [23]. HER-2 overexpression greatly predicts a decrease in disease free and survival rates [24-29] and also correlates with aggressiveness, tumor size and increased proliferation index [30-33].

HER-2 and Breast Cancer

HER-2/neu is a self antigen that is overexpressed in different types of epithelial tumors including breast and is associated with a worse prognosis in several malignancies [34-38]. HER-2/neu is a 185 kDa transmembrane protein that belongs to a family of receptors known as the c-erbB family or EGFR family (Figure 1.1). Overexpression HER-2 has been reported in about 30% of all breast cancers [39] and seems to predict decreased disease free and overall rates of survival. Many therapeutic strategies targeting the HER-2 receptor have been devised and the most outstanding among them are a group of humanized monoclonal antibodies that includes Trastuzumab and Pertuzumab.

Trastuzumab is a growth inhibitory monoclonal Ab that binds the extracellular domain (ECD) of the HER-2 receptor and blocks intracellular signaling. It is currently used in the treatment of HER-2 positive breast cancers [40]. Pertuzumab also binds the ECD of HER-2 thereby preventing dimerization of the receptor and it is currently being evaluated in human clinical trials. HER-2 is an attractive therapeutic target for several reasons. The amount of HER-2 expressed on cancer cells is much higher than in normal adult tissues potentially reducing the toxicity of HER-2 targeting drugs [41]. Tumors with a high expression of HER-2 often show homogenous intense IHC staining [42], signifying that HER-2 targeted therapy will target most cancer cells in a given patient. HER-2 is always observed in metastatic disease suggesting its implication in most aggressive tumors although it is not observed in benign tumors [30, 32, 33, 43-45]. However, evidence suggesting the continuous use of these humanized Abs is lacking due to their instability, repeated administration, and effects of cardiotoxicity, poor penetration across tissue barriers, and their high cost of treatment with potential immunogenicities associated with them [46]. This has led to a renewed interest in the development of vaccines that elicit cellular and humoral HER-2 specific immune responses. It has been demonstrated that HER-2 positive breast cancer patients have pre-existent B and T cells against HER-2 [47, 48]. Our laboratory was the first to propose the use of B cell epitope peptides as HER-2 vaccine candidates [42, 49, 50] before other groups started developing B-cell vaccines [51]. Abs produced against HER-2 peptide vaccines have been shown to prevent HER-2 phosphorylation in a site specific manner [52].

VEGF

Angiogenesis contributes to the development of numerous types of tumors and metastasis. VEGF expression is increased in many different types of cancers and data have shown that tumor cells secrete VEGF [53], which is the most known pro-angiogenic factor today [54]. VEGF and its receptors, VEGFR1 and VEGFR2, are prime targets for anti-angiogenic intervention, which is thought to be one of the most promising approaches in cancer therapy. Blocking angiogenesis will inhibit tumor growth, invasion and metastasis. VEGF can be produced by several types of cells including macrophages and smooth muscle cells but is also overexpressed by tumor cells [55, 56]. VEGFR2 expression was first believed to be exclusive to the endothelial cells but recently, advanced techniques of detection have demonstrated its expression in breast and colon tumor cells [57] showing that VEGF, via VEGFR2 activation, probably plays a role in the formation of new blood vessels in the tumor microenvironment or directly in tumor growth activation in an autocrine pathway.

HER-2 and VEGF Therapeutic Strategies

Many agents have been developed aimed at targeting RTKs and these include therapeutic antibodies to RTK ligands or the receptors themselves and small molecule inhibitors that target the intracellular kinase domains of RTKs [58-60]. Many of the FDA approved therapies targeting both HER-2 (Trastuzumab, Herceptin) and VEGF (Bevacizumab, Avastin) have significant toxicities including cardiac dysfunction and congestive heart failure [61-63] and many of the patients demonstrate disease progression

due to development of resistance. Clinical applications of mAb therapy in general is limited by a number of concerns such as frequency of treatments, associated costs, limited duration of action, undesired immunogenicities, development of resistance, and significant risk of cardiotoxicity [64]. Similarly, the small molecule RTK inhibitors such as Sunitinib which have entered clinical trials alone or in combination with radiotherapy or chemotherapy show problems of efficacy, development of resistance and unacceptable safety profiles that continues to hamper their clinical progress [62]. Despite the success of these drugs, there still remains a high and unmet need for novel molecular cancer therapeutics. Novel therapies targeting these aberrant molecular pathways are urgently needed to offer hope that the effectiveness and duration of response can be greatly improved.

1.3 Targeting Cancer, Angiogenesis and Neovascularization

Angiogenesis is the process whereby new blood vessels are formed from pre-existing ones. All solid tumors need a constant blood and oxygen supply in order to increase in size. Tumors therefore need to turn on the angiogenic switch if they must grow beyond a few millimeters and this switch has been clearly illustrated in many types of cancer [65]. The most pro-angiogenic factor known today is vascular endothelial growth factor or VEGF and its expression is increased in many different types of cancer and most tumor cells secrete VEGF [66]. VEGF has also been implicated in the metastasis of most cancers [67]. VEGF binds to its receptor VEGFR2 before stimulating angiogenesis [68] making this interaction a prime target for anti-angiogenic therapy. Angiogenesis is a very

important process that must be tightly regulated because insufficient or excessive angiogenesis is related to several diseases. Insufficient angiogenesis can result to stroke, delayed wound healing, and coronary artery disease while excessive angiogenesis causes the progression of several pathologies like cancer, atherosclerosis and age-related macular degeneration [69-71]. In order for tumor cells to increase in size, they up-regulate the expression of pro-angiogenic factors that stimulate new blood vessels in and around the tumor [72]. Due to the uncontrolled overexpression of these pro-angiogenic factors like vascular endothelial growth factor (VEGF) and transforming growth factor (TGF), the tumor vasculature is characterized by abnormal blood vessels [71]. Though disorganized, the blood vessels are highly permeable thus contributing to migration and infiltration of tumor cells to other sites which lead to metastasis [73, 74]. Folkman and his group were the first to suggest that angiogenesis was a crucial step in tumor growth and they demonstrated that an angiogenic switch needs to be turned on in order to allow solid tumors to grow beyond a few millimeters [75-77].

Most importantly, overexpression of HER-2 is associated with increased expression of VEGF both at the RNA and protein levels in breast cancer cells and a positive association between HER-2 and VEGF expression in breast cancer patients has been identified [1]. Targeting these two receptors using a combination strategy can therefore interact in a synergistic/additive manner killing tumor cells and retarding tumor development [78-80].

Peptides and peptidomimetics

Peptides are small protein-like chains of amino acids and a peptide mimic is a peptide or a peptide-like molecule that intends to mimic one portion of the entire protein which is

usually the active or binding site of an enzyme [81]. The development of peptides that can block receptor-ligand interactions can be achieved via screening combinatorial libraries of compounds based on structural designs. The most important parameter is to maintain the conformational space and orientation of the active site while retaining maximum flexibility to bind cooperatively with its ligand [82]. Peptide mimics offer the benefits of being water soluble, non-immunogenic, and having an ability to easily cross tissue barriers [83]. The only drawback of using peptides as therapy is their high susceptibility to proteosomal degradation [84]. This problem can be overcome with the use of pseudo and modified peptides. The retro-inverso modification is a reversal of the peptide backbone by inverting the amino acid sequence, as well as amino acid chirality using D-amino acids. The resulting peptide is a topographical equivalent of the parent peptide with the amino acid side chain in similar orientation. Since retro-inverso peptides are synthesized with D-amino acids, and proteases usually recognize L-amino acids, they should be resistant to proteosomal degradation and therefore will increase the bioavailability of the peptide *in vivo* [84, 85].

Synergy between Immunotherapy with HER-2 Peptide Vaccines and Anti-angiogenic Therapy with VEGF Peptide Inhibitors.

The overexpression of HER-2 is associated with increased expression of VEGF both at the RNA and protein level in human breast cancer cells based on transfection of cells with HER-2 [86]. In addition, exposure of HER-2 overexpressing cells to trastuzumab significantly decreases VEGF [87]. Shc, a downstream adaptor protein of the HER-2 signaling pathway has been identified as a critical angiogenic switch for VEGF

production [88]. This clearly suggests that VEGF is a downstream target of the HER-2 signaling pathway. A positive association between HER-2 and VEGF expression in breast cancer patients has been identified [89]. This supports the hypothesis that the effects of HER-2 on tumor cell behavior may be mediated in part through stimulation of angiogenesis. A two-pronged approach to target cancer cells by co-immunizing with defined tumor-associated antigens and angiogenesis associated antigens has been shown to have synergistic effects [90-92].

Combination of Chemotherapy and Anti-angiogenic Agents

Angiogenesis also plays a major role in the growth and metastasis of different types of cancers [93] and VEGF as one of the most pro-angiogenic factors was identified as the main regulator of the process of angiogenesis [94]. Many tumor cells are known to secrete VEGF, which is a key promoter of metastasis. Communication between cancer cells and their microenvironment is therefore critical for the development and metastasis of many tumors [78-80, 94]. The role of macrophages in tumor growth and development is two pronged. They are initially recruited to tumor sites as a response by the immune system but since most tumors are able to evade immune surveillance, these macrophages may promote tumor growth and angiogenesis [95, 96]. Many anti-tumor agents have been developed aimed at targeting receptor tyrosine kinases (RTKs) most of which targets the intracellular kinase domain [97-99]. Trastuzumab (Herceptin) and bevacizumab (Avastin) are humanized monoclonal antibodies that are approved by the FDA for targeting HER-2 and VEGF. The safety profiles of these hmAbs are unacceptable and concerns such as frequency of treatment, associated cost, development of resistance, undesired

immunogenicity and significant risks of cardiotoxicity continue to hamper their clinical use [100]. Many studies have shown that these Abs cause cardiac dysfunction and congestive heart failure [101, 102]. Another drug, paclitaxel (Taxol) is known to bind to the β subunit of tubulin thereby preventing depolymerization and interrupting the cell cycle [103, 104]. Paclitaxel have been used alone or in combination with radiation or other anti-tumor agents in different clinical trials [105, 106]. Other studies have also reported certain antiangiogenic properties with paclitaxel treatment [107, 108]. Despite the success of some of these drugs, there still remains a high and unmet need for novel molecular cancer therapeutics.

Novel Pertuzumab binding conformational B-cell epitopes

Many monoclonal Abs are extensively used as cancer therapeutic agents today because of their ability to interfere with signaling events that lead to cellular proliferation and thereby preventing tumor growth in many ways [109, 110]. There is still widespread concern regarding the use of these drugs due to the emergence of problems of resistance, repeated treatments, associated cost, undesired immunogenicity and toxicity. Active immunization will therefore offer sustained immune responses and many benefits over passive treatment with these Abs. Prediction of novel B-cell epitopes from the ECD of HER-2 has been the focus of our laboratory for the past decade. Through such an approach, we have identified two novel HER-2 vaccine candidates that were translated into a Phase I clinical trial thereby validating our overall strategy. The vaccine was safe and immunogenic in the patients and the Abs were able to prevent HER-2 signaling processes in cancer cells in vitro. A subset of the patients (6 out of 24) showed clinical

responses and some of these patients were given more doses of the vaccine due to their clinical responses [111]. After the publication of the crystal structure of HER-2 complexed with trastuzumab [112] and HER-2 complexed with pertuzumab[113], we synthesized a second generation of peptide vaccines based on the interaction of these abs with the ECD of the HER-2 receptor. We have shown extensively the efficacy of these new vaccine candidates in preclinical studies [114, 115]. Two of the vaccine candidates will be evaluated in a future Phase Ib NCI-funded clinical trial which starting in 2011 at the James Cancer Hospital.

Pertuzumab binds to different epitopes in the extracellular domain near the junction of domains I, II, and III of the HER-2 extracellular domain [113] amino acids **266 - 333**. This interaction of pertuzumab with HER2 was shown to sterically hinder the association of HER2 with other receptors such as HER3 [113] sterically blocking a binding pocket necessary for receptor dimerization and signaling. Based on these studies we have selected one HER-2 peptide mimic pertuzumab-like sequence, 266-296, which has been shown to be a good candidate as a neutralizing, blocking peptide [114] and the contact residues of the peptide with the HER-2 receptor are shown in Figure 1.2.

Design and synthesis of novel VEGF peptide mimics

The crystal structure of the complex between VEGF and the Fab fragment of a humanized antibody bevacizumab (Avastin) (Figure 1.3), and analysis of the contact residues on both sides of the interface was published by Muller *et al*. [116, 117]. Zilberberg *et al*. identified sequence 79-93 of VEGF, which is involved in the interaction with VEGF receptor-2 [118]. It can be seen that although the VEGF residues critical for

antibody binding are distinct from those important for high-affinity receptor binding (Figure 1.3), they occupy a common region on VEGF. This demonstrates that the neutralizing effect of antibody binding results from steric blocking of VEGF–receptor interactions and only a small number of the residues buried in the VEGF–Fab interface are critical for high-affinity binding and are concentrated in one continuous segment of polypeptide loop between β5-β6 [119]. Several residues are important for VEGF receptor binding, including Met 81, Ile 83, Lys 84, Pro 85, Gln 89, and Gly92. We have selected a peptide encompassing residues 76-96 that mimics the VEGFR2 and Avastin overlapping binding sites. The strategy to create a conformational peptide consisting of an anti-parallel β-sheet (and Figure 1.3) required two artificial cysteines to be introduced between Gln79 and Gly92 and between Ile80 and Glu93. The VEGF P3 constructs (cyclic and non-cyclic) were synthesized with L-amino acids. After synthesis and purification of the *VEGF-P3 (NC)* (non-cyclized) peptide, the disulfide bond was formed by oxidation reaction enabling the formation of the twisted anti-parallel β-sheet structure in the *VEGF-P3 (CYC)* (cyclized). Following characterization by HPLC and MS, peptides were kept lyophilized and will be dissolved prior to use in subsequent assays. The VEGF-RI-P4 peptide was synthesized with D-amino acids in reverse order (retro-inverso). Also, after synthesis and purification of the VEGF-RI-P4 (NC), it was subjected to oxidation for disulfide formation VEGF-RI-P4 (CYC), as described from our published studies [42, 115] .

1.4 Hypothesis and Overview of Chapters 2-4

The structures of the HER-2-pertuzumab complex have led to new insights into the mechanistic and biological activities of HER-2 antibodies as well as the process of HER-2 homodimerization and heterodimerization. This in turn has led us to rationally design more effective HER-2 conformational epitopes with potentially increased efficacy to prevent and inhibit tumor growth. To date, no conformational peptides have been designed and tested based on the crystal structure of the HER-2 ECD in complex with the trastuzumab or the pertuzumab Fab fragments or VEGF in complex with VEGFR2 or bevacizumab, hence these studies are novel. Our main hypothesis is that targeting both HER-2 and VEGF pathways will simultaneously kill cancer cells and deprive them of nutrient and blood supply, which will produce greater anti-tumor effects in vitro and in vivo.

Chapter 2 of this dissertation presents studies evaluating the effects of combination treatment with HER-2 and VEGF peptide mimics using both in vitro and in vivo tumor models. The HER-2 epitope 266-296 was shown to elicit antibodies that were able to bind the HER-2 ECD and prevent dimerization. These antibodies were able to prevent HER-2 signaling processes in vitro and immunization with this B-cell epitope was able to significantly inhibit tumor growth in vivo in three different mouse models [114]. We therefore hypothesized that, combination treatment using this peptide and the VEGF peptide mimics should produce greater antitumor effects. As expected, this combination treatment was able to additively inhibit HER-2 signaling processes like proliferation and phosphorylation in vitro and also caused a significant delay in tumor

growth and development using a transplantable mouse model of Balb/c challenged with TUBO cells. This shows that the B-cell epitope was able to mimic the functions of pertuzumab antibody. In Chapter 3, we have evaluated the antitumor effects of immunization with this B-cell eptitope alone as compared to both immunization and treatment with VEGF peptide mimics. We showed that immunization alone was able to inhibit tumor growth in vivo but treatment with VEGF peptides after immunization produced superior anti-tumor effects in our mouse model. The antibodies raised against both HER-2 and VEGF peptides were also able to cause ADCC of cancer cells in vitro. The results of combination treatment with low-dose paclitaxel and our peptides are detailed in Chapter 4. We started by showing that treatment with our HER-2 peptides was as efficient as treatment with paclitaxel or trastuzumab but had no effects of cardiotoxicity as in the case of paclitaxel and trastuzumab. Based on this, we hypothesized and showed that, combining low doses of these chemotherapeutic agents with normal doses of our peptide mimics will produce better responses *in vivo*. The results from the studies indicate that combining low dose chemotherapy with other cancer treatment strategies results to better responses and can increase survival in cancer patients. Overall, targeting HER-2 and VEGF pathways seems to be a better strategy for treating HER-2 positive cancers.

1.5 Figures

Figure 1.1 Epidermal growth factor receptor family members.

All members are composed of three main domains, an extracellular domain, transmembrane domain and an intracellular domain. All members have ligands except HER-2 with no known ligand and is hence known as an orphan receptor.

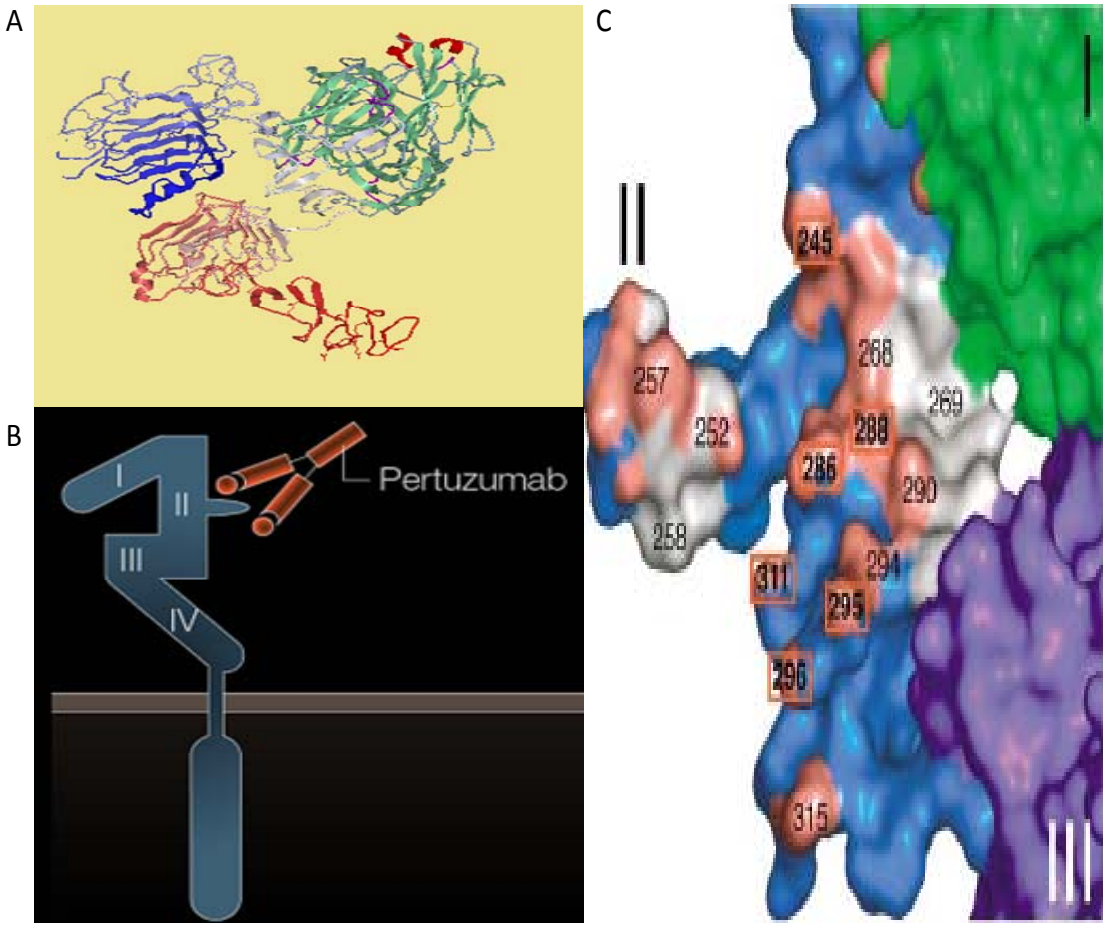


Figure 1.2 The HER-2 extracellular domain and pertuzumab interface.

Diagrams A and B shows HER-2 extracellular sub-domain II in contact with pertuzumab (green in A). Diagram C shows sub-domain II of the HER-2 ECD, and the residues that make contacts with the pertuzumab antibody are shown in red.

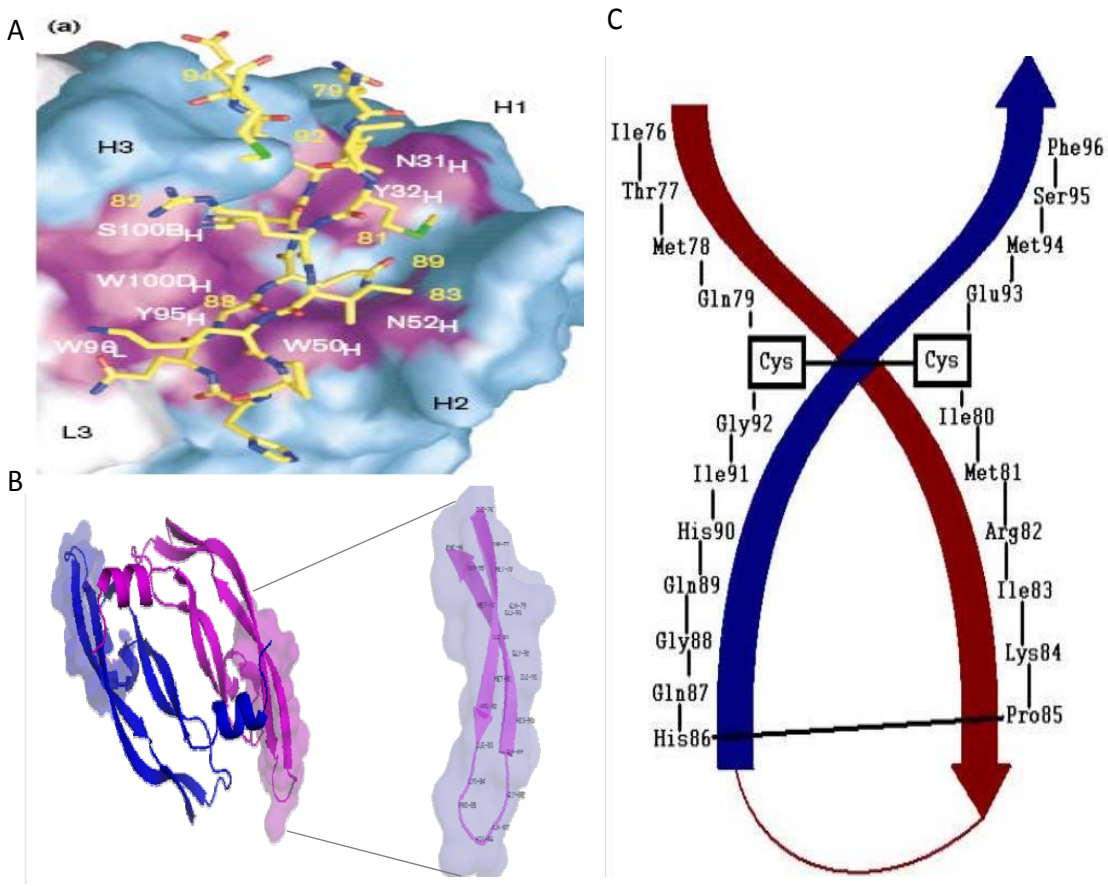


Figure 1.3 Structure of the antibody combining interface between VEGF and anti-VEGF antibody and epitope used for designing VEGF peptide mimics. (A) Shows the contact residues critical for binding to the VEGF antibody bevacizumab and this region is delineated in (B) and known to overlap with the binding of VEGF to VEGFR2. Shown in (C) is the schematic of VEGF peptide mimic that is cyclized to adequately mimic the binding site on VEGF. Arrows show anti-parallel β-sheet orientation with the cross over.

CHAPTER 2

COMBINATION TREATMENT WITH HER-2 AND VEGF PEPTIDE MIMICS INDUCES POTENT ANTI-TUMOR AND ANTI-ANGIOGENIC RESPONSES IN *VITRO* AND IN *VIVO*

2.1 Introduction

HER-2 (human epidermal growth factor receptor-2) is a member of the HER family of receptor tyrosine kinases and is overexpressed in about 30% of invasive breast cancers [120, 121]. HER-2/neu is a self antigen that is over-expressed in multiple epithelial tumors and is associated with markedly aggressive forms of cancer with a worse prognosis than several other malignancies [35, 36]. The HER-2 protein is an important therapeutic target in many cancers for several reasons. The amount of HER-2 expressed in cancer cells is much higher than in normal adult tissues [1], potentially reducing the toxicity of HER-2 targeting drugs. Tumors with a high expression of HER-2 often show homogenous, intense IHC staining [122], signifying that HER-2 targeted therapy would target most cancer cells in a given patient. Additionally, HER-2 overexpression is found in both primary and metastatic sites [78], suggesting that HER-2 targeted therapy may be effective in all disease sites. HER-2 overexpression and amplification is seen in subsets of gastric, esophageal, endometrial, uterine, ovarian, and lung cancers [123-128]. All

four HER receptors [EGFR (HER-1), HER-2, HER-3, and HER-4] share structurally homologous extracellular domains [129]. HER-2 plays a major coordinating role in this network, since each receptor with a specific ligand seems to prefer HER-2 as its heterodimeric partner [130, 131] due to its constitutively "open" conformation. HER-2 containing heterodimers potently amplify signaling because HER-2 reduces the rate of ligand dissociation, allowing strong activation of downstream signaling pathways [132, 133] and those involving the phosphatidylinositol-3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) [134]. This makes HER-2 a very attractive therapeutic target and also suggests that HER-2 targeted therapy will target most cancer cells in a given patient. The overexpression of HER-2 has also been shown in both the primary and metastatic sites [78], which strongly suggests that HER-2 therapy can have potentials in all disease sites.

The upregulation of HER-2 is associated with increased expression of vascular endothelial growth factor, or VEGF at both the RNA and protein level in human breast cancer cells and exposure of HER-2 positive cells to Trastuzumab significantly decreases VEGF expression [135]. Shc, a downstream adaptor protein of the HER-2 signaling pathway has been identified as a critical switch for VEGF production [136] showing that VEGF is a downstream target of the HER-2 signaling pathway. This shows that the effects of HER-2 on tumor cell behavior may be mediated in part through stimulation of angiogenesis. Angiogenesis is the growth of new blood vessels from pre-existing ones and contributes to the development of numerous types of tumors and their metastasis. VEGF, a well known pro-angiogenic factor is secreted by most tumor cells [54]. VEGF expression is increased in many different types of cancer and most tumor cells secrete

VEGF [94]. It is also thought that VEGF is a key promoter of metastasis [137]. VEGF is a 34-42kDa, homodimeric, heparin binding, disulfide-bonded glycoprotein that has several isoforms arising from splice variants [67, 80, 94, 138, 139]. VEGF has three known tyrosine kinase receptors: Flt-1 (VEGF-R1), KDR (VEGF-R2, Flk-1), and Flt-4 (VEGF-R3). VEGF-R1 has a higher affinity for VEGF, but it has been shown that VEGF-R2 is the biologically relevant receptor. Therefore, VEGF and its receptors VEGFR-1 and VEGFR-2 are prime targets for anti-angiogenic intervention, which is thought to be one of the most promising approaches in cancer therapy. Blocking angiogenesis is an attractive strategy to inhibit tumor growth, invasion, and metastasis. Several monoclonal antibodies have been developed to block VEGF from binding to its receptors, of which one, A4.6.1, or Avastin® (Bevacizumab), has been approved by the FDA [140]. Bevacizumab has been tested in several cancer clinical trials [140-142] and showed some promising results in a phase II clinical trial for breast cancer [135]. Other VEGF inhibitors in clinical trials in progress today include the two receptor tyrosine-kinase inhibitors, VEGF-Trap and anti-VEGF-R2 [136, 140]. VEGF stimulates angiogenesis by binding to its receptor VEGFR2, which is expressed by both endothelial and tumor cells [57]. A two-pronged approach to target by co-immunizing with defined tumor associated antigens and angiogenesis associated antigens have been shown to have synergistic effects [57, 143, 144]. All of these shows that combination treatment targeting both HER-2 and VEGF is a promising strategy since angiogenic therapy alone will only delay tumor growth [145] and targeting HER-2 and VEGF will destroy two different tumor dependent sub-pathways.

The work described here stems from work conducted in our laboratories over the past decade in developing effective vaccine strategies for HER-2/neu [41, 42, 49, 50, 146, 147] as well as developing novel therapies based on blockade of receptor:ligand interactions such as B7:CD28 [65, 66, 148]. We have shown that peptide vaccines of the HER-2/neu dimerization loop are effective in inhibiting mammary tumor growth in vivo [65, 66, 148]. We have also developed effective inhibitors of VEGF: VEGFR2 (Vicari et al.). The latter objective was driven by the observation that HER-2 activation induces the expression of VEGF, which is a pro-angiogenic factor making blockade of angiogenesis an attractive and additional strategy to inhibit tumor growth, invasion, and metastasis.

The basic hypothesis in the design of peptide mimics of VEGF and HER-2 is that many proteins exert their biological activity through relatively small regions of their folded surfaces. This approach relies on the premise that the key residues of the binding epitope, in particular side-chain functional groups responsible for a significant portion of the binding affinity to a given receptor/ ligand may be transferred to a much smaller molecule with the contributions to binding largely intact [149]. The strategy for the Retro-inverso (RI) modification of peptides is synthesized using D-amino acids with the amino acid sequence in reverse order, such that the resulting peptide mimetic has a reversal of the peptide backbone but topochemical equivalence to the parent peptide in terms of side-chain orientation.

In this study, we report on the activity of the HER-2-266-296 peptide mimic in combination with two VEGF peptide mimics that were synthesized using L and D amino acids. The VEGF peptides were shown to mimic the binding site of VEGF to its receptor

VEGFR2 (Vicari *et al.*). Combination treatments with both peptides were able to cause superior anti-tumor and anti-angiogenic effects *in vitro* and *in vivo*. This dual therapeutic benefit is clearly demonstrated by the increased proliferation and phosphorylation inhibition as well as by a decrease in cell viability. Combination treatment also caused a greater delay in tumor growth and development in a transplantable tumor model. These results are indicative that the selected inhibitory peptides have great therapeutic effects in targeting both HER-2 and VEGF by eliciting potent anti-tumor and anti-angiogenic effects.

2.2 MATERIALS AND METHODS

Synthesis and characterization of conformational peptides.

Peptide synthesis was performed on a Milligen/Biosearch 9600 peptide solid-phase synthesizer (Bedford, MA) using Fmoc/*t*-But chemistry. Preloaded Fmoc-Val-CLEAR ACID resin (0.35 mmol/g) for the 266-296 and CLEAR AMIDE RESIN for the VEGF peptides (0.32 mmol/gm) (Peptides International, Louisville, KY) were used for synthesis. The 266-296 cyclized epitope was assembled by choosing the regioselective side chain protector Trt on Cys residues 268 and 295 [150], and in the VEGF peptides two cysteines were inserted between amino acid Gln79and Gly92 and between Ile80 and Glu93. Peptides were cleaved from the resin using cleavage reagent B (trifluoroacetic acid-phenol-water: TIS, 90:4:4:2), and crude peptides purified by semi-preparative reversed-phase-HPLC and characterized by electrospray ionization mass.

Circular Dichroism

This was done as previously described [150]. Briefly, aqueous solutions for CD were prepared by dissolving the freeze-dried peptides in an appropriate amount of HPLC water to give a final concentration of 0.5mM and used as stock solution for further dilution. CD spectra were recorded on an AVIV model 62A DS CD instrument. Mean residue ellipticity ($[\theta]_{M,\lambda}$) values were calculated according to the equation; $[\theta]_{M,\lambda} = (\theta \ X \ 100 \ X \ M_r)/(n \ X \ c \ X \ l,$ where θ is the recorded ellipticity (degree); M_r the molecular weight of the peptide; *n*, the number of residues in the peptide; *c*, the peptide concentration (milligrams per milliliter); and *l*, the path length of the cuvette.

Animals.

Female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animal care and use was in accordance with institutional guidelines.

Cell lines and Antibodies.

All culture media, FBS, and supplements were purchased from Invitrogen Life Technologies (San Diego, CA). The human breast tumor cell lines BT-474, SK-BR-3, and MDA-468 were purchased from American Type Culture Collection (Rockville, MD) and maintained according to supplier's guidelines. TUBO cells are a cloned cell line established in vitro from a lobular carcinoma that arose spontaneously in BALB-neuT mouse [151]. Humanized mouse mAb Trastuzumab was generously provided by Genentech, Inc (South San Francisco, CA).

Statistical analysis.

Tumor growth over time was analyzed using Stata's XTGEE (cross-sectional generalized estimating equations) model which fits general linear models that allow you to specify within animal correlation structure in data involving repeated measurements [152]. For other experiments t-test was carried out to observe the statistical relevancy in between different sets of experiments as well as the significant difference between treated and non-treated cells.

Proliferation assay.

BT-474, SK-BR-3, MDA-468 and TS/A cells ($1x10^4$) were plated in 96-well flat-bottom plates overnight. Growth medium was replaced with low sera (1% FCS) medium and the cells were incubated overnight. Media were removed from the wells and replaced with low sera medium containing HER-2 and VEGF mimic peptides at concentrations ranging from 25-150 μg/ml and plates were incubated an additional 1 h at 37°C before adding 10 ng/ml HRG in 1% medium. Plates were incubated for an additional 72h at 37°C before adding MTT (5 mg/ml) to each well. Plates were incubated 2h at 37°C, and 100 μl of extraction buffer (20% SDS, 50% dimethylformamide (pH 4.7)) was added to each well. Plates incubated overnight at 37°C and read on an ELISA reader at 570 nm with 655 nm background subtraction. Inhibition percentage was calculated as 100% x (Untreated cells – Peptide treated cells)/ (Untreated cells).

Phosphorylation assay.

1 x10^6 BT-474 cells were plated in each well of a six well plate and incubated overnight at 37°C. Culture medium was removed and the cell layer washed once with PBS low score (1% FCS). Culture medium was added to the wells and plates incubated overnight. Cells were washed and 50 µg of peptides, anti-peptide Abs and controls in binding buffer (0.2% w/v BSA, RPMI 1640 medium with 10mM HEPES (pH 7.2) was added to the wells and incubated at room temperature for 1h. HRG (5 nM/well) was added and the incubation continued for 10min. Binding buffer was removed and the cell layer washed once with PBS before adding 1ml of RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Plates were rocked at 4°C for 2h. Lysates were removed, spun at 13000 X g and supernatants collected. Protein concentration of each sample was measured by Coomassie plus protein assay reagent kit and lysates were stored at -80°C. Phosphorylation was determined by Duoset IC for human phosphor-ErbB2 according to the manufacturer's directions (R&D Systems).

Viability assay.

This assay was performed just like the proliferation assay but after treatment with the peptide inhibitors, the aCella-TOX reagent was used to estimate the amount of dead cells. After peptide treatment for 72h, the plate was removed and equilibrated to room temperature for 15 minutes before adding 10 µl of lytic agent to the control wells for maximum lysis and incubated for 15min at room temperature. A 100 µl aliquot of the Enzyme Assay reagent containing G3P was then added to all wells followed by 50 µl of the detection reagent. The plate was immediately read using a luminometer.

Peptide treatment in transplantable mouse model.

Balb/c mice (n =5), 5 to 6 weeks of age, were challenged subcutaneously with 1 $x10^5$ TUBO cells that express the rat HER-2/neu and after challenge, mice were treated intravenously weekly with 100 μg of either HER-2 or VEGF peptide mimics or a combination of both as inhibitors for a total 6 treatments. At day zero, mice were given both the TUBO cells and the first treatment. Mice were euthanized at day 39 post TUBO challenge (week 10) and tumors removed. Tumors were measured for tumor volume twice a week using calipers and calculated using the formula (length x $width^2$)/2. All animal handling was done according to the institutional guidelines.

In vivo Matrigel assay.

Liquid matrigel (500 μl) were injected subcutaneously into the flanks of Balb/c mice. The matrigel (BD Bioscience, CA) contained $VEGF_{165}$ at a final concentration of 500 ng/ml to stimulate angiogenesis and VEGF peptides or irrelevant peptides were added at a concentration of 500 μg/ml. All treatment groups contained 3 mice and each mouse had two plugs each on the left and right flanks. After ten days, the mice were sacrificed and matrigel plugs were removed and hemoglobin content was determined using the Drabkin's reagent kit. The matrigel plugs were homogenized in hypotonic lysis buffer (250ul of 0.1% Brij-35 per plug) and centrifuged for 5min at 5000g. The supernatant was incubated at 0.5ml of Drabkin's solution for 15min at room temperature and the absorbance was measured at 540nm with Drabkin's solution as a blank. Since absorbance is proportional to the total hemoglobin content, the relative hemoglobin content was calculated versus the negative and positive controls.

2.3 RESULTS

Selection, design, synthesis and characterization of peptides.

The selection of the VEGF peptide mimic residues 102-122 (numbered as 76-96 in the crystal structure) corresponds to the overlapping VEGF binding sites to VEGFR-2 and Avastin®. Engineering of this peptide sequence has been described in details elsewhere (Vicari et al.). The sequences of both the HER-2 and VEGF peptide mimics are shown in Table 2.1. Briefly, the strategy to create a conformational peptide consisting of an anti-parallel β-sheet is described elsewhere (Vicari et al.) where the sequence was modified in a way that the resulting peptide VEGF-P3 (NC) adopted a conformation very similar to the native structure. It also required two artificial cysteines to be introduced between Gln79 & Gly92, and between Ile80 & Glu93. After synthesis and purification of VEGF-P3 (NC) (non-cyclized) peptide, the disulfide bond was formed by oxidation enabling the formation of the twisted anti-parallel β-sheet structure in the VEGF-P3(CYC). The retro-inverso (RI) peptide analog VEGF-RI-P4 was synthesized using D-amino acids with the amino acid sequence in reverse order, such that the resulting peptide mimic has a reversal of the peptide backbone but a topochemical equivalence to the parent peptide in terms of side-chain orientation [149, 153, 154]. The rationale behind the retro-inverso peptidomimetic is that it should present similar activity with the advantage of higher bioavailability [155]. Figure 2.1A shows a schematic representation of the retro-inverso peptide mimics.

The choice for the HER-2 peptide mimic sequence spanning residues 266-339 (Table 2.1) was determined first on the basis of the crystal structure of the Fab of pertuzumab bound to the subdomain II of HER-2 ECD [113] and extensive immunogenic studies as described in (Allen *et al*., 2007). The HER-2 peptide mimic 266-296 was selected based on the criteria that antibodies elicited against the peptide was capable of inhibiting dimerization of HER-2 due to its ability to bind and recognize the HER-2 ECD [114]. Similar strategies to the VEGF peptide mimic was used to design the HER-2 peptide mimic RI-HER-2 (CYC). All synthetic peptides were successfully synthesized in our laboratory using well established protocols, purified by reverse phase HPLC using a Vydac C-4 column and acetonitrile-water (0.1 % TFA) gradient system and characterized by MALDI mass spectrometric analysis. As shown in Figure 2.1B the CD spectrum is typical of β-sheet structure.

Antiproliferative effects of peptides.

The antiproliferative effects (Figure 2.2) of the peptides were tested using four different cell lines (BT-474 and SK-BR-3: HER-2^{high}, MDA-468: HER-2^{low} and TS/A: HER-$2^{negative}$) in the presence of HRG to activate the HER-3 receptor. Unlike trastuzumab that is specific to HER-2 positive cells, pertuzumab is known to act on cells by disrupting ligand dependent receptor complexes independent of HER-2/neu expression [110]. The cells were incubated with the peptides before being exposed to HRG. It was found that both the HER-2 and VEGF peptides were able to inhibit tumor growth and the effect was concentration dependent (Figure 2.2). Four different cell lines were used to show that the effects of the peptide are dependent on HER-2 expression since higher inhibition was

observed in cases of high HER-2 expression. BT-474 and SK-BR-3 both have high HER-2 expression but the level of HER-1 and HER-3 (HER-2 dimerization partners) in SK-BR-3 are respectively ten times and two times higher than in BT-474 [156]. This probably explains why the percentage inhibition is by far greater in SK-BR-3 cells than in BT-474 cells (Figure 2.2). The HER-2-266-296 also showed inhibitory effects on HER-2 negative cells (TS/A cells which originated from a mammary adenocarcinoma that arose spontaneously in a BALB/c female retired breeder. This is because the peptide was designed based on the binding of HER-2 to pertuzumab, which has been shown to inhibit HER-2 negative cells (36).

To determine whether combination treatment would have a significant bearing on inhibition of proliferation as compared to individual treatment, mixtures of the HER-2 and VEGF peptide mimics were used. As shown in Figure 2.3, we observed an increase in rate of inhibition when both peptides were used as compared to single treatments (Figure 2.3). Statistical analysis showed a significant difference between the treated and untreated cells in all five concentrations (25, 50, 75, 100 and 150 μg) with P* values of < 0.001 using the 95% confidence intervals. Irrelevant peptide did not show antiproliferative effects while Trastuzumab (positive control) showed antiproliferative effects only on cells that express the HER-2 receptor (Figures 2.2 and 2.3).

Effects of peptide treatment on breast cancer cell viability.

We next evaluated the effects of combination treatment on tumor cell survival *in vitro*. The MTT proliferation assay simply shows that the peptides are able to prevent the cells from growing but does not show whether the cells are being killed by the peptide. This

was tested using the acella-TOX reagent kit where dead or dying cells released the enzyme GAPDH and measuring the activity of this enzyme will give an estimate of the cell viability after treatment. The results showed that the peptide treatment was able to cause a decrease in cell viability and combination treatment caused a further decrease in viability of at least 40% compared to single treatment (Figure 2.4). There was a statistically significant difference between treatment with HER-2 or VEGF peptides and the untreated group with P* values < 0.05 using the 95% confidence interval. The difference was most significant in the case of the combination treatment with both HER-2 and VEGF peptide mimics with P* values < 0.001 when using the 95% confidence interval when compared to the untreated. Finally, when comparing the single and combination treatment, we also obtained a significant difference with P** values < 0.001 using the same confidence interval. Treatment with irrelevant peptide showed no statistical difference with untreated cells.

Peptide inhibition of Phosphorylation.

The main mode of action of Pertuzumab is to inhibit phosphorylation. This is due to the fact that antibody sterically blocks the dimerization domain of HER-2 thereby preventing the formation of dimers with other HER receptors and thus interrupting downstream signaling. As shown in Figure 2.5, the peptides were able to prevent phosphorylation of the HER-2 protein and single treatment with the HER-2 peptide alone caused a 38% inhibition rate while the VEGF peptide with L and D amino acids caused an inhibition rate of 28% and 39%, respectively. Combination treatments led to dramatic increases in rate of inhibition of 67 % and 70% for combining HER-2 + VEGF-

P3-CYC and HER-2 +VEGF P4-CYC respectively (Figure 2.5). All peptide treatments were compared to the positive control AG825 (Calbiochem), a HER-2 specific phosphorylation inhibitor. Statistical analysis also showed a significant difference between the treated and untreated groups with P* values of < 0.001 using the 95% confidence intervals. Also, comparing the single and combination treatments also showed a statistical significant difference between the two treatments with P** values of < 0.001. The cells treated with the irrelevant peptide were similar to untreated cells.

Transplantable tumor challenge models.

In order to determine the ability of the peptides to inhibit tumor growth *in vivo*, we used a rat neu-expressing tumor challenge model. The rat neu has a 97% similarity to that of the human HER-2 266-296 sequence with only one disparate amino acid [135]. To investigate the efficacy of peptide treatment, we challenged groups of BALB/c mice (n=5) with TUBO cells derived from tumors of BALB-neuT transgenic mice [94, 157] followed by weekly treatment with either HER-2 or VEGF peptide mimics or a combination of both and monitored tumor growth up to 5-6 weeks post challenge.

As shown in Figure 2.6, combination treatment with HER-2-266-296 (L) peptide mimic with VEGF peptide mimics (VEGF-P3-CYC-(L) or VEGF-P4-(D) produces greater antitumor effects as compared to single HER-2 or VEGF peptide mimic treatment. Mice that were treated with the HER-2 peptide alone was significantly different from the group treated with combination of HER-2 and VEGF peptide mimics (*$P<0.001$). There was also a significant delay in unset of tumor development in the case

of combination treatment (around day 25) as compared to single treatment (around day 18).

Next we compared *in vivo* anti-tumor effects with all D- retro-inverso peptide mimics. As shown in Figure 2.7. combination treatment with HER-2 D amino retro inverso peptide and the VEGF-P4 peptide shows greater inhibitory effects than treatment with HER-2 peptide alone. Mice treated with the combination was significantly different from those treated with either the HER-2 peptide alone or the VEGF peptide alone (*$P<0.001$). There was also a delay in unset of tumor development in the case of combination treatment (around day 28) as compared to single treatment (around day 21). These groups also produced a delay in tumor burden and the group treated with HER-2 and D-amino acid VEGF peptide was 20% tumor free at the end of the experiment (Figure 2.8A). The same combination treatment group using the D-amino acid VEGF peptide and HER-2 peptide produced the most statistical significant reduction in the percent tumor weight ($P^{**}<0.001$) using ANOVA analysis (Figure 2.8B). There was no significant difference or delay in tumor growth between the untreated and the irrelevant peptide and tumor growth and development followed a similar pattern.

These results strongly indicate that combination treatment with HER-2 and VEGF peptide mimics produced a statistically significant reduction in tumor growth and development *in vivo* and also showed more potent anti-tumor effects in *in vitro* assays indicating that targeting both HER-2 and VEGF is a more attractive strategy than targeting only one of the pathway. Also, the retro inverso D-amino acid peptide mimics gave better results than the L-amino acid peptides in both the cases of single and

combination treatments. Additionally as shown in Figure 2.9, combination treatment causes a decrease in tumor burden as illustrated by the % tumor weight in the mice treated with both peptides. The best results were obtained in the case of combination of both the D-amino acids peptides of HER-2 and VEGF with a significant value of *P<0.001 as compared to their individual treatments with *P<0.0047 when compared to those untreated.

In vivo Matrigel angiogenesis assay.

To determine whether the VEGF peptides were able to inhibit angiogenesis *in vivo,* we performed an *in vivo* matrigel assay in which BALB/c mice were injected with matrigel containing VEGF and treated with peptides. Figure 2.10 shows the decrease in hemoglobin content in the case of treatment with VEGF peptide mimics while the irrelevant peptide had no effect since it was not different from the untreated. These results show that the peptides are able to prevent blood flow to the plugs due to limited angiogenesis.

2.4 DISCUSSION

Work in our laboratory over the past two decades has focused mostly on the development of B-cell epitope vaccines that activate both the humoral and cellular arms of the immune system resulting in the production of high affinity anti-peptide antibodies with enhanced antitumor activities. HER-2/neu is an oncoprotein that is overexpressed in many types of tumors and is associated with highly aggressive forms of cancers [158,

159]. HER-2 is thus an important therapeutic target and therapeutic modalities have been devised that target the receptor and downstream molecular pathways. We have successfully translated our extensive HER-2 preclinical studies [49], [50] to the clinic in a phase 1 trial [111]. Structural studies of HER-2 in complex with the anti-HER-2 antibodies Trastuzumab/Herceptin and Pertuzumab/Omnitarg have significantly provided new insights into how these drugs function [113]. We have designed several conformational peptides based on the binding of the ECD of HER-2 with pertuzumab [114] and trastuzumab [115] as vaccine candidates which are the subject of an upcoming clinical trial at the James Cancer Hospital (Trial 0105)

Many therapeutic modalities targeting receptors tyrosine kinases (RTK) and downstream molecular pathways have been devised and the most outstanding among these are the ErbB and vascular endothelial growth factor receptor (VEGFR) families of kinases [67, 141, 160]. Many agents have been developed aimed at targeting RTKs and these include therapeutic antibodies to RTK ligands or the receptors themselves and small molecule inhibitors that target the intracellular kinase domains of RTKs [58-60]. Many of the FDA approved therapies targeting both HER-2 (Trastuzumab, Herceptin®) and VEGF (Bevacizumab, Avastin®) have significant toxicities including cardiac dysfunction and congestive heart failure [61-63] and the patients demonstrate disease progression due to development of resistance. Clinical applications of mAb therapy in general is limited by a number of concerns such as frequency of treatments, associated costs, limited duration of action, undesired immunogenicities, development of resistance, and significant risk of cardiotoxicities [64]. Similarly, the small molecule RTK inhibitors such as Sunitinib which have entered clinical trials alone or in combination with

radiotherapy or chemotherapy show problems of efficacy, development of resistance and unacceptable safety profiles that continues to hamper their clinical progress [62]. Despite the success of these drugs, there still remains a high and unmet need for novel molecular cancer therapeutics. Novel therapies targeting these aberrant molecular pathways are urgently needed that can offer hope that the effectiveness and duration of response can be greatly improved.

In the present work, we were interested in extending our approaches by evaluating combination therapy with peptide mimics designed to inhibit the interaction between receptor and its ligand as immunotherapeutic strategies. Given the present state of small molecule inhibitors and their unacceptable safety profiles we believe that rationale synthetic peptide approach may offer a viable safety alternative. Peptide mimics offer the benefits of being water soluble, non-immunogenic, and the ability to easily cross tissue barriers [83]. One of the major drawbacks of using peptides as therapy is their high susceptibility to proteosomal degradation [84]. Because retro-inverso peptides are synthesized with D-amino acids and proteases usually recognize L-amino acids, they should be resistant to proteosomal degradation, and therefore will increase the bioavailability of the peptidomimetic therapeutic in vivo [84, 85, 148]. Our interest in VEGF stems from the fact that overexpression of HER-2 is associated with increases expression of VEGF at both the RNA and protein level in breast cancer cells and a positive association between HER-2 and VEGF expression in breast cancer patients has been identified [74]. Targeting these two receptors using a combination strategy can therefore interact in a synergistic/additive manner killing tumor cells and retarding tumor development [57, 143, 144]. Thus, combination therapy targeting both HER-2 and

VEGF is a very promising strategy since anti-angiogenic therapy alone will only delay tumor growth [145] and targeting HER-2 and VEGF will destroy two different tumor dependent pathways. We have successfully designed peptide mimics of VEGF to inhibit angiogenesis. This peptide was synthesized and tested in a number of different studies using cancer cells, HUVECs and animal models (vicari *et al.*) resulting in the choice of VEGF-P3-CYC.

The retro-inverso analog of the VEGF peptide was designed and synthesized using D-amino acids with the intent that it would be superior to the L-amino-acid peptide mimic when used *in vivo.* Pertuzumab binds to different epitopes in the HER-2 extracellular domain amino acids 266 to 333 near the junction of domains I, II, and III [113]. This interaction was shown to sterically hinder the association of HER-2 with other receptors such as HER-3 sterically blocking a pocket necessary for receptor dimerization and signaling. Based on these studies, we have selected one HER-2 peptide mimic pertuzumab-like sequence 266-296 which has been shown to be a good candidate for a neutralizing peptide [114].

We evaluated the antiproliferative effects of the peptides or their combinations on different cell lines. Trastuzumab has been shown to be specific to only HER-2 positive cells [49] and this was observed in our results where no inhibition was observed with the TS/A(HER-2 negative) cell line. There was also a reduction in % inhibition in the case of MDA-468 (HER-2 low) as compared to BT-474 and SK-BR-3 (HER-2 high) cells. This indicates that the peptides were effective in inhibiting HER-2 cancer cells. The HER-2-266 peptide showed inhibitory effects also on the HER-2 negative cell line

(TS/A) and this is because it is the pertuzumab-like peptide and pertuzumab is also effective in cells that are independent of HER-2 [49]. After showing some level of specificity to the HER-2 receptor, we tested the effects of combination treatment with both HER-2 and VEGF peptides. We noticed that there was an increase in proliferation inhibition when combination treatment was used and the treated groups were statistically different from the untreated while the irrelevant peptide had no statistical effects on the cells (Figures 2.2 & 2.3).

We also evaluated the effects of combination treatment on cell viability and the results obtained showed that single treatment with HER-2 or VEGF peptides gives a viability of about 70% while combination treatment with both peptides reduces the viability to less than 25% (Figure 2.4). The difference was statistically significant between the single and combination treatment with P** values of < 0.001. HER-2 is known to dimerize with its partner HER-1 and HER-3 leading to receptor phosphorylation and intracellular signaling and pertuzumab mainly functions by sterically blocking this receptor from binding to its partners and is therefore classified as a dimerization inhibitor [161, 162]. We therefore wanted to investigate the effects of peptide treatment on phosphorylation and the results also indicated and increased in phosphorylation inhibition from less than 40% in the case of single treatments to about 70% in the case of combination treatment and the difference between these two treatments were statistically significant with P** values of < 0.001 (Figure 2.5).

In order to evaluate the effects of peptide treatment in vivo, we used a transplantable mouse model. BALB/c mice were challenged with TUBO cells and treated with peptides

and their combinations. The results obtained indicated a statistical significance of $*p <$ 0.001 between the group treated with the peptides and their combinations and the group treated with the irrelevant peptide or untreated (Figure 2.6). Combination treatment with HER-2 D amino retro inverso peptide and the VEGF-P4 peptide showed greater inhibitory effects than treatment with HER-2 peptide alone (Figure 2.7) . Combination treatment increases the survival free rates and unset of tumor emergence since 20% of mice in the group treated with HER-2-L and VEGF-P4 remained tumor free at the end of the experiment (Figure 2.8A) while 40% of mice in the group treated with HER-2-D and the VEGF-P4 also remained tumor free at the end of the experiment (Figure 2.8B). Combination treatment causes a decrease in tumor burden as illustrated by the % tumor weight in the mice treated with both peptides. The best results were obtained in the case of combination of both the D-amino acids peptides of HER-2 and VEGF with a significant value of *P<0.001 as compared to their individual treatments with *P<0.0047 when compared to the untreated (Figure 2.9).

Efficient *in vivo* angiogenesis assays to assess and compare anti-angiogenic activity are a prerequisite for the discovery and characterization of anti-angiogenic targets. When VEGF are mixed with Matrigel and injected subcutaneously into mice, endothelial cells migrate into the gel plug. These endothelial cells form vessel-like structures, a process that mimics the formation of capillary networks. To delineate whether our results in BALB/c mice were due to inhibition of angiogenesis, we carried out experiments in which we injected matrigel, matrigel containing VEGF and used P3, P4 and an irrelevant peptide as inhibitors (Figure 2.10). The results are clear in that there were significant inhibition of hemoglobin content in the plugs treated with the VEGF peptides. This

indicates that angiogenesis was significantly inhibited and points to the importance of targeting VEGF and tumor angiogenesis for the treatment of human cancer.

In summary, these data greatly illustrates that the peptide mimics have potent anti-tumor activity and combination treatment with both HER-2 and VEGF peptides mimics produces additive effects. This shows that, targeting the two different proteins will produce greater antitumor and anti-angiogenic effects both in vitro and in vivo. Also from the *in vivo* studies, the best result was obtained in the case of combination of the D-amino peptide mimics of HER-2 and VEGF. This shows that the D-amino peptide probably had a greater stability to *in vivo* since it cannot be degraded by proteases in the blood. In conclusion the innovative approaches we have described in this paper may have significant implications and provide viable safe alternatives to present treatment and holds tremendous potential for the treatment of various solid tumor types. Tailored treatments consisting of combinations of vaccines, targeted therapies, angiogenesis inhibitors and metronomic chemotherapy will hopefully result in better patient outcomes with little toxicity events. Integration of novel agents targeting VEGF, HER-2 and EGFR with the goal to predict which specific targeted agent combination will most likely benefit individual patients remains a formidable challenge but certainly an attainable goal in the future.

2. 5 Tables and Figures

Designation	Peptide	Sequence	M.Wt. (da)
HER-2-266-CYC	***266-296*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{266}LH**C**PA *LVTYNTDTFESMPNPEGRYTFGAS**C**V*^{296}*-CONH2*	2925
VEGF-P3-CYC	***76-96*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{76}ITMQ^{79}-C-^{92}GIHQGQHPKIRMI^{80}-C-^{93}EMSF^{96}-*CONH2*	2527
VEGF-RI-P4-CYC	***96-76*** *peptide with D amino acids and one disulfide bond*	*CH_3CONH-*(D)-^{96}FSME^{93}-**C**-^{80}IMRIKPHQGQHIG^{92}-**C**-^{79}QMTI^{76}-*CONH2*	2527

Table 2.1: Amino acid sequences and molecular weight of HER-2 and VEGF peptide mimics. Sequences of amino acids are represented from N to C terminal except for the retro inverso peptides RI-HER-2-CYC and VEGF-RI-P4-CYC that were synthesized in the reverse order and using D amino acids. All peptides were synthesized on CLEAR amide resin, using Fmoc/t-But chemistry. All peptides were acetylated on resin using Acetyl-Imidazole (4x) in DMF for 4 hr. Cysteine residues are underlined to indicate the locations of the disulfide bonds.

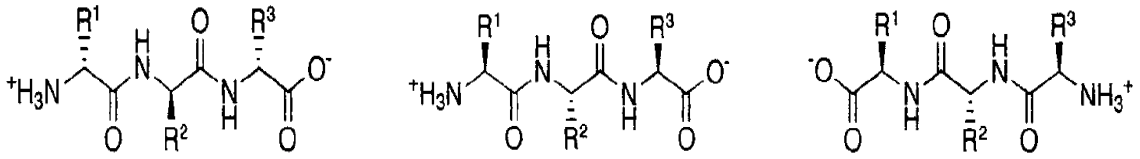


Figure 2.1A Schematic representation of the effects of retro-inverso peptides. Natural orientation of parent L-amino acid peptide (**I**). Reversed side chain orientation in D-amino acid peptide (**II**). Retro-inverso peptide with restored side chain orientation (**III**).

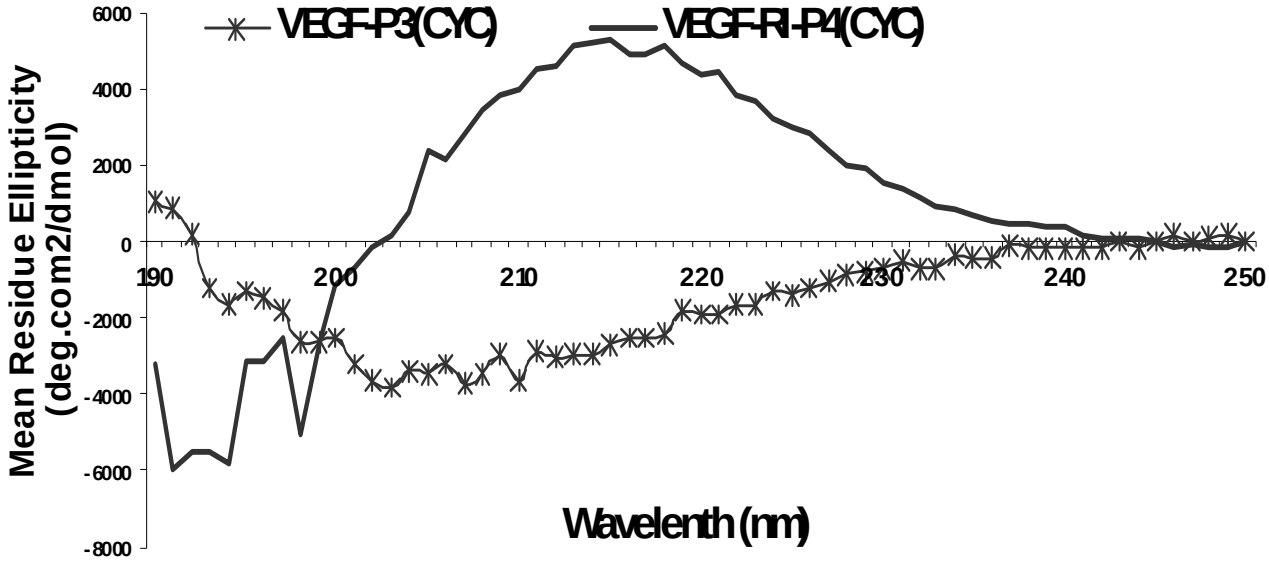
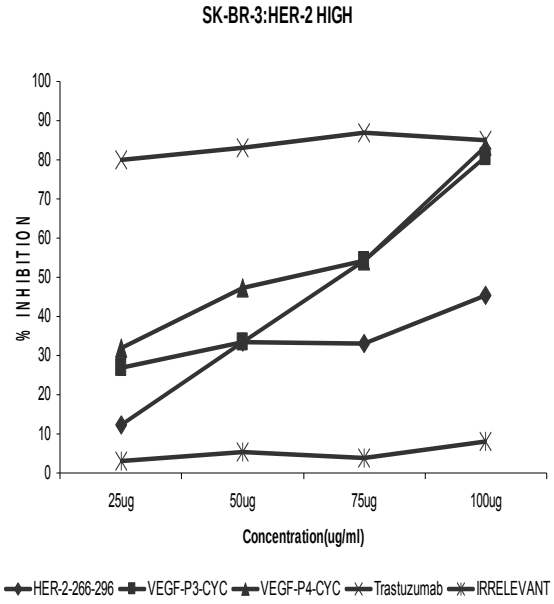
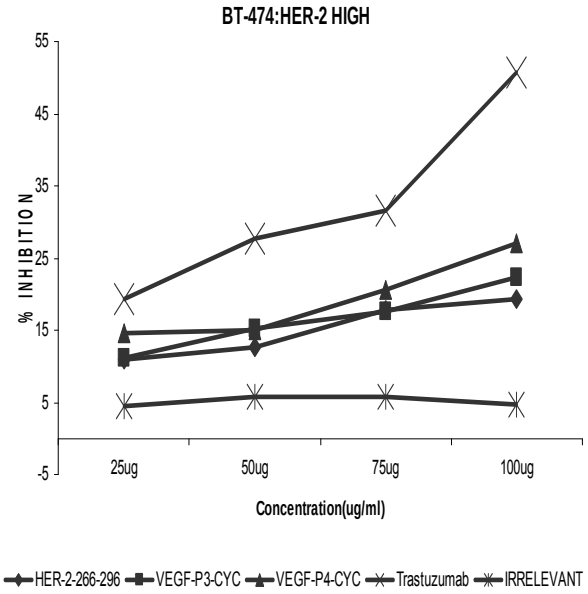
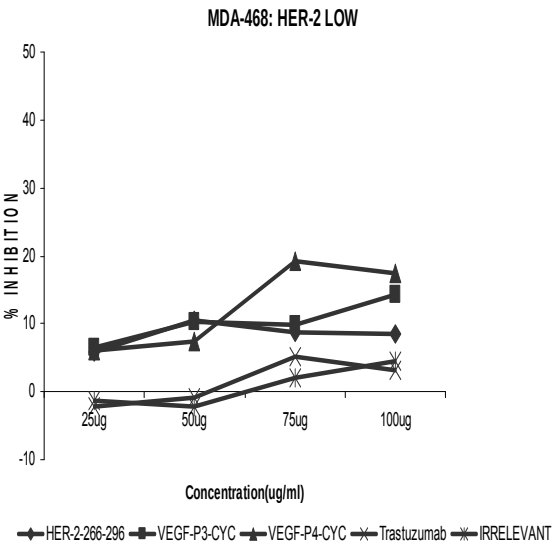


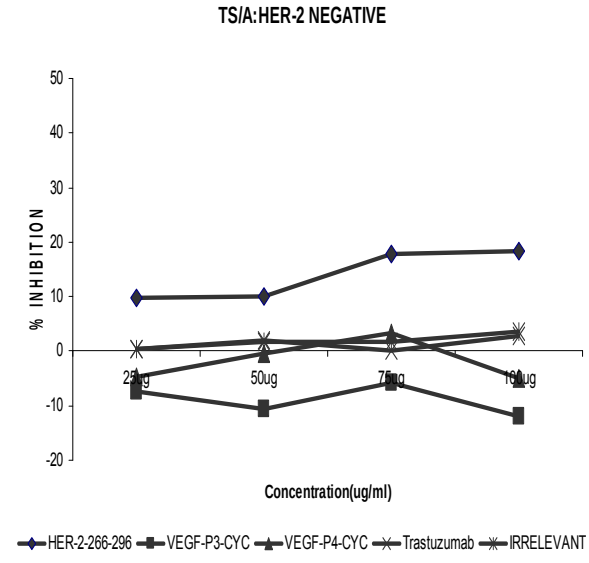
Figure 2.1B CD spectra of retro-inverso VEGF peptide mimic. CD spectroscopy measurements were made of 100 μM peptide solutions in either water. Spectra characteristic of β-turn is observed by the shifting of θ mimina towards 217nm. Inversion of the elipticity in the spectra was result of inverted chirality by using D-amino acid (RI) resulting in a mirror image profile.

Figure 2.2. **Anti-proliferative effects of HER-2 and VEGF peptide mimics used as single treatments.** BT474, SK-BR-3, MDA-468 and TS/A cells were incubated with HER-2 peptide, VEGF peptides, Trastuzumab and irrelevant peptide. Bioconversion of MTT was used to estimate the number of active tumor cells remaining after 3 days. Peptides were added at four different concentrations using the above-mentioned cell lines. The proliferation inhibition rate was calculated using the formula (ODnormal Untreated - OD peptides or Ab)/ODnormal untreated x 100.


SK-BR-3:HER-2 HIGH
% INHIBITION
100
90
80
70
60
50
40
30
20
10
0
25ug
50ug
75ug
100ug
Concentration(ug/ml)
HER-2-266-296
VEGF-P3-CYC
VEGF-P4-CYC
Trastuzumab
IRRELEVANT


BT-474:HER-2 HIGH
% INHIBITION
55
45
35
25
15
5
-5
25ug
50ug
75ug
100ug
Concentration(ug/ml)
HER-2-266-296
VEGF-P3-CYC
VEGF-P4-CYC
Trastuzumab
IRRELEVANT


MDA-468: HER-2 LOW
% INHIBITION
50
40
30
20
10
0
-10
250g
500g
75ug
100ug
Concentration(ug/ml)
HER-2-266-296
VEGF-P3-CYC
VEGF-P4-CYC
Trastuzumab
IRRELEVANT


TS/A:HER-2 NEGATIVE
% INHIBITION
50
40
30
20
10
0
-10
-20
50ug
Concentration(ug/ml)
HER-2-266-296
VEGF-P3-CYC
VEGF-P4-CYC
Trastuzumab
IRRELEVANT

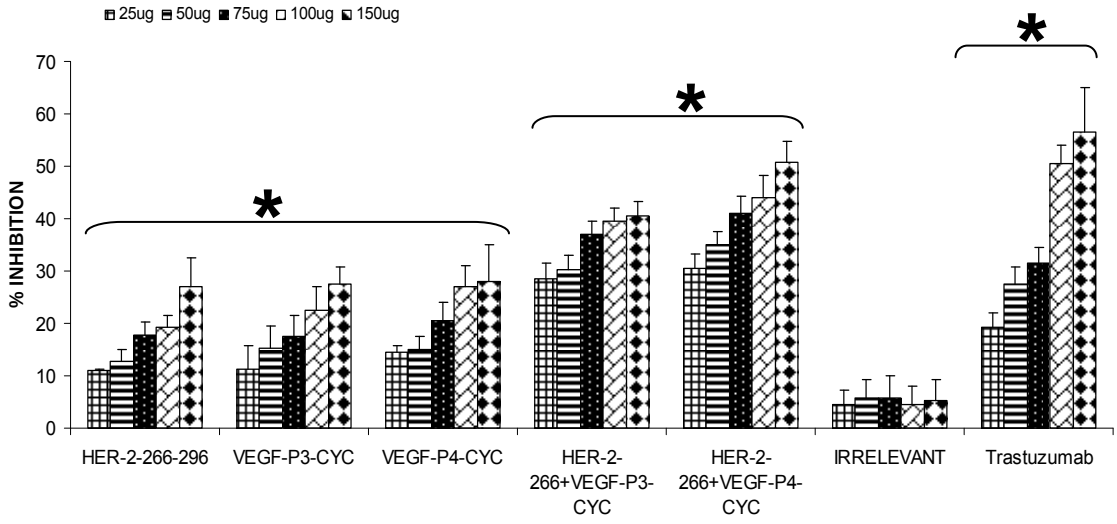


Figure 2.3. **Anti-proliferative effects of combination treatment with HER-2 and VEGF peptide mimics.** BT-474 cells were treated in the same manner as in Figure 2.2 but treated with HER-2 peptide, VEGF peptides or combination of both. Trastuzumab and irrelevant peptide were used as positive and negative controls. Rate of inhibition was calculated using the same formula as above and all results represents the average of three different experiments. Statistical analysis was done using the ANOVA model with *$P<0.001$ when compared to the untreated. Error bars represent SD of the mean.

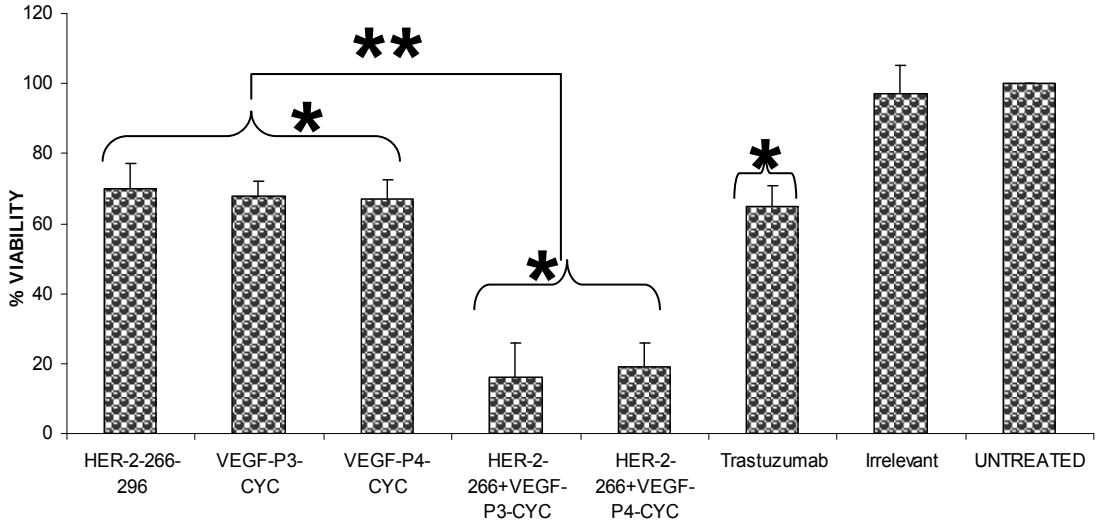


Figure 2.4 Effects of combination treatment on cancer cell viability (A) BT474 cells were incubated with media alone, HER-2 peptide, VEGF peptides, trastuzumab, and irrelevant peptide (100µg each). The number of viable cells remaining after three days was determined using the aCella-TOX reagent kit and all instructions were done according to manufacturer's instructions. Cell viability is expressed as a percentage of untreated cells. Data points represent the mean of three independent experiments. Error bars represent SD. Results represent average of three different experiments. Statistical analysis was done using the random effect linear regression model and *$P<0.001$ when compared to the untreated and **$P<0.001$ when comparing single treatments to combination treatments.

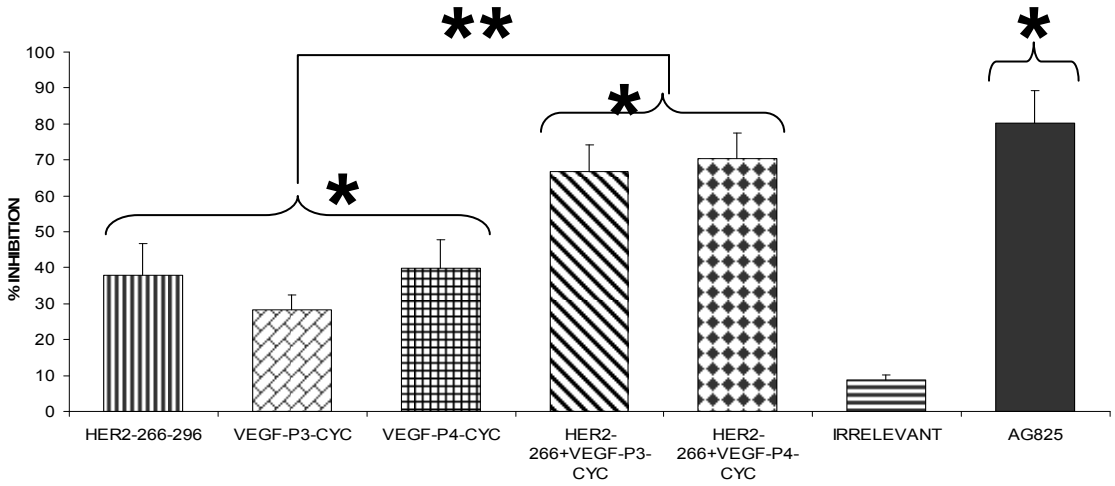


Figure 2.5 Effects of combination treatment on HER-2 phosphorylation

BT-474 cells were incubated with 100ug of HER-2 and VEGF peptides before being exposed to HRG (HER-3 activating ligand) for 10 minutes and lysed. Phosphorylated HER-2/neu was determined by indirect ELISA and percent inhibition was calculated as in (A) above. AG825 (Calbiochem) a potent HER-2 phosphorylation inhibitor was used as a positive control. Results represent average data from three different experiments. Error bars represent SD of the mean. Statistical analysis was done using the random effect linear regression model and *P<0.001 when compared to the untreated and **P<0.001 when comparing single treatments to combination treatments**.**

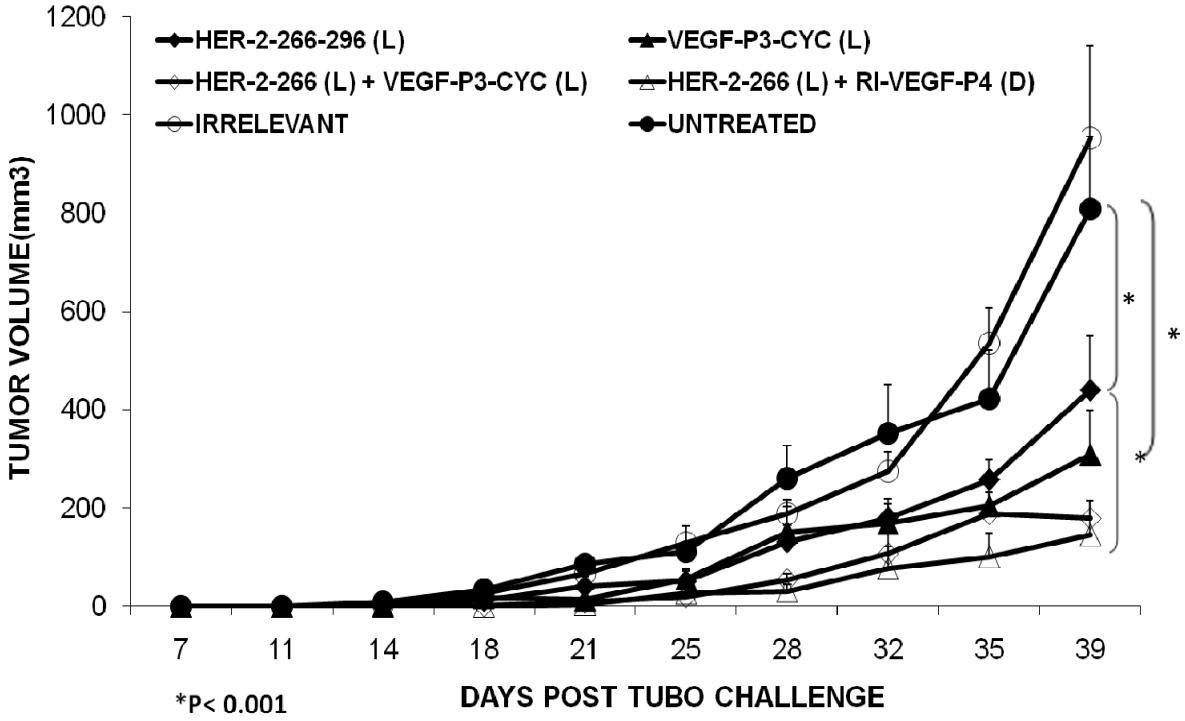


Figure 2.6 In vivo anti-tumor effects of combination treatment with HER-2 L-amino acid peptide. Wild type BALB/c mice (*n* =5), at the age of 5-6 weeks was challenged with TUBO cells that were derived from BALB-*neuT* mice which are transgenic for the rat HER-2/neu oncogene, and were treated intravenously with HER-2 and VEGF peptide mimics, and scrambled irrelevant peptide, Tumor measurements were performed twice a week using calipers. The data are presented as the average tumor size per group and are reported as mm^3 for combination treatment with HER-2 L amino acid VEGF peptide mimics.

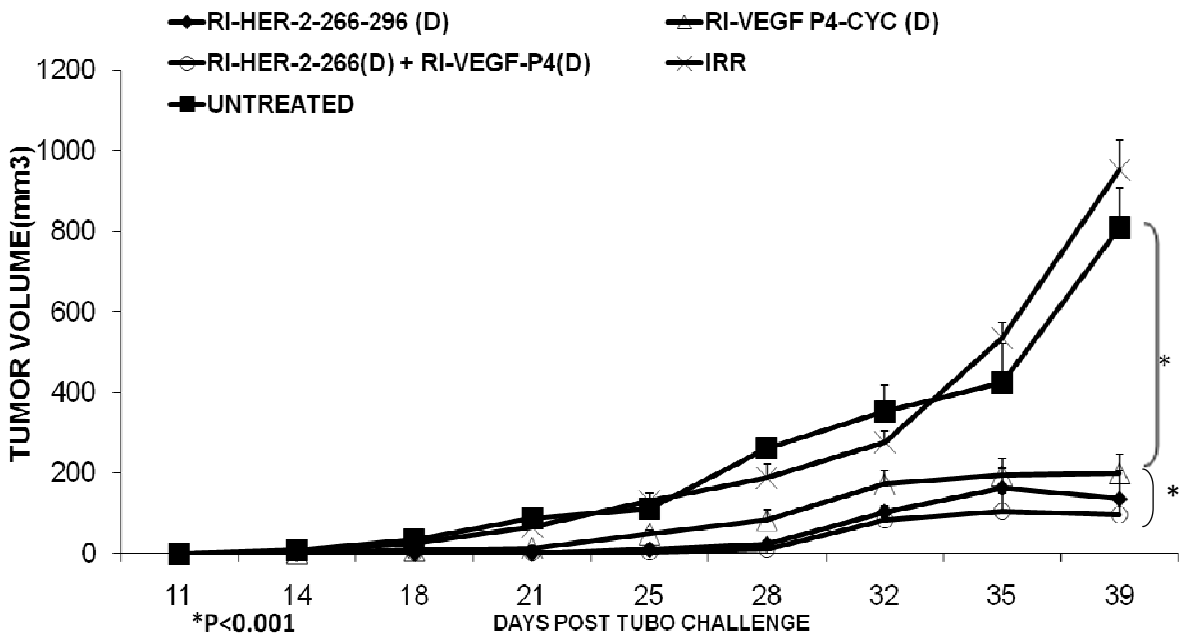
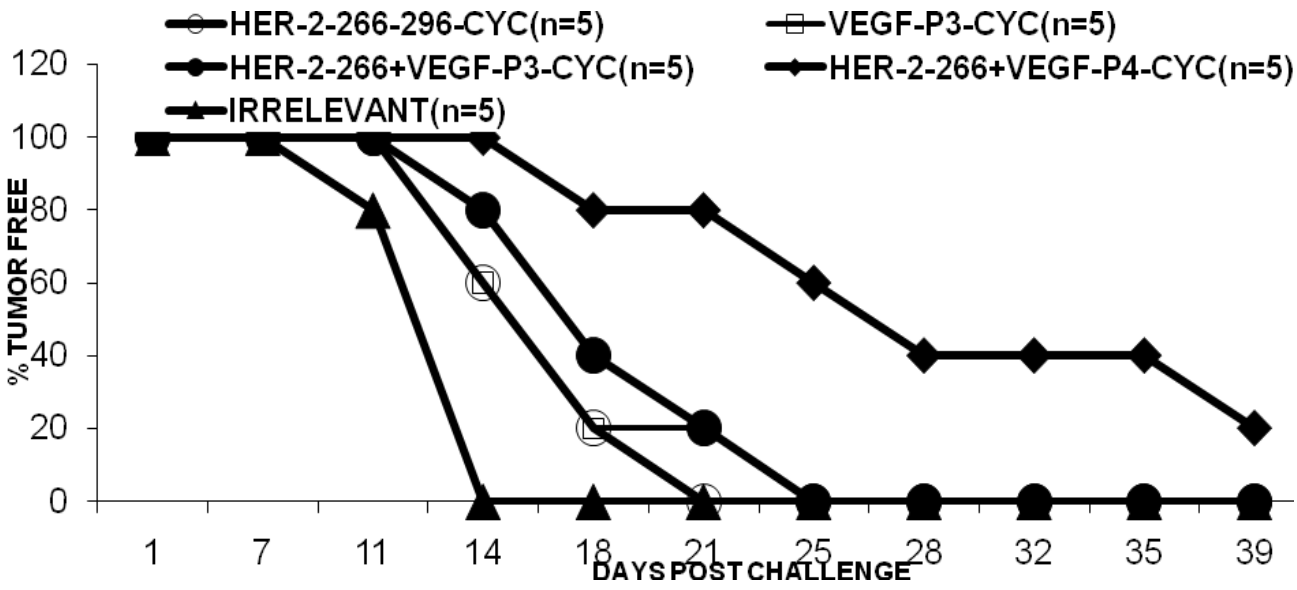


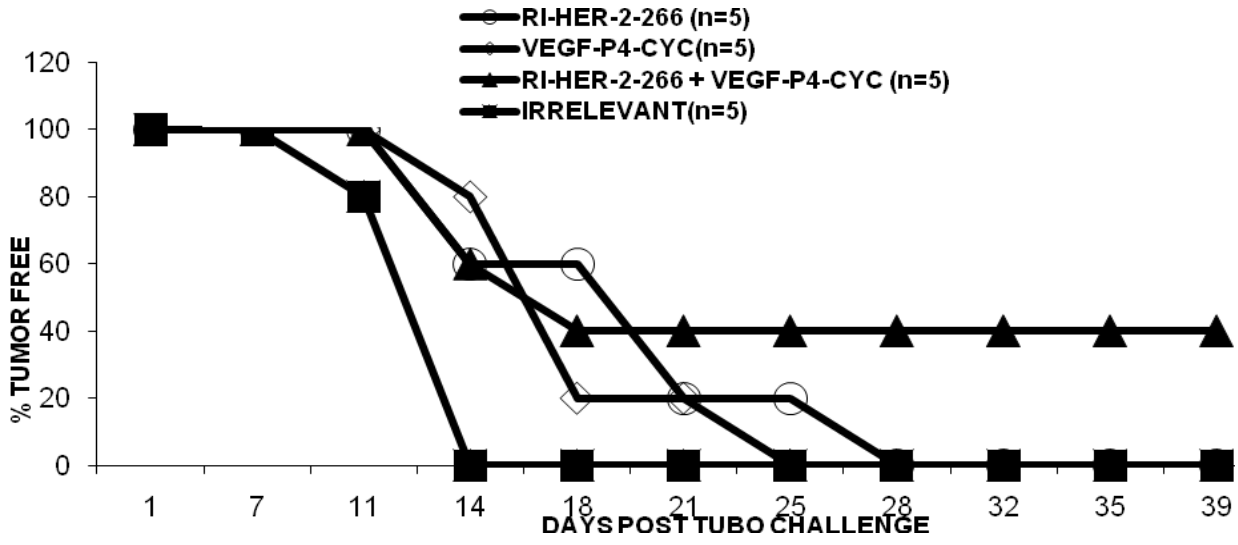
Figure 2.7. **In vivo anti-tumor effects of combination treatment with HER-2 D-and VEGF D-amino acid peptide.** Wild type BALB/c mice (*n* =5), at the age of 5-6 weeks was challenged with TUBO cells that were derived from BALB-*neuT* mice which are transgenic for the rat HER-2/neu oncogene, and were treated intravenously with HER-2 and VEGF peptide mimics, and scrambled irrelevant peptide, Tumor measurements were performed twice a week using calipers. The data are presented as the average tumor size per group and are reported as mm^3 for combination treatment with HER-2 D amino acid VEGF peptide mimics.

Figure 2.8 Tumor free survival rates for combination treatments with HER-2 and VEGF peptide mimics. Mice were monitored for tumor development twice weekly in both cases of combination treatment with L-amino HER-2 peptide (A) and D-amino HER-2 peptide (B). In both cases, combination treatment showed the best survival free rates as compared to single treatments and untreated.

A


HER-2-266-296-CYC(n=5)
VEGF-P3-CYC(n=5)
HER-2-266+VEGF-P3-CYC(n=5)
HER-2-266+VEGF-P4-CYC(n=5)
IRRELEVANT(n=5)
% TUMOR FREE
120
100
80
60
40
20
0
1
7
11
14
18
21
25
28
32
35
39
DAYS POST CHALLENGE

B


RI-HER-2-266 (n=5)
VEGF-P4-CYC(n=5)
RI-HER-2-266 + VEGF-P4-CYC (n=5)
IRRELEVANT(n=5)
% TUMOR FREE
120
100
80
60
40
20
0
1
7
11
14
18
21
25
28
32
35
39
DAYS POST TUBO CHALLENGE

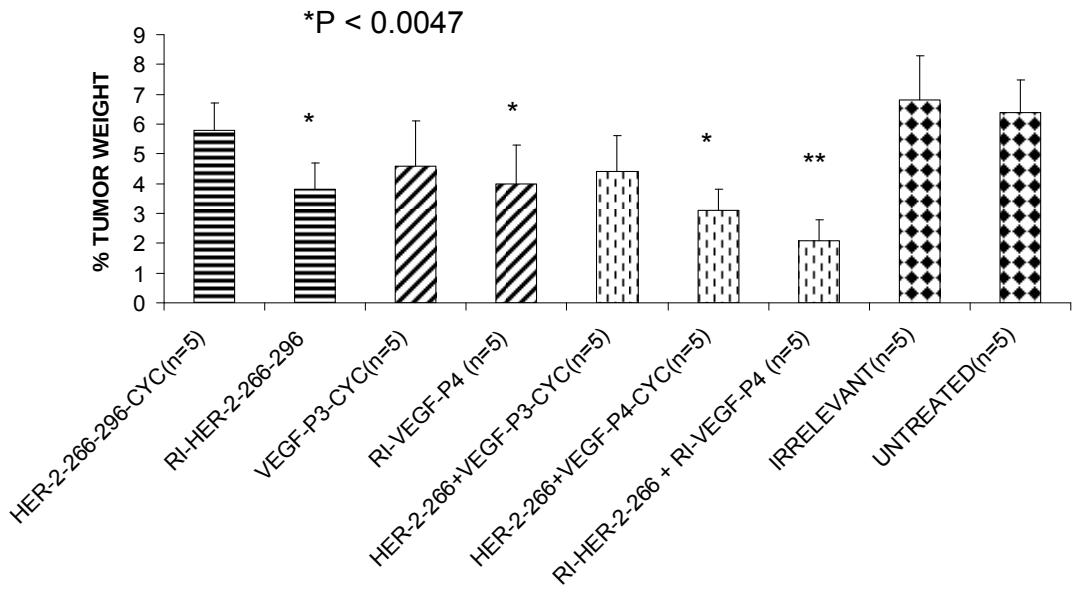
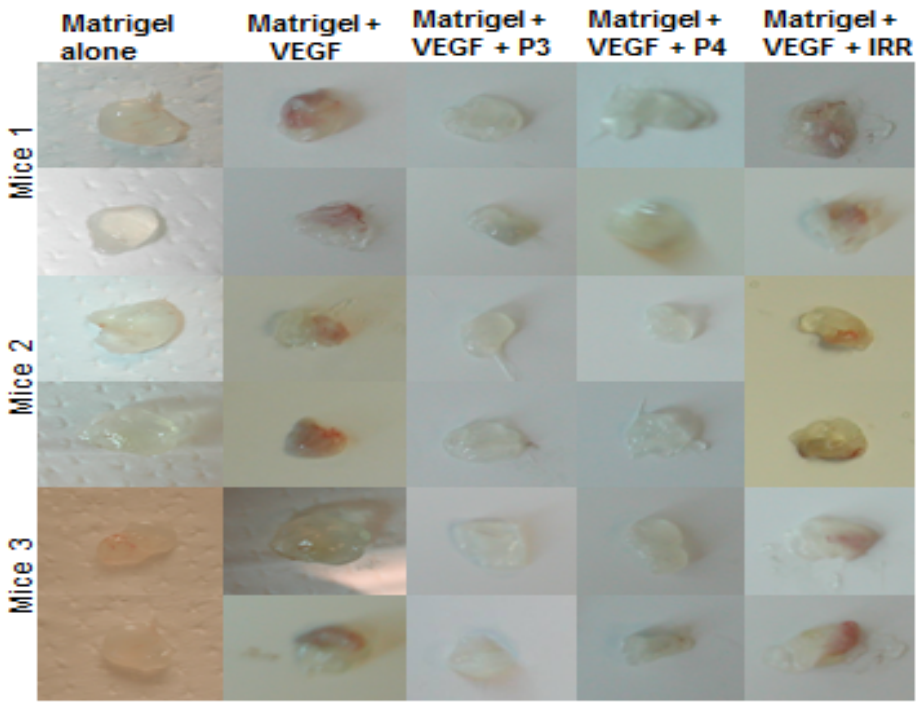


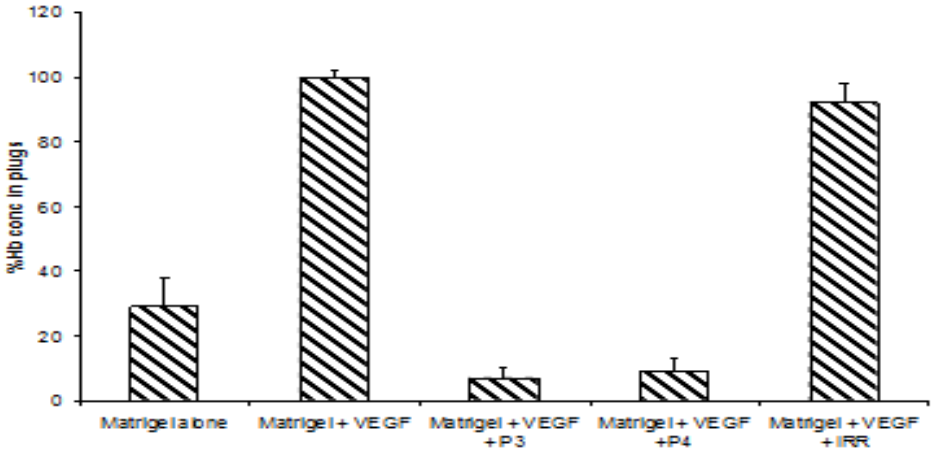
Figure 2.9 Effects of combination treatment on tumor weight. Wild type BALB/c mice (*n* =5), after TUBO challenged and treatment were sacrificed at day 39 and the weight of the mice with the tumors were measured and recorded. The tumors were then extracted and also measured and the data was used to calculate the % tumor weight. Results showed a greater reduction in % tumor weight in the cases of combination treatment with the best results observed in the case of combination with both retro inverso D-amino acid peptides. Error bars represent mean standard deviations.

Figure 2.10. Inhibition of VEGF dependent angiogenesis in Matrigel plugs by VEGF peptides. A. Lane 1: Matrigel containing PBS alone (-control), Lane 2: VEGF (+ control), Lane 3: VEGF + P3 peptides, Lane 4: VEGF +P4, and Lane 5: VEGF+ IRR(irrelevant peptide) were subcutaneously administered to Balb/c mice on the right and left flanks and ten days later the plugs were removed and photos taken as shown. B. Hemoglobin content was determined using Drabskin's method. Each group contained three mice. The error bars represents standard deviation of the mean.

A


Matrigel alone
Matrigel + VEGF
Matrigel + VEGF + P3
Matrigel + VEGF + P4
Matrigel + VEGF + IRR
Mice 1
Mice 2
Mice 3

B


%Hb conc in plugs
120
100
80
60
40
20
0
Matrigel alone
Matrigel + VEGF
Matrigel + VEGF + P3
Matrigel + VEGF + P4
Matrigel + VEGF + IRR

CHAPTER 3

IMMUNIZATION WITH HER-2 PEPTIDE VACCINES FOLLOWED BY TREATMENT WITH VEGF PEPTIDE MIMICS INDUCES POTENT ANTI-TUMOR AND ANTI-ANGIOGENIC RESPONSES IN VITRO AND IN VIVO.

3.1 Introduction

The oncoprotein HER-2 is a ligandless member of the HER family of receptors [163] and other members of this family are HER-1, HER-3 and HER-4. The absence of a HER-2 ligand makes it a preferred dimerization partner with other HER receptors. All members of the HER family have an extracellular domain, a single transmembrane domain and a cytoplasmic portion that contains a conserved tyrosine kinase domain flanked by a carboxyl terminal tail with autophosphorylation sites [129] HER-2 is known to regulate the formation of neuromuscular synapses and is also important in muscle spindle development [164]. High levels of HER-2 causes dysregulation of the HER network leading to transformation, tumorigenesis and resistance to cytotoxic effects of TNFα [6, 165]. HER-2 overexpressing breast cancers are biologically different from other breast cancers and are known to be resistant to hormonal agents, and have increased ability to metastasize to other organs of the body like the lung and brain [166]. HER-2 upregulation is not only limited to breast cancers as its amplification has been reported in subsets of gastric, esophageal, ovarian, uterine, endometrial and lung cancers [123-126]. HER-2 upregulation is always accompanied by VEGF upregulation both at the RNA and protein level [54] and most drugs that target HER-2 are known to also down-regulate VEGF

expression [135]. This implies that, the effects of HER-2 may partly be mediated by upregulation of VEGF. Immunization with both tumor and angiogenesis associated antigens showed synergistic effects [57]. Tumor cells are known to up-regulate the expression of VEGF and its receptors thereby stimulating angiogenesis [55, 56]. Targeting HER-2 alone might not be enough to completely kill tumor cells and interrupting VEGF signaling alone will only delay tumor growth thereby allowing tumor cells to develop other mechanisms of stimulating angiogenesis [145]. All of this has led us to postulate that, targeting HER-2 and VEGF will produce additive effects since two different compartments of tumor cells are involved. Our strategy therefore involves immunization with HER-2 peptide epitopes and treatment with VEGF peptide mimics. Humanized monoclonal antibodies like Trastuzumab and Pertuzumab are known to target two different sub-domains of the extracellular domain of HER-2 [113] and despite some impressive clinical results with these abs, there are side effects with mAb therapy including cardiotoxicity. Also the low half-life of these abs makes treatment very expensive since multiple doses are required within a short period of time, which increases the probability of toxicity. We are therefore proposing active immunotherapy whereby the body is being trained to produce high specific abs against tumor cells as opposed to passive immunotherapy where large amounts of abs and other immune cells are administered to the patient. The main advantage of active immunization with peptide epitopes lies in the fact that, there is generation of immunological memory, which is absent in passive immunization. The main aim of this study is based on the hypothesis that combination of anti-angiogenic therapy and tumor immunotherapy of cancer may be synergistic.

In this study, we used the MVF-HER-2 266 peptide which has been shown to be immunogenic in both rabbits and mice and also have potent anti-tumor effects in vitro and in vivo [114]. We therefore report the in vitro effects of combination treatment with both HER-2 and VEGF anti-peptide abs that were raised in rabbits and also the anti-tumor effects in vivo of active immunization with MVF-HER-2-266 and treatment with VEGF peptide mimics. Immunization with the HER-2 peptide epitope and treatment with the D-amino acid VEGF peptide mimic produced superior anti-tumor and anti-angiogenic effects in vivo.

3.2 Materials and Methods.

Synthesis and characterization of conformational peptides.

Peptide synthesis was performed on a Milligen/Biosearch 9600 peptide solid phase synthesizer (Bedford, MA) using Fmoc/*t*-But chemistry. Preloaded Fmoc-Val-CLEAR ACID resin (0.35 mmol/g) for the 266-296 and CLEAR AMIDE RESIN for the VEGF peptides (0.32 mmol/gm) (Peptides International, Louisville, KY) were used for synthesis. The 266-296 cyclized epitope was collinearly synthesized with the promiscuous T_H epitope MVF and assembled by choosing the regioselective side chain protector Trt on Cys residues 268 and 295 [150], and in the VEGF peptides two cysteines were inserted between amino acid Gln79and Gly92 and between Ile80 and Glu93. Peptides were cleaved from the resin using cleavage reagent B (trifluoroacetic acid: phenol: water: TIS, 90:4:4:2), and crude peptides purified by semi-preparative reversed-phase HPLC and characterized by electrospray-ionization mass spectroscopy [167]. Intramolecular disulphide bonds were formed using iodine oxidation as described

[150] and disulfide bridge formation was further confirmed by maleimide-PEO_2-biotin reaction and subsequent analysis using electrospray-ionization mass spectroscopy. Peptides that were used for immunization both in rabbits and mice were collinearly synthesized with the promiscuous T_H epitope MVF (MVF-HER-2-266-296, MVF-VEGF-P3-CYC and MVF-P4-CYC) (Table 1) while those that were used for intravenous treatment of mice after vaccination was synthesized without any MVF (VEGF-P3-CYC and VEGF-P4-CYC).

Circular Dichroism

This was done as previously described [150]. Briefly aqueous solutions for CD were prepared by dissolving the freeze-dried peptides in appropriate amount of HPLC water to give a final concentration of 0.5mM and used as stock solution for further dilution. CD spectra were recorded on an AVIV model 62A DS CD instrument. Mean residue ellipticity ($[\theta]_{M,\lambda}$) values were calculated according to the equation $[\theta]_{M,\lambda} = (\theta \text{ X } 100 \text{ X } M_r)/(n \text{ X } c \text{ X } l)$. Where θ is the recorded ellipticity (degree); M_r the molecular weight of the peptide; n, the number of residues in the peptide; c, the peptide concentration (milligrams per milliliter); and l, the path length of the cuvette.

Animals

Female New Zealand white outbred rabbits were purchased from Harlan (Indiana, IN). Female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animal care and use was in accordance with institutional guidelines.

Cell lines and Antibodies.

All culture media, FBS, and supplements were purchased from Invitrogen Life Technologies (San Diego, CA). The human breast tumor cell lines BT-474 and MDA-468 were purchased from American Type Culture Collection (Rockville, MD) and maintained according to supplier's guidelines. TUBO cells were a cloned cell line established in vitro from a lobular carcinoma that arose spontaneously in BALB-neuT mouse [151]. Humanized mouse mAb Trastuzumab was generously provided by Genentech, Inc (South San Francisco, CA).

Peptide immunization and antibody purification.

Mice and rabbits were immunized s.c. at multiple sites with a total of 1 mg (rabbits) or 100 μg (mice) of peptide dissolved in H_2O with 100μg of a muramyl dipeptide adjuvant, nor MDP (N–acetyl-glucosamine-3 yl–acetyl L–alanyl–D–isoglutamine). Peptides were emulsified (50:50) in Seppic Montanide ISA 720 vehicle. The same dose of booster injections was administered twice at three and six weeks. Sera were collected, and complement was inactivated by heating to 56ºC for 30 min. High-titered sera were purified on a protein A/G agarose column (Pierce) and eluted antibodies were concentrated and exchanged in PBS using 100 kDa cut-off centrifuge filter units (Millipore, Billerica, MA). The concentration of antibodies was determined by Coomassie plus protein assay reagent (Pierce).

BALB/c mice were immunized in the same manner described, commencing at 5-6 weeks of age. After the second boost, the transgenic mice received two subsequent

boosters at 3-week intervals (Figure 3.5). Tumor size (length and width) in each of ten mammary glands was measured twice weekly with Venier calipers beginning at 18 weeks of age. Individual tumors were calculated by the formula (length x width^2)/2. All mice were euthanized at day 39 after TUBO challenge

ELISA.

To determine the ability of the peptide Abs to bind various peptides, a specific ELISA was performed. Ninety-six well plates were coated with 100 µl of peptide at 2 µg/ml in PBS overnight at 4°C. Nonspecific binding sites were blocked for 1 h with 200 µl of PBS-1% BSA, and plates were washed with PBT. Rabbit sera (1:500 dilution) was added to antigen-coated plates in duplicate wells, serially diluted 1:2 in PBT, and incubated for 2 h at room temperature. After washing the plates, 100 µl of 1:500 goat antihuman IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL) were added to each well and incubated for 1 h. After washing, the bound antibody was detected using 50 µl of 0.15% H_2O_2 in 24 mM citric acid and 5 mM sodium phosphate buffer (pH 5.2) with 0.5 mg/ml of ABTS as the chromophore. Color development was allowed to proceed for 10 min, and the reaction was stopped with 25 µl of 1% SDS. Absorbance was determined at 410 nm using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Ab titers were defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. All data represent the average of duplicate samples.

Immunization and Peptide treatment in transplantable mouse model.

Balb/c mice 5 to 6 weeks of age were immunized with 100μg of MVF-HER-2-266 three times at three week intervals. Two weeks after the third immunization, the mice were challenged with 1 $x10^5$ TUBO cells and after challenge, mice were treated intravenously with 100μg of either VEGF-P3-CYC, VEGF-P4-CYC or irrelevant peptide as inhibitors. Treatment was done weekly for six consecutive weeks. Mice were euthanized at week 10 and tumors removed. Tumors were measured for tumor volume twice a week using calipers and calculated using the formula (length x $width^2$)/2.

Statistical analysis.

Tumor growth over time was analyzed using Stata's XTGEE (cross-sectional generalized estimating equations) model which fits general linear models that allow the possibility to specify within animal correlation structure in data involving repeated measurements [168]. For other experiments t-test was carried out to observe the statistical relevancy in between different sets of experiments as well as the significant difference between treated and non treated cells.

Proliferation assay.

BT-474 and MDA-468 ($1x10^4$) were plated in 96-well flat-bottom plates overnight. Growth medium was replaced with low sera (1% FCS) medium and the cells were incubated overnight. Media were removed from the wells and replaced with low sera medium containing anti-HER-2 peptide and anti-VEGF mimic peptides antibodies at concentrations ranging from 25-100ug/ml and plates were incubated an additional 1 h at

37°C before adding 10ng/ml HRG in 1% medium. Plates were incubated for an additional 72h at 37°C before adding MTT (5 mg/ml) to each well. Plates were incubated 2h at 37°C, and 100µl of extraction buffer (20% SDS, 50% dimethylformamide (pH 4.7)) was added to each well. Plates incubated overnight at 37°C and read on an ELISA reader at 570 nm with 655 nm background subtraction. Inhibition percentage was calculated as 100% x (Untreated cells – Peptide treated cells)/ (Untreated cells).

Phosphorylation assay

1 x10^6 BT-474 cells were plated in each well of a six well plate and incubated overnight at 37°C. Culture medium was removed and the cell layer washed once with PBS low score (1%FCS). Culture medium was added to the wells and plates incubated overnight. Cells were washed and 50ug of anti-peptide Abs and controls in binding buffer (0.2% w/v BSA, RPMI 1640 medium with 10mM HEPES (pH 7.2) was added to the wells and incubated at room temperature for 1h. HRG (5nM/well) was added and the incubation continued for 10min. Binding buffer was removed and the cell layer washed once with PBS before adding 1ml of RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Plates were rocked at 4°C for 2h. Lysates were removed, spun at 13000 X g and supernatants collected. Protein concentration of each sample was measured by Coomassie plus protein assay reagent kit and lysates were stored at -80°C. Phosphorylation was determined by Duoset IC for human phosphor-ErbB2 according to the manufacturer's directions (R$D Systems).

ADCC

We used the bioluminescence cytotoxicity assay (aCella-TOX™ Mountain View, CA) and all procedures were performed according to the manufacturer's instructions. Briefly, The BT-474 target cells (1x 10^4/well) were plated on a 96 well plate and anti-peptide abs were added to the wells containing the target cells. The plate was incubated at 37°C for 15 minutes to allow opsonization of antibody to occur. Effectors cells (hPBMCs from red cross) were then added to the wells at three different E:T ratios (100:1, 20:1 and 4:1) and the plate incubated at 37°C for 3 hours . The plate was then removed and equilibrated to room temperature for 15 mins before adding 10µl of lytic agent to the control wells for maximum lysis and incubated for 15min at room temperature. 100µl of the Enzyme Assay reagent containing G3P was then added to all wells followed by 50µl of the detection reagent. The plate was immediately read using a luminometer.

Viability assay

This assay was performed just like the proliferation assay but after treatment with the anti-peptide antibodies as inhibitors, the aCella-TOX reagent was used to estimate the amount of dead cells. After peptide treatment for 72h, the plate was removed and equilibrated to room temperature for 15mins before adding 10µl of lytic agent to the control wells for maximum lysis and incubated for 15min at room temperature. 100µl of the Enzyme Assay reagent containing G3P was then added to all wells followed by 50µl of the detection reagent. The plate was immediately read using a luminometer.

3.3 Results

Selection, design and characterization of peptides.

The crystal structure of the complex between VEGF and the Fab fragment of a humanized antibody [169], and analysis of the contact residues on both sides of the interface was published by Muller *et al*., [117, 119]. Zilberberg *et al*., also identified that the sequence 79-93 of VEGF is involved in the interaction with VEGF receptor-2 [118]. Although the VEGF residues critical for antibody binding are distinct from those important for high-affinity receptor binding, they occupy a common region on VEGF demonstrating that the neutralizing effect of antibody binding results from steric blocking of VEGF–receptor interactions and only a small number of the residues buried in the VEGF–Fab interface are critical for high-affinity binding and are concentrated in one continuous segment of polypeptide loop between β5-β6. Several residues are important for VEGF receptor binding, including Met 81, Ile 83, Lys 84, Pro 85 , Gln 89, and Gly92 [117, 119, 170]. We have selected to use a peptide encompassing residues 102-122 (numbered as 76-96 in the crystal structure) which mimics the overlapping VEGF binding sites to VEGFR-2 and Avastin. The strategy to create a conformational peptide consisting of an anti-parallel β-sheet is shown in Table 1, where the sequence was modified in a way that the ends were twisted to generate VEGF-P3(NC). It also required two artificial cysteines to be introduced between Gln79 and Gly92, and between Ile80 and Glu93. After synthesis and purification of VEGF-P3 (NC) (non-cyclized) peptide, the disulfide bond was formed by oxidation reaction enabling the formation of the twisted anti-parallel β-sheet structure in the VEGF-P3 (CYC) (cyclized). The retro-

inverso (RI) peptide analog VEGF-RI-P4 was synthesized using D-amino acids with the amino acid sequence in reverse order, such that the resulting peptide mimic has a reversal of the peptide backbone but a topochemical equivalence to the parent peptide in terms of side-chain orientation [149, 154, 171]. The rationale behind the retro-inverso peptidomimetic is that it should present similar activity with the advantage of higher bioavailability [155].

HER-2-266-296 peptide (Table 3.1) was synthesized based on the crystal structure of the Fab of pertuzumab bound to the ECD of HER-2/neu. This reveals that pertuzumab binds to subdomain II of the HER-2 ECD [113]. The 266-333 region of HER-2 was selected for the design of the peptides with the objective of eliciting abs against the peptide capable of inhibiting dimerization of HER-2 with other members of the EGFR family. The peptide can also be use to directly block dimerization due to its ability to bind and recognize the HER-2 ECD [114]. Peptides that were used for immunization both in rabbits and mice were collinearly synthesized with the promiscuous T_H epitope MVF while those that were used for intravenous treatment of mice after vaccination was synthesized without any MVF (Table 3.1)

Antigenicity and Immunogenicity of Peptides.

Earlier studies in our lab have shown that the MVF-HER-2-266 was highly immunogenic in both rabbits and mice [114]. We also observed high antibody titers with the MVF-VEGF-P3 peptide (Vicar et al,) and in our present study we showed that the D-amino acid VEGF peptide (VEGF-P4-CYC) is also immunogenic though not as much as the L- amino acid counterparts which is probably due to the fact that D-amino acids

are not natural so not easily recognized by the body. We had to do up to six immunizations with the D-amino acid peptide before we could obtain higher abs titers (Figure 3.1) while with the L-amino acids only four immunization are enough to produce higher titers [114].We therefore used the abs raised against these peptides to test their effects on cancer cells in vitro.

Antiproliferative effects of anti- peptide Abs.

The antiproliferative effects of the antibodies raised against the peptides in rabbits were tested using two different cell lines (BT-474, HER-2^{high} and MDA-468, HER-2^{low} (Figure 3.2) in the presence of HRG to activate the HER-3 receptor. Unlike trastuzumab that is specific to HER-2 positive cells, pertuzumab is known to act on cells by disrupting ligand dependent receptor complexes independent of HER-2/neu expression [110]. The cells were incubated with the anti-peptide antibodies followed by exposure to HRG. Results indicate that the antibodies raised against both the HER-2 peptides and VEGF peptides were able to inhibit tumor growth in a concentration dependent manner (Figure 3.2). We used two different cell lines to show that the effects of the anti peptide Abs was dependent on HER-2 expression since higher inhibition was observed in cases of high HER-2 expression (Figure 3.2). We also tested the effects of combination treatment with both HER-2 and VEGF anti-peptide Abs and the results showed an increase in rate of inhibition when both anti-peptide Abs were used as compared to single treatments (Figure 3.3). Normal rabbit IgG did not show antiproliferative effects while Trastuzumab (positive control) showed antiproliferative effects only on cells that express the HER-2 receptor (Figure 3.2).

Effects of anti-peptide Abs on HER-2 specific Phosphorylation.

The main mode of action of Pertuzumab is to inhibit phosphorylation. This is due to the fact that it sterically blocks the dimerization domain of HER-2 thereby preventing the formation of dimers with other HER receptors and thus interrupting downstream signaling. We have tested the effects of the anti-peptide Abs on HER-2 phosphorylation and the results obtained showed that these anti-peptide Abs were able to prevent phosphorylation of the HER-2 protein and single treatment with the HER-2 anti-peptide Abs alone caused a 30% inhibition rate while combination with the VEGF anti-peptide Abs increased the inhibition from 30% to about 75% (Figure 3.4). All treatments were compared to the positive control AG825 (Calbiochem), a HER-2 specific phosphorylation inhibitor. The negative control (normal rabbit IgG) showed no significant inhibitory effects on HER-2 phosphorylation.

Effects of anti-peptide Abs on breast cancer cell viability.

We next evaluated the effects of combination treatment with both HER-2 and VEGF anti-peptide Abs on tumor cell survival *in vitro*. This was done using the acella-TOX reagent kit where dead or dying cells released the enzyme GAPDH and measuring the activity of this enzyme will give an estimate of the cell viability after treatment. The results obtained showed that the Abs were able to cause a decrease in cell viability and combination treatment caused a further decrease in viability of at about 25% compared to single treatment (Figure 3.5).

Ability of anti-peptide antibodies to mediate ADCC.

It has been well documented that *in vivo* the Fc portions of antibodies can be of foremost importance for efficacy against tumor targets [172].When Fc binding is reduced or completely removed, Trastuzumab loses virtually all *in vivo* efficacy [173]. We have therefore measured the ability of anti-peptide antibodies to mediate ADCC *in vitro*. Anti-peptide antibodies elicited in rabbits against the HER-2 and VEGF peptides were tested. To study this, we used the bioluminescence cytotoxicity assay (aCella-TOX^{TM}) and all procedures were done according to the manufacturer's instructions. This method is very advantageous in that non-radioactive reagents are used and it is very sensitive in measuring the GAPDH enzyme released by dead or dying cells. The effector cells are normal human PBMCs from healthy donors while the target cells are BT-474 cells that overexpress HER-2. The results from these assays showed that combination treatments with peptide mimics induced a more potent response than just single treatments (Figure 3.6). Trastuzumab was used as a positive control while normal mouse and rabbit IgG were used as negative controls.

Transplantable tumor challenge models

We used a rat neu-expressing tumor challenge model which is produced by challenging wild type Balb/c mice with TUBO cells. The rat neu has a 97% similarity to that of the human HER-2 266-296 sequence with only one disparate amino acid [150]. To investigate the efficacy of both immunization and peptide treatment, we immunized BALB/c mice with 100μg of MVF-HER-2-266 peptide three times at three weeks intervals and two weeks after the third immunization (Figure 3.7), mice were challenged

with TUBO cells derived from tumors of BALB-neuT transgenic mice [66]. Groups of mice (n=5) were treated with either VEGF peptides, irrelevant peptide or left untreated. Results obtained indicates that immunization with MVF-HER-2 and treatment with VEGF peptide mimics caused statistically significant (P**<0.001)greater delay in tumor growth and development (Figure 3.8A & 3.8B). The groups that were immunized with MVF-HER-2 peptide and treated with the irrelevant peptide or just immunization alone also showed a delay in tumor growth and development though the difference was not statistically significant since the P* value was = 0.082 using the 95% confidence intervals (Figure 3.8A) when compared to the untreated. Most interestingly, there was a significant difference between immunization alone and immunization and treatment with the VEGF peptides. In both cases, the P* values were < 0.001 but in the case of the D-amino VEGF peptide mimic (MVF-HER-2 + P4), there was a greater delay in tumor growth as compared to the L-amino acid VEGF peptide (MVF-HER-2 + P3) (Figure 3.8B). At the end of the experiment, some of the mice were tumor free and this was observed in the case of both immunization with MVF-HER-2 and treatment with the D-amino acid VEGF peptide (MVF-HER-2 + P4) where 40% of the mice (2 out of 5) did not develop tumors (Figure 3.9A). We also measured the tumor weights after the experiment and calculated the % tumor weights and the results indicated a statistical difference between all treatments except the irrelevant with the untreated. The P*** value was < 0.001 in the case of both immunization with MVF-HER-2 and treatment with the D-amino acid VEGF peptide while the P** value was < 0.002 in the case of immunization and treatment with the L-amino acid VEGF peptide. In the case of immunization alone, the difference was also statistically significant with a P* of 0.044.

We also compared the group of immunization with HER-2 alone to that of both immunization and treatment with VEGF peptides and observed a statistically significant difference using the 95% confidence interval with a P# value of 0.018 (Figure 3.9B). Physical observation of the tumors showed a decrease in size in the case of the treated and also a decrease in blood since the tumors were less red in color especially in the cases of treatment with the VEGF peptide mimics (Figure 3.10). Also there was a great evidence of a decrease in blood flow to the tumors and normalization of the tumor vasculature in the case of immunization with MVF-HER-2 and treatment with VEGF peptides (Figure 3.11 C&D) while immunization and treatment with irrelevant peptide only decreases tumor size but has no effect on blood supply(Figure 3.11B). Results from these studies strongly suggest that targeting both HER-2 and VEGF is a better strategy that can completely prevent tumor growth and development. Also, the retro inverso D-amino acid peptide produced better results than the L-amino acid peptide in both the cases of single and combination treatments as illustrated in Figures 3.8A, 3.8B, 3.9A, and 3.11.

3.4 Discussion

The receptor HER-2 has been shown to be upregulated in many types of cancers especially breast [129]. Weak immune responses have been detected in patients with HER-2 positive cancers indicating that the receptor is weakly immunogenic. Humanized monoclonal antibodies like Trastuzumab, Pertuzumab and Bevacizumab have been developed to treat different types of cancers. Despite their approval by the FDA, a lot of

concerns still exist with passive immunotherapy using these antibodies. There is the requirement of repeated treatment with high dosing and also high cost, the immunogenicity of these antibodies resulting in the production of anti-idiotypic antibodies and the development of resistance due to loss of immunodorminant epitopes. Above all there is high level of toxic side effects like cardiotoxicity associated with these treatments. Immunization or treatment with peptides offers the opportunity of stimulating the body's immune response leading to immunological memory. Peptides are relatively safe, non toxic, cheaper and highly specific. The only drawback associated with peptides is their ability to be degraded by proteases in the body. This can however be overcome by using D-amino acids that cannot be recognized by proteases. The peptide can be synthesized with a reversal of the peptide chirality and using D-amino acids resulting in a topographical equivalent of the parent peptide.

The overexpression of HER-2 is associated with increased expression of VEGF at both the RNA and protein levels in human breast cancer cells and exposure of HER-2 positive cells to trastuzumab significantly decreases VEGF[135]. Shc, a downstream adaptor protein of the HER-2 signaling pathway, has been identified as a critical angiogenic switch for VEGF production [136] showing that VEGF is a downstream target of the HER-2 signaling pathway. This shows that, the effects of HER-2 on tumor cell behavior may be mediated in part through stimulation of angiogenesis. A two pronged approach to target cancer cells by co-immunizing with defined tumor associated antigens and angiogenesis associated antigens have been shown to have synergistic effects [57, 143, 144]. All of these show that, combination therapy targeting both HER-2 and VEGF is a very promising strategy since antiangiogenic therapy alone will only

delay tumor growth [145] and targeting HER-2 and VEGF will interupt two different tumor dependent pathways.

During the past decade, work in our laboratory was mainly focused on the development of B-cell vaccines targeting the HER-2 epitope. The association between HER-2 and VEGF and the Folkman's idea that tumor growth is angiogenesis dependent attracted us to targeting these two different proteins. Our main hypothesis is that immunization with HER-2 peptide epitopes will produce highly specific antibodies that will fight cancer cells and treatment with VEGF peptides will be able to prevent angiogenesis thereby preventing tumor growth due to decrease in blood and oxygen supply. We therefore hypothesized that targeting these two sub pathways will most efficiently prevent the establishment of tumors. We have designed several peptides based on the binding of the ECD of HER-2 with pertuzumab [150] and after several in vitro and in vivo studies, the HER-2 266-296 was shown to produce superior anti-tumor effects. Abs raised against this peptide was also able to recognize HER-2 and also inhibit tumor growth both in vitro and in vivo [150]. Another set of peptides were also synthesized based on the binding of VEGF to its receptor VEGFR2 and after several studies using cancer cells and animal models (vicari et al.), the VEGF-P3-CYC was selected for further studies. The retro-inverso analog of the VEGF peptide was synthesized using D-amino acids. The peptides were immunogenic though the D-amino acid peptide needed more booster immunizations before higher titers could be obtained (Figure 3.1).

We evaluated the antiproliferative effects of the anti-peptide Abs or their combinations on different cell lines. Trastuzumab has been shown to be specific to only HER-2 positive

cells and this was observed in our results (Figure 3.2) where little inhibition was observed with the MDA-468 (HER-2 low) cell line as compared to the BT-474 cell line (HER-2 high). The anti- peptide abs were effective in inhibiting HER-2 cancer cells. The HER-2-266 peptide abs showed some inhibitory effects on the HER-2 low cell line (MDA-468) (Figure 3.2). This is probably due to the fact that the peptide was synthesized using the pertuzumab epitope so Abs raised against this peptide should be able to function like pertuzumab so should have some inhibitory effects in cells independent of HER-2 since pertuzumab is also effective in cells that are independent of HER-2. We also evaluated the in vitro effects of combination treatment with both HER-2 and VEGF anti-peptide abs on cell proliferation and viability, and the results illustrates that combination treatment produced greater anti-tumor effects than single treatments alone. (Figures 3.2 and 3.3).

HER-2 is known to dimerize with its partner HER-1 and HER-3 leading to receptor phosphorylation and intracellular signaling and pertuzumab mainly functions by sterically blocking this receptor from binding to its partners and is therefore classified as a dimerization inhibitor [161, 162]. We therefore investigated the effects of the anti-peptide abs on phosphorylation and the results also showed an increase in phosphorylation inhibition from less than 35% in the case of single treatments to about 75% in the case of combination treatment (Figure 3.3). We also observed increased inhibition of phosphorylation and decrease in cell viability with combination of HER-2 and VEGF abs (Figure 3.4 and 3.5). One of the main mode of action of Abs is to cause ADCC, so we also evaluated the ability of anti-peptide abs to cause ADCC of BT-474 cells. Results showed that the anti-peptide abs were able to cause ADCC and their effects

were comparable to that of the positive control Trastuzumab (Figure 3.6). Also in the case of combination treatment with both anti-HER-2 and anti-VEGF peptide abs, there was an increase in ADCC as compared to single treatments. The combination treatment was greater than that of Trastuzumab.

In order to evaluate the effects of peptide treatment in vivo, we used a transplantable mouse model. BALB/c mice were immunized with MVF-HER-2 peptide before being challenged with TUBO cells and treated with VEGF peptides. The results obtained showed significant differences between the treated and untreated groups and also a delay in tumor growth and development, with a decrease in tumor weight. The case of immunization with MVF-HER-2 and treatment with VEGF-P4 produced the best results and 40% of the mice in this group remained tumor free at the end of the experiment (Figure 3.8 and 3.9). The VEGF peptide treatment also appeared to cause a decrease in blood flow to the tumors thereby limiting their size increase and normalization of the tumor vasculature (Figure 3.10 and 3.11). The results strongly suggest that tumor growth and development can be completely prevented by targeting both the tumors and preventing blood supply. This is because the tumor cells are genetically unstable so they constantly change their form thereby developing resistance but the tumor vasculature is genetically stable [174]. Targeting both the genetically stable vasculature will be able to prevent the tumors that develop resistance overtime from growing thereby producing greater inhibitory effects. Active immunization with HER-2 peptide epitopes and treatment with VEGF peptide mimics is a better strategy than immunization alone. Also, the D-amino acid peptide produces greater inhibitory effects probably due to its longer half-life in vivo due to inability of proteases to recognize it.

3.5 Tables and Figures

Designation	Peptide	Sequence	M.Wt. (da)
HER-2-266-CYC	***266-296*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{266}LH**C**PA *LVTYNTDTFESMPNPEGRYTFGAS**C**V*^{296}*-CONH2*	2925
VEGF-P3-CYC	***76-96*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{76}ITMQ^{79}-C-^{92}GIHQGQHPKIRMI^{80}-C-^{93}EMSF^{96}*-CONH2*	2527
VEGF-RI-P4-CYC	***96-76*** *peptide with D amino acids and one disulfide bond*	*CH_3CONH-*(D)-^{96}FSME^{93}**-C-**^{80}IMRIKPHQGQHIG^{92}-**C-**^{79}QMTI^{76}*-CONH2*	2527

Table 3.1: Amino acid sequences and molecular weight of HER-2 and VEGF peptide mimics. Sequences of amino acids are represented from N to C terminal except for the retro inverso peptide VEGF-RI-P4-CYC that was synthesized in the reverse order and using D amino acids

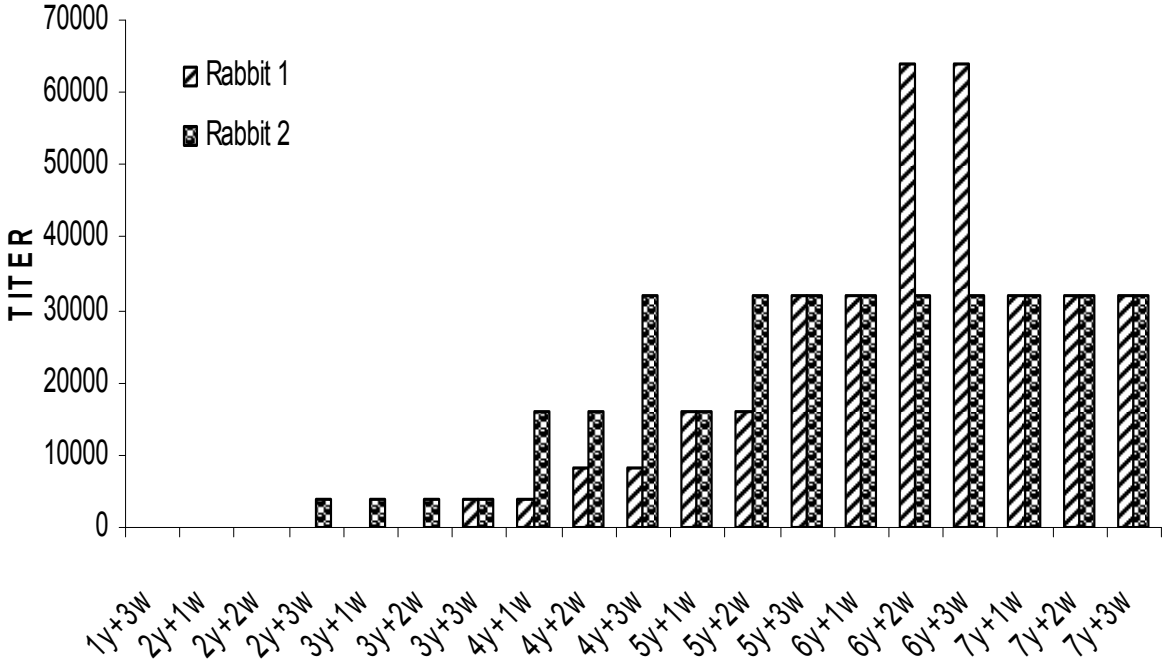
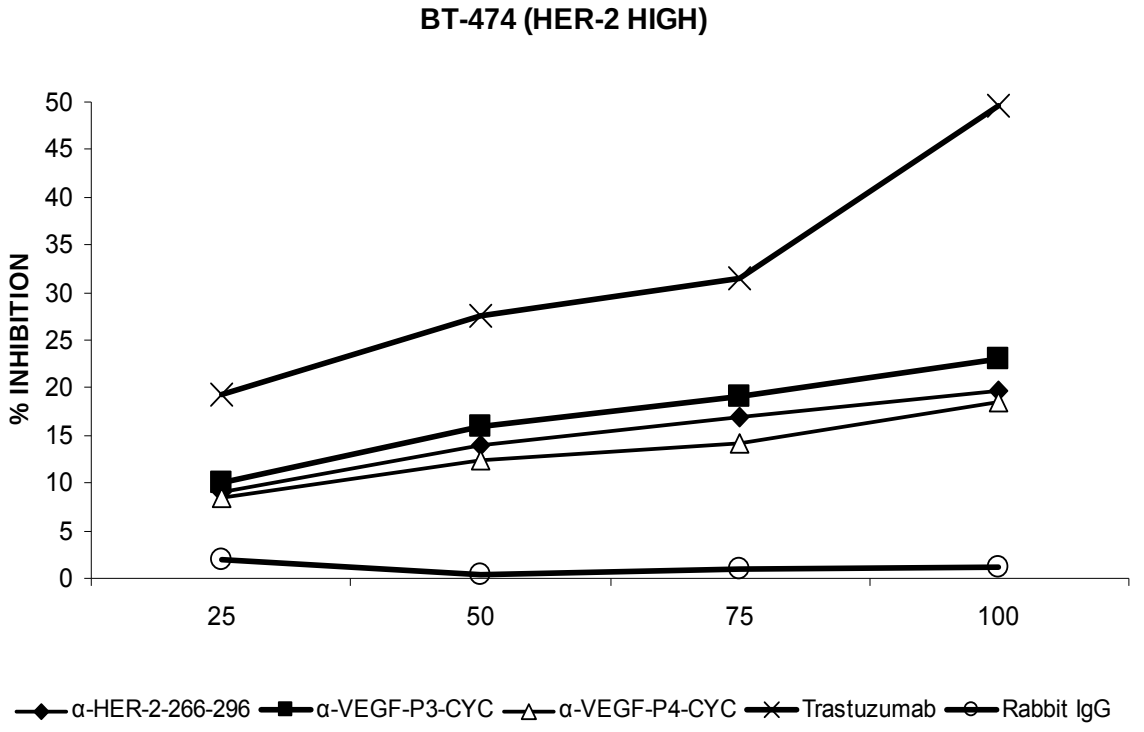
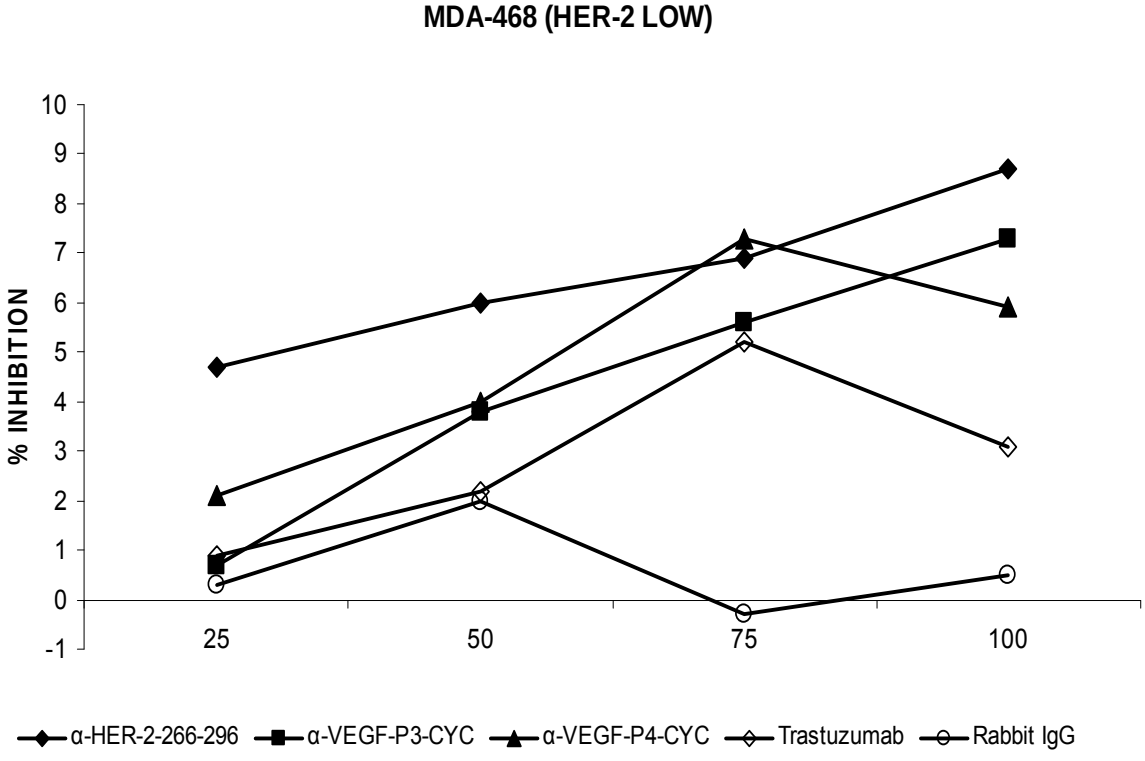


Figure 3.1: Antibody responses elicited by peptide vaccines in outbred rabbits. Two rabbits were immunized with MVF-VEGF-P4-CYC peptides. Blood was drawn weekly, and sera surveyed for peptide specific antibodies by ELISA. The results of each individual rabbit are shown and titers are defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. 2y + 3w indicate the antibody titer in blood drawn three weeks after the second immunization.

Figure 3.2: Anti-proliferative effects of HER-2 and VEGF peptide abs. BT474 and MDA-468 cells were incubated with HER-2 peptide Abs, VEGF peptides Abs, Trastuzumab and normal rabbit IgG. Bioconversion of MTT was used to estimate the proliferation rate of cells after 3 days. Peptide Abs were added at four different concentrations using the above mentioned cell lines. The proliferation inhibition rate was calculated using the formula (ODnormal Untreated - OD peptides or Ab)/ODnormal untreated x 100.Results represent average of three different experiments. Trastuzumab and normal rabbit IgG were used as positive and negative controls respectively.


BT-474 (HER-2 HIGH)
% INHIBITION
50
45
40
35
30
25
20
15
10
5
0
25
50
75
100
α-HER-2-266-296
α-VEGF-P3-CYC
α-VEGF-P4-CYC
Trastuzumab
Rabbit IgG


MDA-468 (HER-2 LOW)
% INHIBITION
10
9
8
7
6
5
4
3
2
1
0
-1
25
50
75
100
α-HER-2-266-296
α-VEGF-P3-CYC
α-VEGF-P4-CYC
Trastuzumab
Rabbit IgG

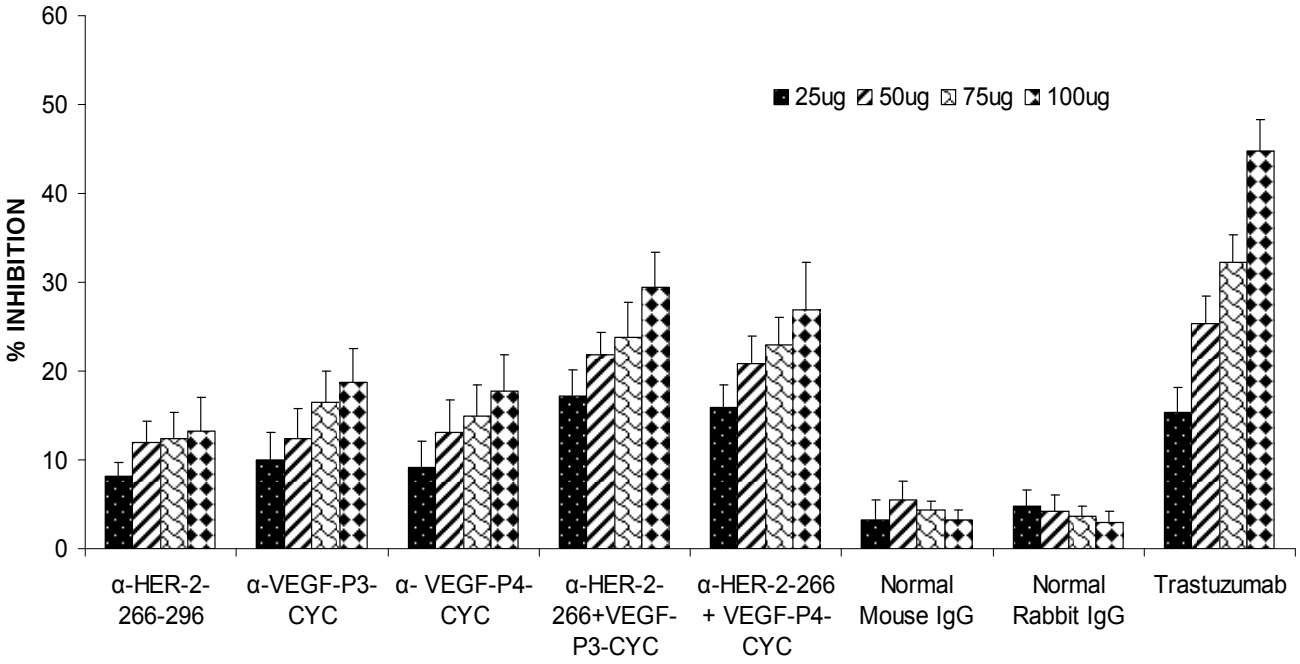


Figure 3.3: Anti-proliferative effects of combination treatment with HER-2 and VEGF peptide mimics. BT474 and cells were incubated with HER-2 peptide Abs, VEGF peptide Abs, Trastuzumab, normal rabbit and mouse IgG or combination of HER-2 and VEGF peptide Abs. Bioconversion of MTT was used to estimate the number of active tumor cells remaining after 3 days. Peptides were added at four different concentrations using the above mentioned cell lines. The proliferation inhibition rate was calculated using the formula (ODnormal Untreated - OD peptides or Ab)/ODnormal untreated x 100. Error bars represent SD. Trastuzumab and normal IgG were used as positive and negative controls respectively. All results represent the average of three different experiments. Error bars represent SD of the mean. Results represent average of three different experiments.

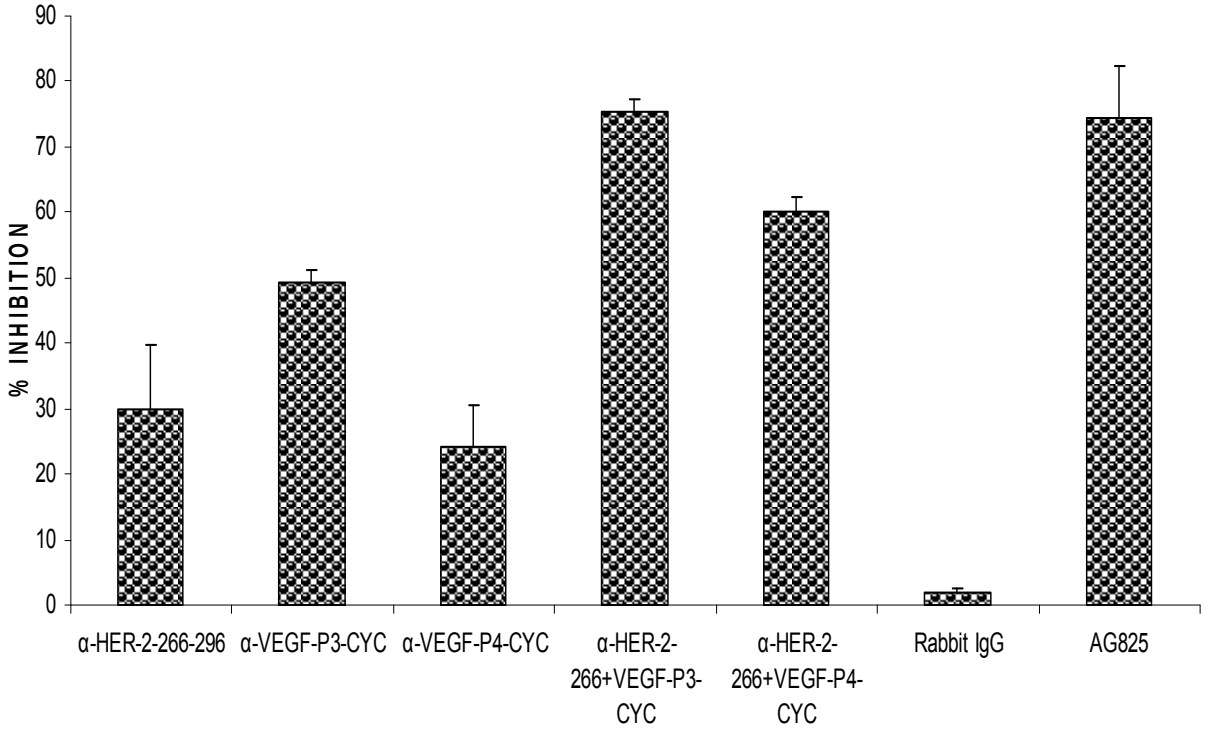


Figure 3.4. Effects of combination treatment on HER-2 phosphorylation

BT-474 cells were incubated with 100µg of HER-2 and VEGF peptide Abs before being exposed to HRG (HER-3 activating ligand) for 10 minutes and lysed. Phosphorylated HER-2/neu was determined by indirect ELISA and percent inhibition was calculated using the formula (ODnormal Untreated - OD peptides or Ab)/ODnormal untreated x 100. Error bars represent SD. All results represent the average of three different experiments. Error bars represent SD of the mean. AG825 (Calbiochem) a potent HER-2 phosphorylation inhibitor was used as a positive control while normal rabbit IgG was used as a negative control.

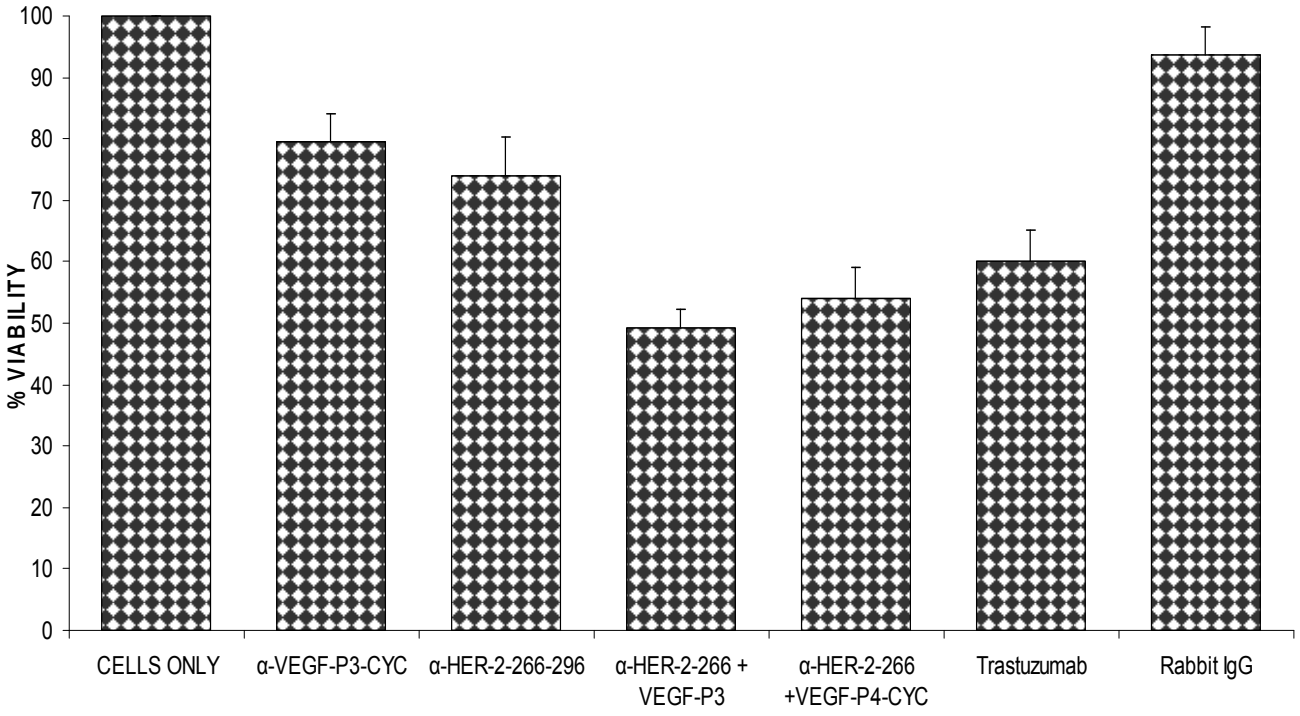


Figure 3.5. Effects of combination treatment on cell viability

BT474 cells were incubated with media alone, HER-2 peptide Abs, VEGF peptide Abs, trastuzumab, and normal rabbit IgG. The number of viable cells remaining after three days was determined using the aCella-TOX reagent kit and all steps were carried out according to manufacturer's instructions. Cell viability is given as a percentage of untreated cells. Data points represent the mean of three independent experiments. Trastuzumab and normal rabbit IgG were used as positive and negative controls respectively. Error bars represent SD. Results represent average of three different experiments.

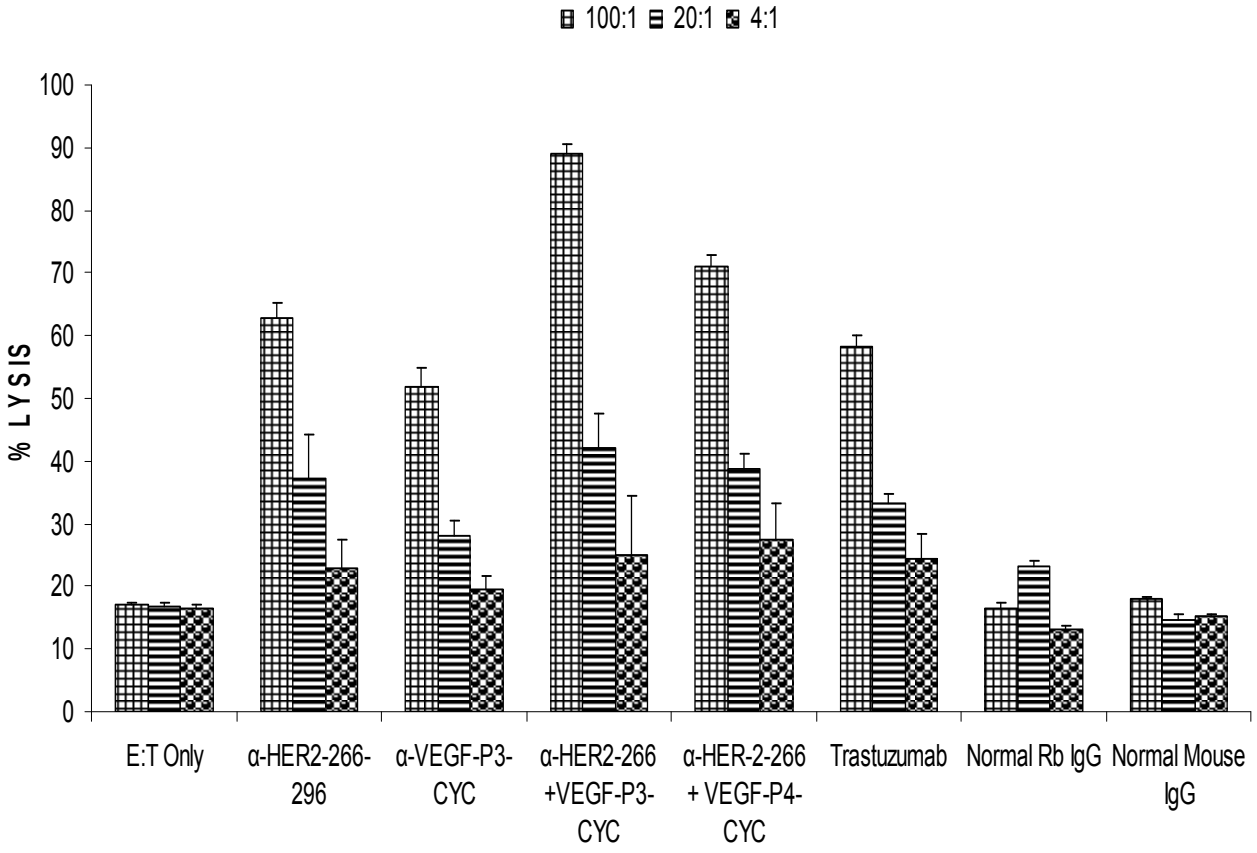


Figure 3.6. Anti-peptide antibodies induces ADCC

Anti-peptide Abs raised in rabbits are capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC). Target cell line BT474 was coated with 50μg of purified anti-peptide antibodies from rabbits, normal rabbit IgG, normal mouse IgG or trastuzumab and then cultured in the presence of human PBMC effector cells to give an effector: target ratio of 100:1, 20:1, and 4:1 in triplicates. After treatment, the number of dead or dying cells was measured by measuring the release of the G6PD. Bars represent SD of mean. Results represent average data from three different experiments with each treatment performed in triplicate.

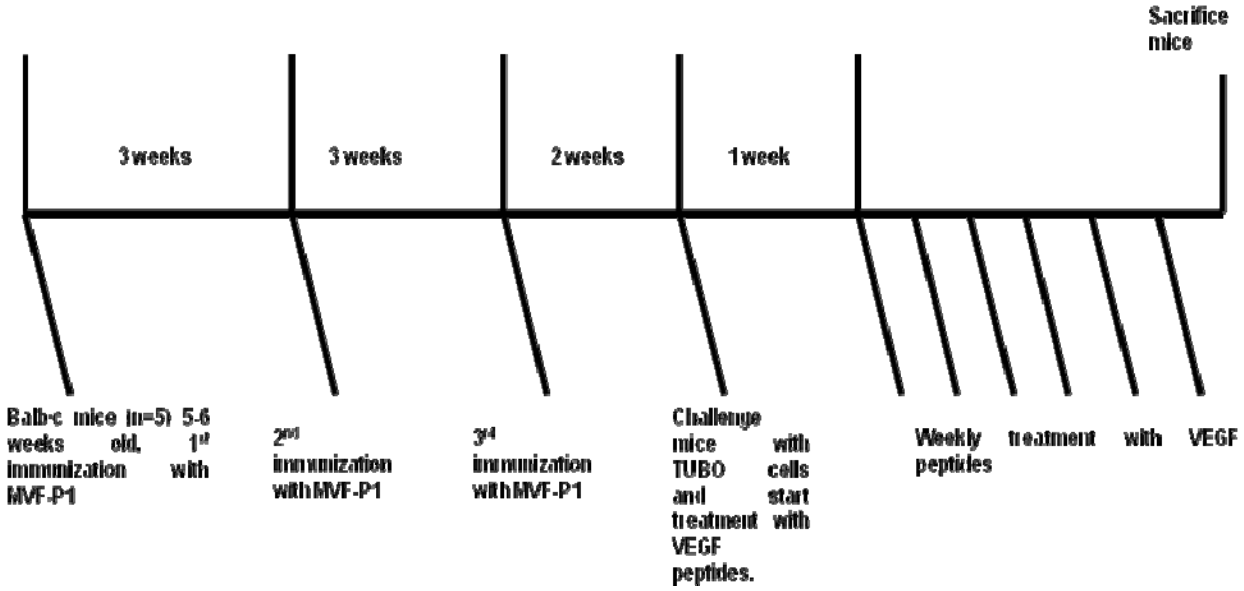


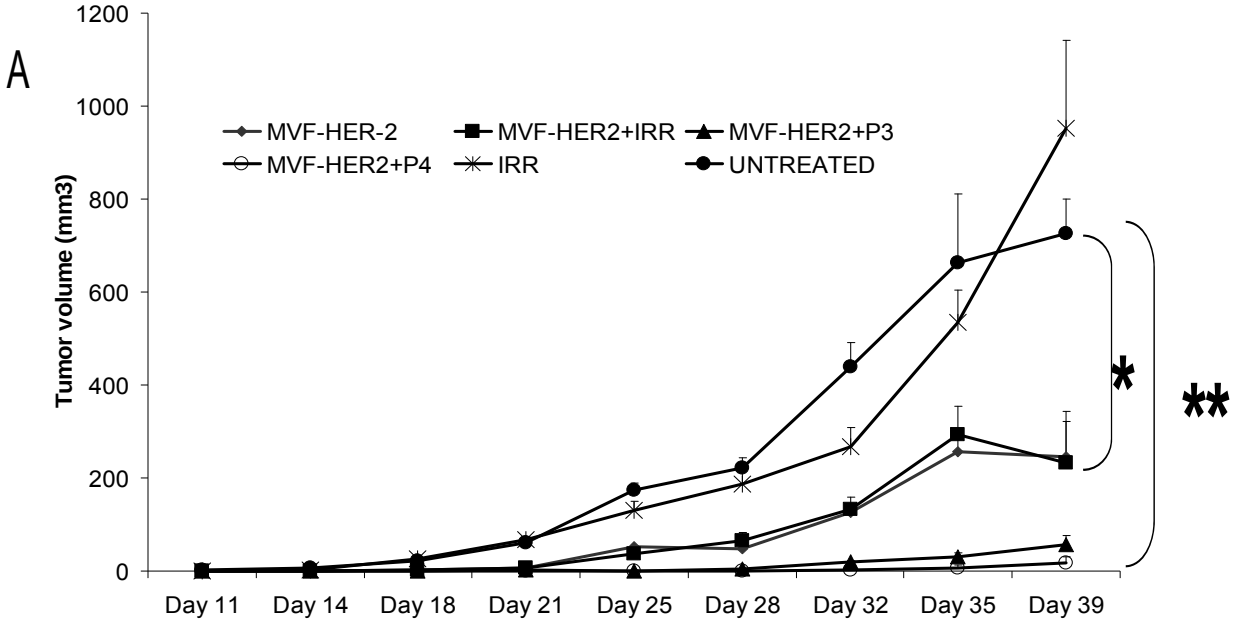
Figure 3.7 Immunization and treatment scheme for Balb/c mice.

Mice were immunized subcutaneously with 100μg of MVF-HER-2-266 three times at three weeks intervals. Two weeks after the third immunization, mice were challenged with TUBO cells and treated weekly with VEGF peptide mimics and irrelevant peptide for 6 weeks.

Figure 3.8: Effects of immunization and peptide treatment in a transplantable tumor model.

A: Wild type BALB/c mice (*n* =5), at the age of 5-7 weeks were immunized subcutaneously three times at three weeks intervals with 100μg of MVF-HER-2-266-296 emulsified in ISA720. After immunization, mice were then challenged with TUBO cells that were derived from BALB-*neuT* mice which are transgenic for the rat HER-2/neu oncogene, and were treated intravenously with VEGF peptide mimics and scrambled irrelevant peptide. Tumor measurements were performed twice a week using calipers. The data are presented as the average tumor size per group and are reported as mm^3 for immunization with MVF-HER-2 and treatment with VEGF peptides and irrelevant. Results show a statistical significant difference between the group immunized with MVF-HER-2 and treated with the VEGF peptide mimics (** $P< 0.001$) while the group that was immunized and treated with the irrelevant peptide showed a non-significant P value of 0.082 when compared to the untreated.

B: Comparison of the effects of immunization with MVF-HER-2 alone with that of immunization with MVF-HER-2 and treatment with VEGF peptide mimics. There is a significant difference between immunization plus irrelevant treatment and immunization plus treatment with VEGF peptide mimics (*$P<0.001$). Also there was a greater delay in tumor growth in the case of the D-amino acid VEGF peptide mimic (MVF-HER-2 + P4) as compared to the case of the L-amino acid VEGF peptide (MVF-HER-2 + P3).


A
1200
1000
800
600
400
200
0
Tumor volume (mm3)
MVF-HER-2
MVF-HER2+IRR
MVF-HER2+P3
MVF-HER2+P4
IRR
UNTREATED
*
**
Day 11
Day 14
Day 18
Day 21
Day 25
Day 28
Day 32
Day 35
Day 39

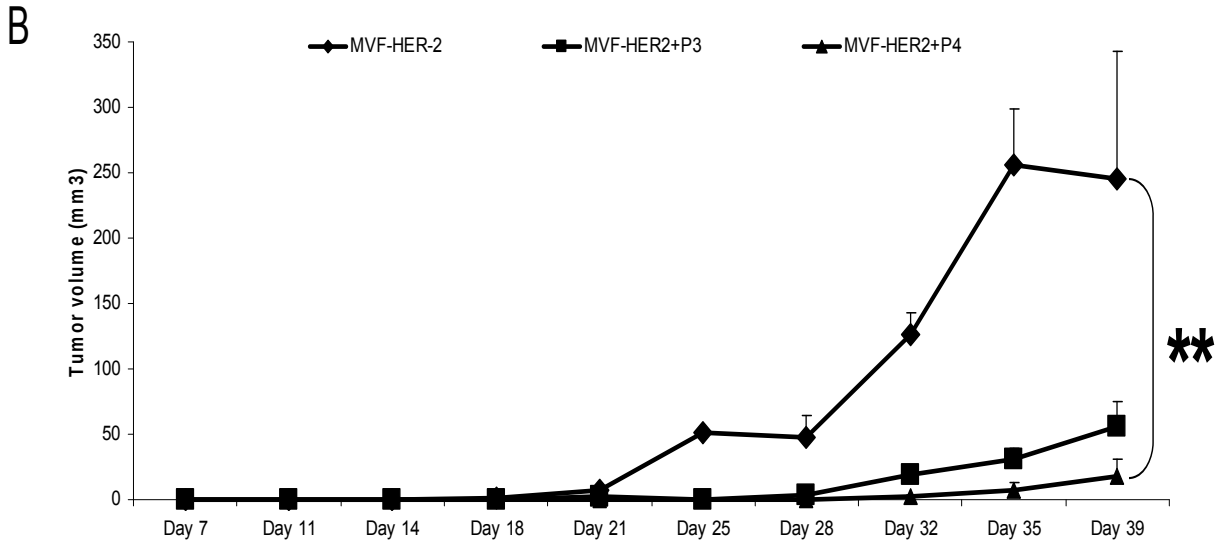
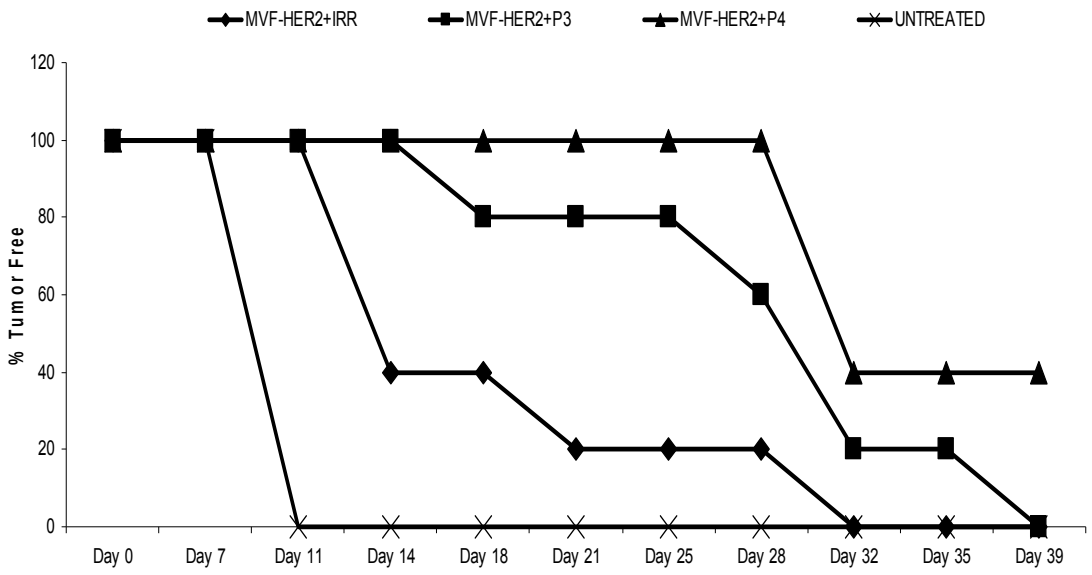

B
350
300
250
200
150
100
50
0
Tumor volume (mm3)
MVF-HER-2
MVF-HER2+P3
MVF-HER2+P4
**
Day 7
Day 11
Day 14
Day 18
Day 21
Day 25
Day 28
Day 32
Day 35
Day 39

Figure 3.9: Effects of immunization peptide treatment on survival rates and percentage tumor weight.

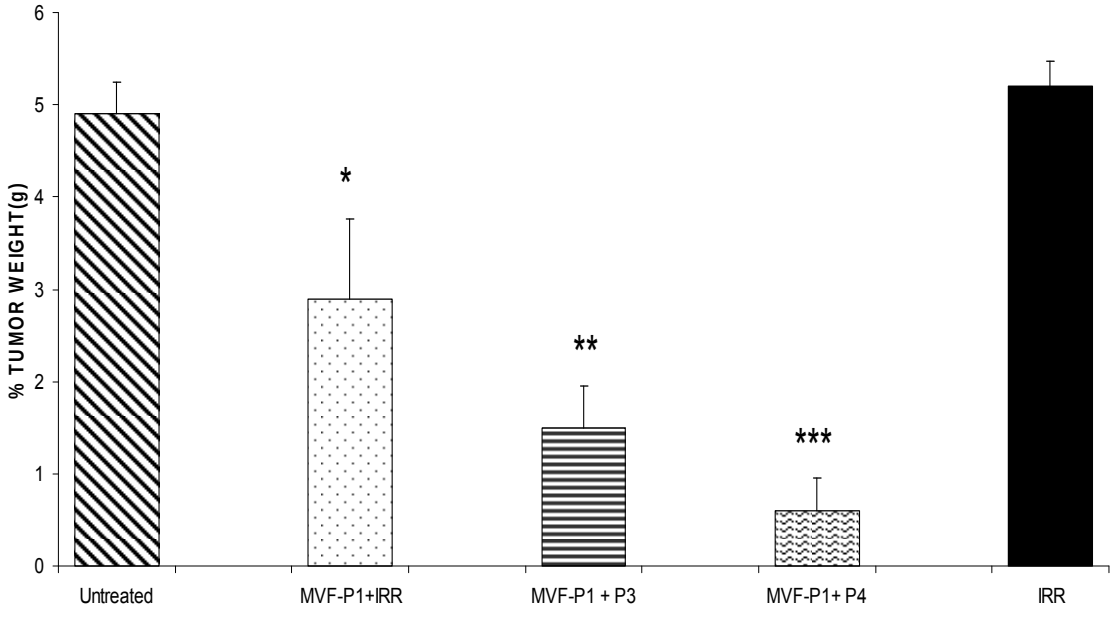
A: Shows the effects of immunization and treatment on tumor development. Results show that immunization with MVF-HER-2 and treatment with VEGF-P4 (D-amino acid VEGF peptide) produced the best results since 40% of the mice (2 out of 5) remained tumor free at the end of the experiment. There was also a greater delay in onset of tumor development in the case of VEGF-P3 peptide as compared to the MVF-HER-2 immunization alone.

B: Effects of peptide treatment on % tumor weight. After treatment, the tumors were removed and weighed and the results show a significant difference between the treated groups and the untreated. The group that was immunized with MVF-HER-2 and treated with irrelevant peptide showed a P* value of 0.044. In the case of MVF-HER-2 + VEGF-P3, the P** value was 0.002 while in the case of MVF-HER-2 +VEGF-P4, the P*** value was <0.001.

A


MVF-HER2+IRR
MVF-HER2+P3
MVF-HER2+P4
UNTREATED
% Tumor Free
120
100
80
60
40
20
0
Day 0
Day 7
Day 11
Day 14
Day 18
Day 21
Day 25
Day 28
Day 32
Day 35
Day 39

B


% TUMOR WEIGHT(g)
6
5
4
3
2
1
0
*
**
***
Untreated
MVF-P1+IRR
MVF-P1 + P3
MVF-P1+ P4
IRR

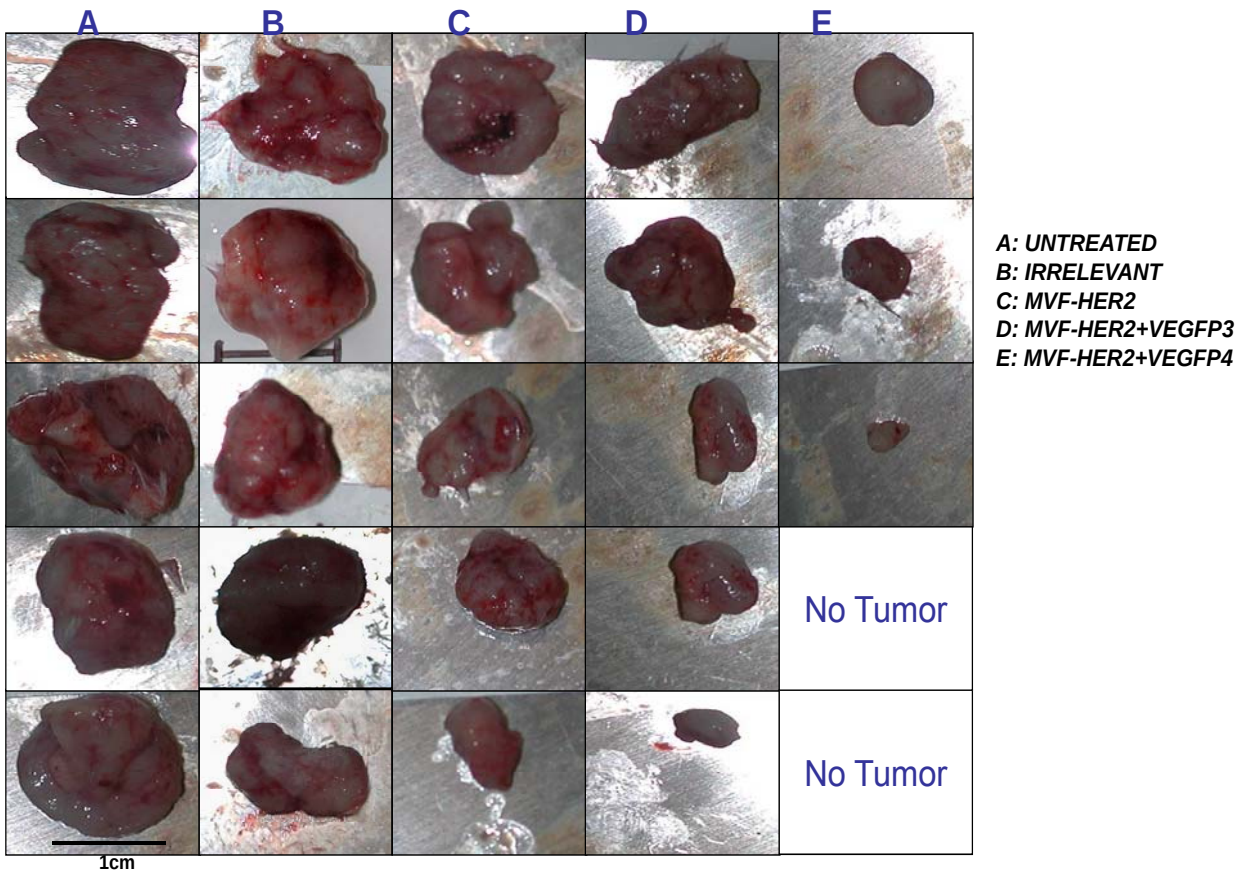


Figure 3.10: Effects of immunization and peptide treatment on tumor size

After treatment, all mice were euthanized according to institutional guidelines and the tumors were extracted and pictures taken.

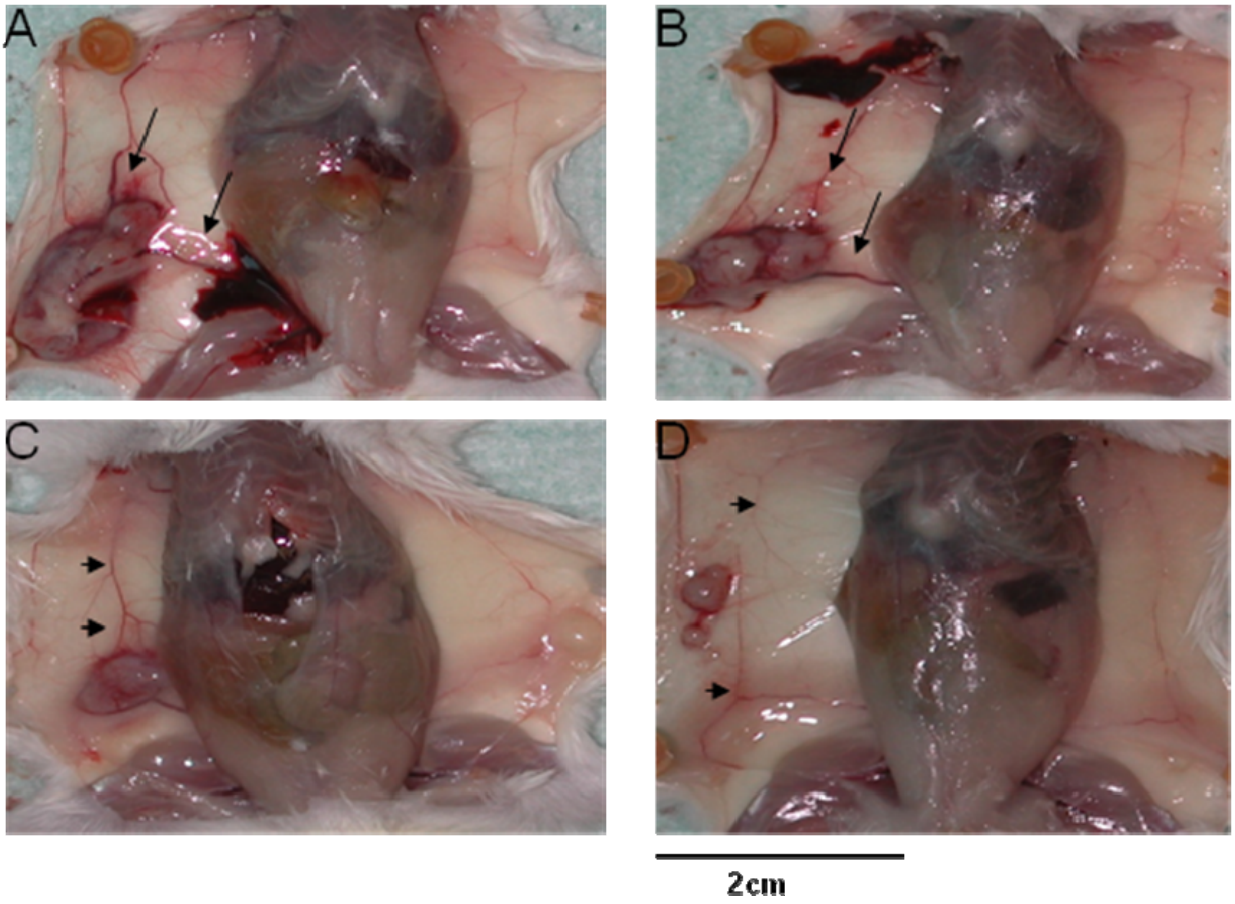


Figure 3.11. Effects of immunization and peptide treatment on tumor vasculature. Representative photos from different treatment groups at day 39 after TUBO challenge. After three immunizations, Balb/c mice were challenged with TUBO cells and treated with VEGF-P3-CYC **(C)** and VEGF-P4-CYC **(D).** Arrow heads shows vascular normalization due to treatment with anti-angiogenic VEGF peptides **(C&D).** Non-immunized and untreated (A) and Immunization with MVF-HER-2 and treatment with Irrelevant (B) represent controls. Arrows show increase blood flow to the tumors **(A&B)** leading to increase in size.

CHAPTER 4

LOW DOSE PACLITAXEL IN COMBINATION WITH HER-2 OR VEGF PEPTIDE MIMICS ADDITIVELY INHIBITS TUMOR GROWTH IN BOTH TRANSPLANTABLE AND TRANSGENIC MOUSE MODELS OF HUMAN BREAST CANCER

4.1 Introduction

HER-2/neu is an oncoprotein that is overexpressed in many epithelial tumors and is associated with highly aggressive forms of cancer [50]. Most solid tumors cannot grow beyond a few millimeters without the angiogenic switch being turn on [175] making angiogenesis suppression a very attractive strategy for targeting tumors. The most well known proangiogenic factor today is VEGF [80] and its overexpression is reported in many different types of cancers [79, 176]. HER-2 upregulation is always accompanied by increase expression of VEGF at both the RNA and protein level in most cancer cells [1, 177]. Targeting these two proteins using novel peptide mimics in a combination strategy can therefore interact in a synergistic/additive manner killing tumor cells and retarding tumor development [78, 80].

One of the greatest challenges of using most chemotherapeutic agents today is to minimize toxicity which has led to the suggestion that combination treatment with low dose chemotherapy and anti-angiogenic agents will reduce toxicity and augment antitumor activity [178]. Anti-angiogenic agents causes normalization of the tumor

vasculature thereby increasing the drug accessibility to the tumor leading to better efficacy [179]. Many studies have shown greater response rates with the use of a combination approach in many preclinical settings [180-182]. Paclitaxel is one of the most widely used chemotherapeutic agents in the treatment of various types of cancers [104, 183]. Generally speaking, paclitaxel exerts its effects by inhibiting mitosis and causing apoptosis of most tumor cells [184, 185]. Extensive studies have been done with paclitaxel alone or in combination with other anti-cancer agents in different types of tumors and in most of the studies, the results showed that combining paclitaxel with these agents seems to yield better response rates [186, 187]. Due to its usage in many types of cancers and its superior antitumor effects [188-190], we wanted to test the effects of low dose paclitaxel in combination with our HER-2 or VEGF peptide mimics in a transgenic mouse model of human breast cancer. We used the PyMT (polyma middle T oncoprotein) transgenic mouse model that starts to develop mammary tumors at around five weeks of age and the different stages of development are similar to that of human breast cancer making it a very useful model for the disease [191]. We hypothesized that, combination treatment with peptide mimics that target either HER-2 or VEGF with low dose paclitaxel will inhibit angiogenesis and cause tumor shrinkage producing better response rates than the single agents alone. In addition, we studied the mechanism of action of these combination treatments by looking at the changes in blood vessel density and the amount of actively dividing cells. We also assessed the cardiotoxicity effects of equal doses of single treatments with the HER-2 peptides in comparison with paclitaxel and Trastuzumab by measuring serum levels of cardiac troponin I after treatment.

4.2 Materials and Methods

Drugs.

Paclitaxel (Taxol) was purchased from the Ohio State University Pharmacy and Trastuzumab (Herceptin) was a kind gift from Dr. William Carson.

Synthesis and characterization of conformational peptides.

Peptide synthesis was performed on a Milligen/Biosearch 9600 peptide solid phase synthesizer (Bedford, MA) using Fmoc/*t*-But chemistry. Preloaded Fmoc-Val-CLEAR ACID resin (0.35 mmol/g) for the 266-296 and CLEAR AMIDE RESIN for the VEGF peptides (0.32 mmol/gm) (Peptides International, Louisville, KY) were used for synthesis. The 266-296 cyclized epitope was assembled by choosing the regioselective side chain protector Trt on Cys residues 268 and 295 [150], and in the VEGF peptides two cysteines were inserted between amino acid Gln79and Gly92 and between Ile80 and Glu93. Peptides were cleaved from the resin using cleavage reagent B (trifluoroacetic acid: phenol: water: TIS, 90:4:4:2), and crude peptides purified by semi preparative reversed-phase-HPLC and characterized by electrospray ionization mass.

Circular Dichroism

This was done as previously described [150]. Briefly aqueous solutions for CD were prepared by dissolving the freeze-dried peptides in appropriate amount of HPLC water to give a final concentration of 0.5mM and used as stock solution for further dilution. CD spectra were recorded on an AVIV model 62A DS CD instrument. Mean residue ellipticity ($[\theta]_{M,\lambda}$) values were calculated according to the equation $[\theta]_{M,\lambda} = (\theta\ X\ 100\ X$

$M_r)/(n X c X l)$. Where θ is the recorded ellipticity (degree); M_r the molecular weight of the peptide; *n*, the number of residues in the peptide; *c*, the peptide concentration (milligrams per milliliter); and *l*, the path length of the cuvette.

Cardiotoxicity Studies.

Groups of female mice (n=5) that are heterozygous for the PyMT transgene were treated intravenously with 300µg of either Trastuzumab, Paclitaxel, HER-2 L amino acid peptide or the D-amino acid HER-2 peptide. Treatment was started from week four till week eleven and tumor sizes were measured twice weekly using calipers and tumor volume calculated using the formula Volume = Length X width^2/2. At the end of the treatment, blood was collected via retro-orbital bleeding and serum samples were used to measure the concentration of Cardiac Troponin I that was present after treatment. This was measured using the highly sensitive mouse Cardiac troponin I ELISA kit from Life Diagnostics.

In vivo antitumor studies in a transgenic mouse model.

Animal breeding was performed in our facility following the institutional guidelines. Female Fvb/n mice that are heterozygous positive for the PyMT transgene were used for the studies. The mice were maintained in a sterile animal facility for the duration of the study. The mice were treated intravenously with the drugs or peptide or a combination of both starting at week four and weekly treatments were continued until week eleven. The treatment included low dose paclitaxel alone (60µg) or peptides alone (300µg) or combination of both. In the combination studies groups of tumor bearing mice (N=5)

received a combination treatment of 60µg of Paclitaxel +300µg of peptide. All the mice were euthanized at 11 weeks of age or if the tumor burden becomes unbearable based on the evaluation of the University animal technician. Tumor sizes were measured twice weekly using calipers and tumor volume was calculated using the formula Volume = Length X width^2/2.

In vivo antitumor studies in a transplantable mouse model.

Female Balb/c wild type mice 5-6 weeks old were purchased from the Jackson laboratory. The mice were maintained in a sterile animal facility for the duration of the study. The mice were subcutaneously challenged with 1 X 10^5 TUBO cells. On the day of TUBO challenge, the mice were intravenously treated with 100µg of either HER-2 or VEGF peptides or 20µg of paclitaxel. In the combination studies groups of tumor bearing mice (N=5) received a combination treatment of 20µg of paclitaxel +100µg of peptide. The mice were then treated weekly for a total of 6 weeks and palpable tumors were measured twice weekly. All the mice were euthanized at 7 weeks after TUBO challenge or if the tumor diameter reaches 1cm. Tumor sizes were measured using calipers and tumor volume was calculated using the formula Volume = Length X width^2/2.

Statistical analysis.

Tumor growth over time was analyzed using Stata's XTGEE (cross-sectional generalized estimating equations) model which fits general linear models that allow the possibility to

specify within animal correlation structure in data involving repeated measurements [152, 168].

Immunohistochemistry.

Tumors excised from mice were fixed in formalin and embedded in paraffin. Paraffin embedded tissue was cut into 4 micron sections and placed on positively charged slides. Slides with specimens were then placed in a 60 °C oven for 1 hour, cooled, and deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 minutes in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase.

Prior to addition of primary antibody, slides were blocked with 10% normal rabbit serum for 30 minutes. An enzymatic digestion was performed with proteinase K for 10 minutes at 37°C. The primary antibodies used included rat anti-mouse F4/80 for macrophages, rat anti-mouse Ki-67 for actively dividing cells, and rabbit anti-mouse CD3 for blood vessels. The primary antibody was added at a dilution ranging from 1:5 to 1:2500 and incubated for 1 hour at room temperature. The secondary antibody used was Vector rabbit anti-rat, mouse adsorbed or goat anti-rabbit at a dilution of 1:200 and incubated for 30 minutes. The detection system used was Vectastain Elite for 30 minutes. The substrate chromogen used was DAB+. The slides were then counterstained with Richard Allen hematoxylin, dehydrated through graded ethanol solutions and coverslipped.

Images were viewed at all ranges and acquired at x40 or x20 original magnification with a Nikon Eclipse E400 microscope and Image-Pro Plus v.5.0 software. To determine

significant differences between CD3-positive staining, three random fields at x40 of each treatment groups were counted. The immunohistochemical detection protocol is detailed as published [192]. Total staining was analyzed using the NIH Image J software and the staining index was calculated as the percentage area occupied by the positive cells to the total area occupied by all the cells.

4.3 Results

Selection, design, synthesis and characterization of peptides.

The selection of the VEGF peptide mimic residues 102-122 (numbered as 76-96 in the crystal structure) corresponds to the overlapping VEGF binding sites to VEGFR-2 and Avastin. Engineering of this peptide sequence has been described in details elsewhere (Vicari et al,). The sequences of both the HER-2 and VEGF peptide mimics are shown in Table I. Briefly, the strategy to create a conformational peptide consisting of an anti-parallel β sheet is described elsewhere (Vicari et al.) where the sequence was modified in a way that the resulting peptide VEGF-P3(NC) adopted a conformation very similar to the native structure. It also required two artificial cysteines to be introduced between Gln79 & Gly92, and between Ile80 & Glu93. After synthesis and purification of VEGF-P3 (NC) (noncyclized) peptide, the disulfide bond was formed by oxidation enabling the formation of the twisted [149, 154]. The rationale behind the retro-inverso peptidomimetic is that it should present similar activity with the advantage of higher bioavailability anti-parallel β-sheet structure in the VEGF-P3(CYC). The retro-inverso

(RI) peptide analog VEGF-RI-P4 was synthesized using D-amino acids with the amino acid sequence in reverse order, such that the resulting peptide mimic has a reversal of the peptide backbone but a topochemical equivalence to the parent peptide in terms of side-chain orientation[66].

Antitumor effects of HER-2 peptide mimics are comparable to that of paclitaxel and Trastuzumab.

The antitumor effects of the HER-2 peptide mimics were compared with that of paclitaxel by treating female Fvb/n mice that are positive for the PyMT transgene with 300μg of the peptides, trastuzumab or paclitaxel. We found that both the L and D-amino acid version of the HER-2 peptide mimics were able to inhibit tumor growth and the effects were comparable to that of paclitaxel and trastuzumab with a significant value of <0.001 when compared to the untreated (Figure 4.1). Since the mice develop multiple tumors but we only measured the sizes of the largest tumor, we also looked at the percentage tumor weights after the individual treatments and our results showed that peptide treatments just like trastuzumab and taxol were able to cause a significant reduction in percentage tumor weight (P*<0.001) when compared to the untreated (Figure 4.2).

Cardiotoxic effects of HER-2 peptide treatment in comparison with Trastuzumab and Paclitaxel.

One of the major drawbacks of the current methods of cancer treatment is the problem of cardiotoxicity. Trastuzumab has been shown to cause cardiotoxic effects in many cancer

patients [193, 194] and other studies have also demonstrated the need to optimize the treatment regimen for paclitaxel due to its numerous side effects as a result of its non specificity[195, 196]. We therefore investigated the cardiotoxic effects of our peptide treatments and compared them with that of trastuzumab and paclitaxel. In order to achieve this, we measured the levels of cardiac troponin I in the serum collected via retro-orbital bleeding from the mice. This is because any toxicity to the heart will cause a release of this protein to the circulation. Our findings showed that treatment with trastuzumab and taxol caused a significant increase in serum levels of cardiac troponin I ($P^*<0.001$) while the HER-2 peptide mimics showed no significant increase ($P^*=0.25$) when compared to the untreated (Figure 4.3)

Combination treatment with low dose paclitaxel and HER-2 or VEGF peptide mimics produces superior anti-tumor effects in a transgenic mouse model.

From the previous cardiotoxicity results, we hypothesized that combination treatment with low doses of these drugs and our peptides will yield synergistic/additive effects. In order to achieve this, we used 60µg of paclitaxel in combination with 300µg of our HER-2 or VEGF peptide mimics using the same PyMT transgenic mouse model of human breast cancer [191]. Results obtained showed that treatment with our HER-2 peptide mimics alone produced a significant reduction in tumor growth and development with a P value of <0.005 and when combined with low doses of paclitaxel, we obtained an even better significant value of <0.001 (Figure 4.4) indicating additive antitumor effects. On the other hand, VEGF peptide mimics were able to inhibit tumor growth and development but the effects were not statistically significant ($^*P<0.195$) but when

combined with low doses of paclitaxel we saw a better significant value of <0.001(Figure 4.5). The results shows that, low dose paclitaxel or VEGF peptide mimics alone had no significant effect on tumor growth but their combination produced outstanding anti-tumor effects in our transgenic mouse model of human breast cancer. In the case of percentage tumor weights, though both single and combination treatment gave as significant value of <0.001, the percentage tumor weight was lower (<10%) in the cases of combination treatment and greater (>10%) in the single treatment groups (Figure 4.6).

Anti-tumor effects of combination treatment with HER-2 and VEGF peptide mimics in a transgenic mouse model.

In order to determine the ability of the peptide combination (HER-2 and VEGF) to inhibit tumor growth in vivo, we used the same PyMT transgenic mouse model. As shown in Figure 7A, treatment with HER-2 peptides alone had significant effects on tumor growth (*P<0.005) while the VEGF peptide mimics showed no significant reduction though there was a delay in tumor development when compared to the untreated. Combination treatment with HER-2 and VEGF peptide mimics produced greater antitumor effects as compared to single HER-2 or VEGF peptide mimics (**P<0.001). There was also a significant delay in unset of tumor development in the case of combination treatment (around week 10) as compared to single treatment (around week 8.5). Next we looked at the effects of combination treatment on the tumor burden by measuring the percentage tumor weight. The groups treated with combination of both HER-2 and VEGF peptides showed a percentage tumor weight of less than 10 while in the case of single treatments, the % tumor weight was greater than 10 in all the cases (Figure 4.8). The best result was

obtained in the case of combination treatment with the D-amino acid HER-2 and VEGF peptide mimics (Figure 4.7&4.8).

Anti-tumor effects of peptide mimics and low dose paclitaxel in a transplantable mouse model.

We evaluated the anti-tumor and antiangiogenic activities of the peptides alone and in combination with paclitaxel. We used wild-type Balb/c mice that were challenged with TUBO cells that were derived from the Balb-*neu*T transgenic mice [46] which are very aggressive tumor cells. The peptides alone were able to cause a delay in onset of tumor development (*P<0.001) (Figure 4.10 & 4.11) and there was an even greater delay when the peptides were combined with paclitaxel. The degree of inhibition observed with the combination treatment was greater than that observed with either peptide alone or paclitaxel alone since there was a marked reduction in tumor volume. Also there was an increase in the percentage tumor survival free rate (Figure 4.12 & 4.13) with one of the combination treatment showing a 20% survival free rate at the end of the experiment (Figure 4.12). We also examined the effects of treatment on the % tumor weight and results showed that single treatments (peptides or paclitaxel) caused a significant reduction of tumor burden (*P<0.005) (Figure 4.14) and combined treatment with paclitaxel caused a marked reduction in tumor burden (**P<0.001).

Combination treatment decreases the number of actively dividing cells in both transplantable and transgenic mouse models.

Paclitaxel is generally considered as a mitotic inhibitor [103, 104]. In order to clarify the anti-tumor mechanisms of the peptides and compare them with that of paclitaxel, we examined the effects of the different treatments on the number of actively dividing cells in both transgenic and transplantable mouse models. Figure 4.9 and 4.16 shows the results of the amount of dividing cells, using Ki-67 staining in transgenic and transplantable tumors respectively. The number of cells greatly decreased in the case of single agent treatment and there was a further decrease in the case of combination treatment.

Combination treatment significantly decreases the microvascular density in a transplantable mouse model.

To further clarify the mechanisms of action of these peptides, we examined the vessel density of the tumors using anti-CD31 antibody. The number of microvessels positive for anti-CD31 staining in the groups treated with the peptides or in combination with paclitaxel was significantly lower than the control untreated group in the transplantable model (Figure 4. 17).

Combination treatment recruits tumor associated macrophages in a transplantable mouse model.

The role played by macrophages in the growth and metastasis of tumors is still unclear. Some studies have even proposed a dual role for tumor associated macrophages.

Initially, they may be recruited to the tumor site as mediators of the immune response but since tumor cells are able to evade immune surveillance, the macrophages promotes tumor growth by secreting growth factors and inducing angiogenesis [95, 145]. In order to further evaluate the anti-tumor mechanisms of these peptides, we quantified the amount of macrophages in the tumor sections by looking for the presence of F4/80+ cells. Results obtained showed that both paclitaxel and the HER-2 peptide mimics were able to cause an increase in the number of infiltrating macrophages (Figure 4.18). The VEGF peptides showed no significant effects on the number of macrophages as compared to the controls but in the cases of combination of paclitaxel with both HER-2 and VEGF peptide mimics, there was a further increase in F480+ cells as compared to paclitaxel alone (Figure 4.18). The combination treatment resulted in an increase in the number of tumor associated macrophages in the transplantable tumor model only.

4.4 DISCUSSION

The main focus of our laboratory is to develop HER-2 and VEGF peptide vaccines that can stimulate the human immune system to produce high affinity antibodies with antitumor effects. HER-2/neu is implicated in highly aggressive forms of cancer [158, 159]. Trastuzumab and Pertuzumab are two humanized monoclonal antibodies that have been shown to bind to the extracellular domain of HER-2 thereby interrupting different signaling mechanisms [113]. VEGF and its receptors are also highly implicated in different forms of cancer and this has led to the development of Bevacizumab which binds to VEGF [61, 197]. Also, upregulation of HER-2 has been shown to increase

VEGF both at the RNA and protein levels [94, 137, 157]. Many of the FDA approved monoclonal antibodies that target both HER-2 and VEGF have been shown to have undesirable toxic profiles [62, 63]. We have designed B-cell epitopes from the HER-2 protein and have successfully translated our preclinical studies into the clinic [111]. We are now directing our focus toward the combination of our HER-2 and VEGF peptides with low dose of chemotherapy.

In our present study, we evaluated the effects of combination treatment with minimal doses of paclitaxel and our peptides. We began by showing that, both paclitaxel and trastuzumab caused significant toxic effects to the heart while our Her-2 peptides were relatively safe when equal doses were administered (Figure 4.3). Besides the safety profiles associated with our peptides, peptide mimics offer the benefits of being water soluble, non-immunogenic and they have the ability to easily cross tissue barriers [83]. The only drawback associated with peptides is their susceptibility to proteosomal degradation but this has been overcome with the development of retro-inverso D-amino acid peptides [84, 85].

We evaluated the antitumor effects of the peptides in comparison to that of paclitaxel and trastuzumab. We noticed that all the treatments showed similar and comparable antitumor effects with the same significant *P value of <0.001 (Figure 4.1) but both paclitaxel and trastuzumab showed significant toxic effects to the heart as showed by the amounts of Cardiac troponin I that was present in the mouse serum (Figure4. 3). Based on these results, we postulated that combining low doses of these drugs with our peptides may yield additive effects in vivo. Many studies have also shown that low doses

of chemotherapy are relatively non-toxic and yields greater anti-tumor effects when combined with other methods of treatment like radiation therapy [198-200]. Results obtained from combining low doses of paclitaxel with either HER-2 or VEGF peptide mimics showed increase antitumor effects (Figure 4.4&4.5) as compared to single treatments. We also evaluated the effects of combination treatment with equal amounts of HER-2 and VEGF peptide mimics and we obtained a significant inhibition of tumor growth in vivo (**P<0.001) as compared to single treatment with HER-2 (*P<0.005) (Figure 4.6). Immunohistochemical studies also showed a great reduction in the amount of actively dividing cells in the cases of combination treatment when compared to single treatments in both tumor models (Figure 4.9 & 4.16).

In conclusion, our results greatly illustrate that the peptide mimics showed potent anti tumor effects when combined with low doses of paclitaxel. Also the peptides are relatively safe when compared to paclitaxel and trastuzumab. In the case of combining both HER-2 and VEGF peptide mimics, the D-amino acid peptides showed better results than the L-amino acid counterparts. Our results show that combining our peptide mimics with low dose chemotherapy seems to be more beneficial and safer since the peptides are relatively non toxic and offer several benefits. Combination treatment aimed at targeting angiogenesis and metronomic chemotherapy will result to better patient survival and little or no toxicity.

4.5 Tables and Figures

Designation	Peptide	Sequence	M.Wt. (da)
HER-2-266-CYC	***266-296*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{266}LH**C**PA *LVTYNTDTFESMPNPEGRYTFGAS**C**V*^{296}*-CONH2*	2925
VEGF-P3-CYC	***76-96*** *peptide with one disulfide bond*	*CH_3CONH-*(L)-^{76}ITMQ^{79}-C-^{92}GIHQGQHPKIRMI^{80}-C-^{93}EMSF^{96}*-CONH2*	2527
VEGF-RI-P4-CYC	***96-76*** *peptide with D amino acids and one disulfide bond*	*CH_3CONH-*(D)-^{96}FSME^{93}-**C**-^{80}IMRIKPHQGQHIG^{92}-**C**-^{79}QMTI^{76}*-CONH2*	2527

Table 4.1: Amino acid sequences and molecular weight of HER-2 and VEGF peptide mimics. Sequences of amino acids are represented from N to C terminal except for the retro inverso peptides RI-HER-2-CYC and VEGF-RI-P4-CYC that were synthesized in the reverse order and using D amino acids. All peptides were synthesized on CLEAR amide resin, using Fmoc/t-But chemistry. All peptides were acetylated on resin using Acetyl-Imidazole (4x) in DMF for 4 hr. Cysteine residues are underlined to indicate the locations of the disulfide bonds.

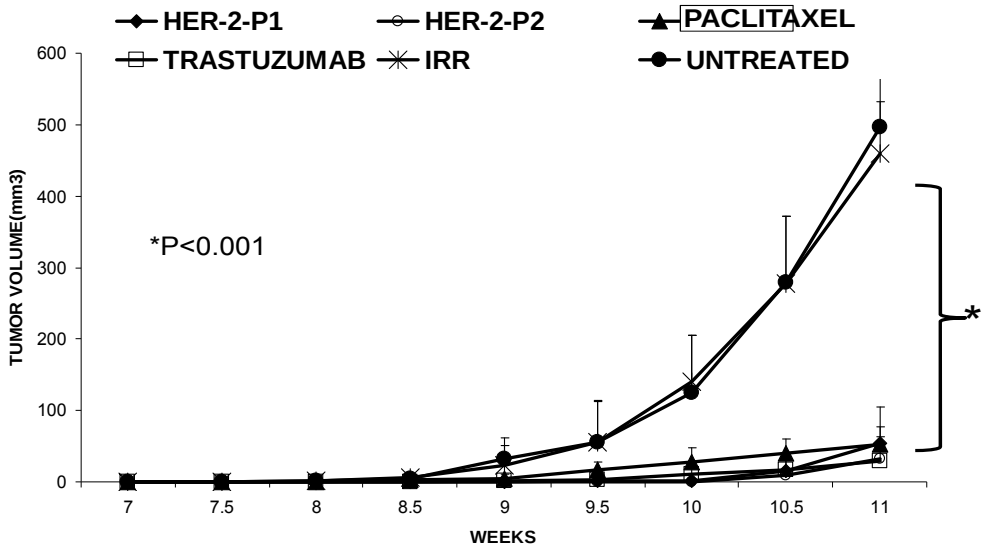


Figure 4.1 Inhibition of tumor growth and development in a transgenic PyMT mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with equal concentrations of HER-2 peptide mimics, Trastuzumab and paclitaxel. Mice were treated intravenously weekly from week four to week eleven. Results showed a delay in tumor growth and development in the treated groups and the effect was equally evident in the case of treatment with taxol, trastuzumab and the peptides (*P<0.001). Tumor measurement was performed using calipers and error bars represent standard deviations from the mean.

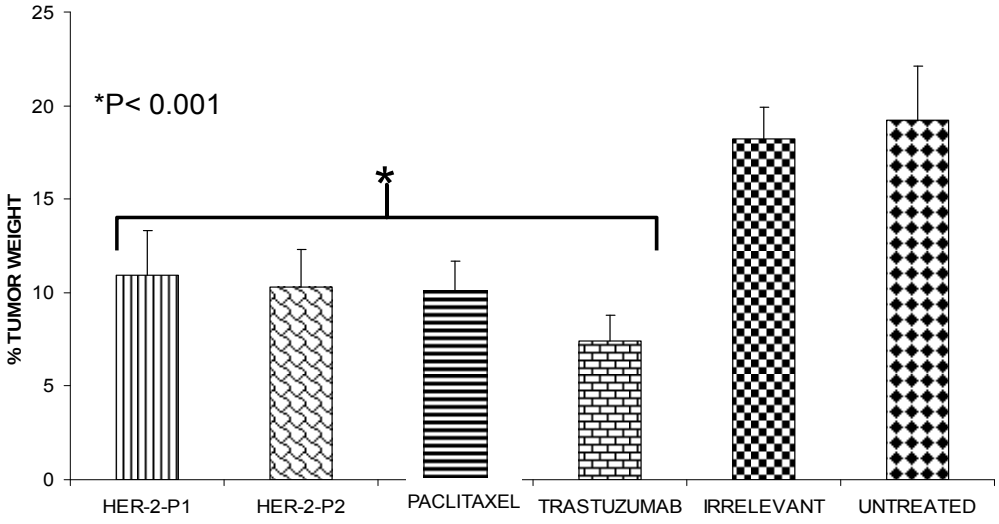


Figure 4.2 Effects of treatment on percentage tumor weight in the PyMT transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with equal concentrations (300µg) of HER-2 peptide mimics, Trastuzumab and paclitaxel. Mice were treated intravenously weekly from week four to week eleven. Results showed a significant reduction in tumor weight in the treated groups and the effect was equally evident in the case of treatment with paclitaxel, trastuzumab and the peptides (*P<0.001). Error bars represent standard deviations from the mean.

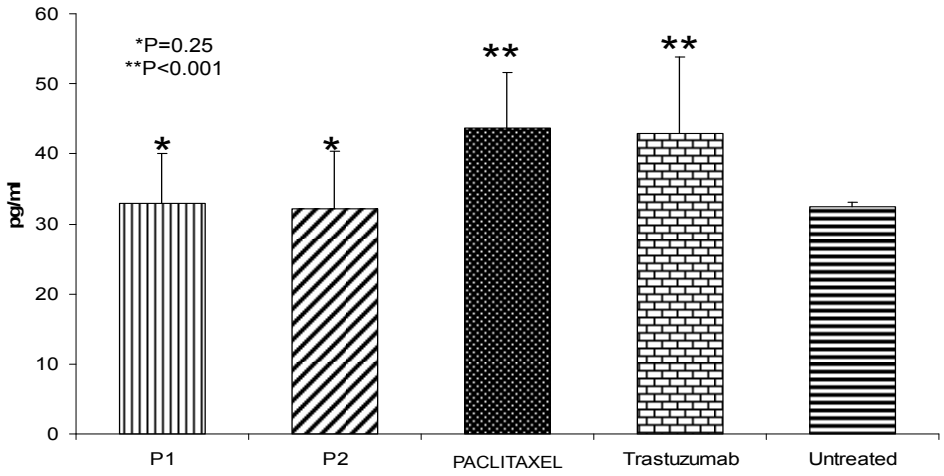


Figure 4.3 Cardiotoxic effects of HER-2 peptide treatment in comparison with Trastuzumab and paclitaxel. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with equal concentrations (300μg) of HER-2 peptide mimics, Trastuzumab and paclitaxel. Mice were treated intravenously weekly from week four to week eleven. At the end of treatment, blood was collected via retro-orbital bleeding and serum levels of Cardiac troponin I was measured using ELISA. Results showed a significant increase in serum levels of cardiac troponin I in the groups treated with paclitaxel and trastuzumab (**P<0.001) while there was no significant effect in the case of treatment with the HER-2 peptides (*P=0.25) when compared to untreated. Error bars represent standard deviations from the mean.

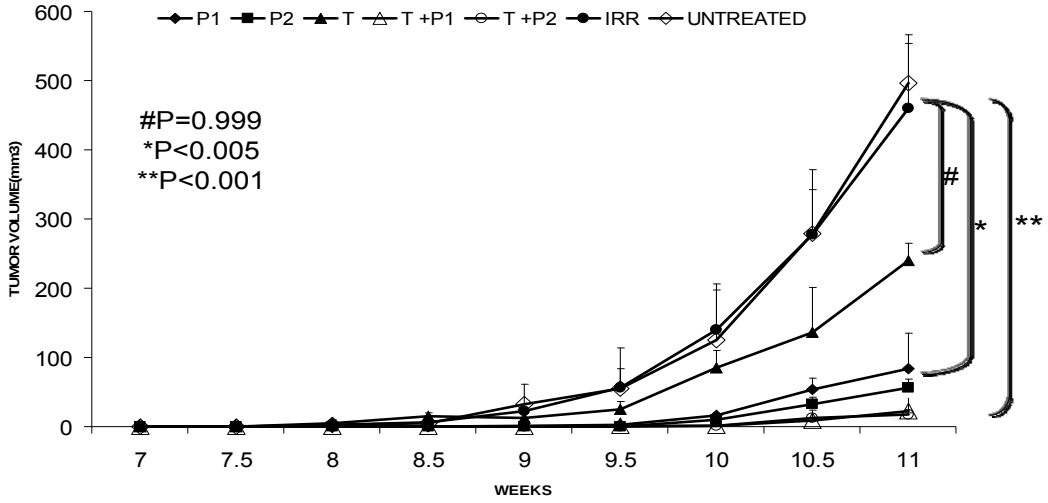


Figure 4.4 Effects of Combination treatments with low dose paclitaxel (T) and HER-2 peptide mimics in a transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with low dose paclitaxel alone (60µg) or in combination with HER-2 peptide mimics (300µg). Mice were treated intravenously weekly from week four to week eleven. Tumor sizes were measured twice weekly using vernier calipers. Results showed a significant delay in tumor growth and development in the groups treated with peptides alone (*P<0.005) and an even better response was observed when these peptides were combined with low doses of paclitaxel (**P<0.001). Low dose paclitaxel alone showed no significant effect (#P=0.999) when compared to untreated. Error bars represent standard deviations from the mean.

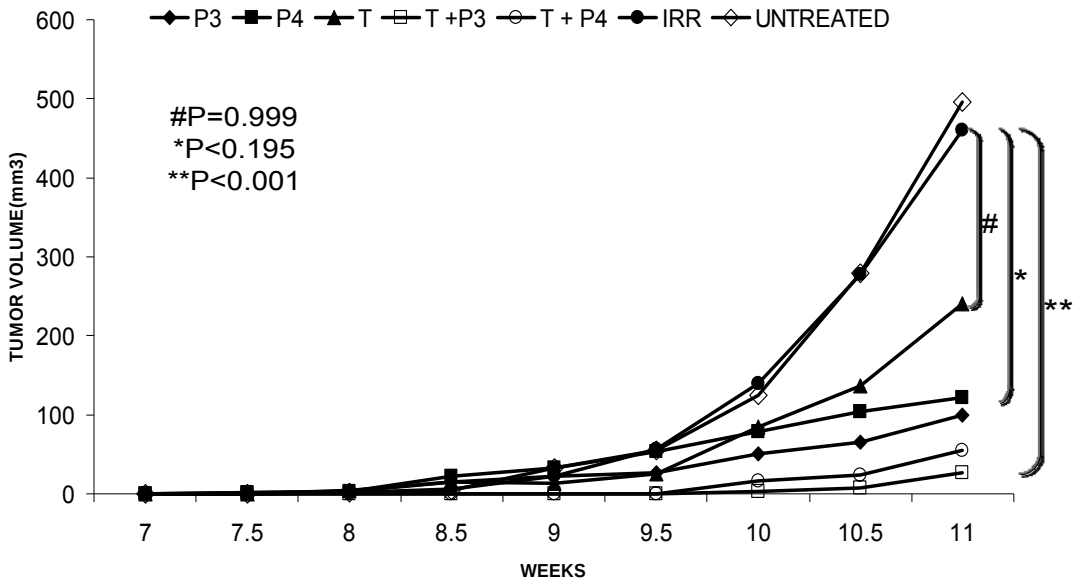


Figure 4.5 Effects of Combination treatments with low dose paclitaxel (T) and VEGF peptide mimics in a transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with low dose paclitaxel alone (60μg) or in combination with VEGF peptide mimics (300μg). Mice were treated intravenously weekly from week four to week eleven. Tumor sizes were measured twice weekly using vernier calipers. Results showed an insignificant delay in tumor growth and development in the groups treated with peptides alone (*P<0.195) and a better response was observed when these peptides were combined with low doses of paclitaxel (**P<0.001). Low dose taxol alone showed no significant effect (#P=0.999) when compared to untreated. Error bars represent standard deviations from the mean.

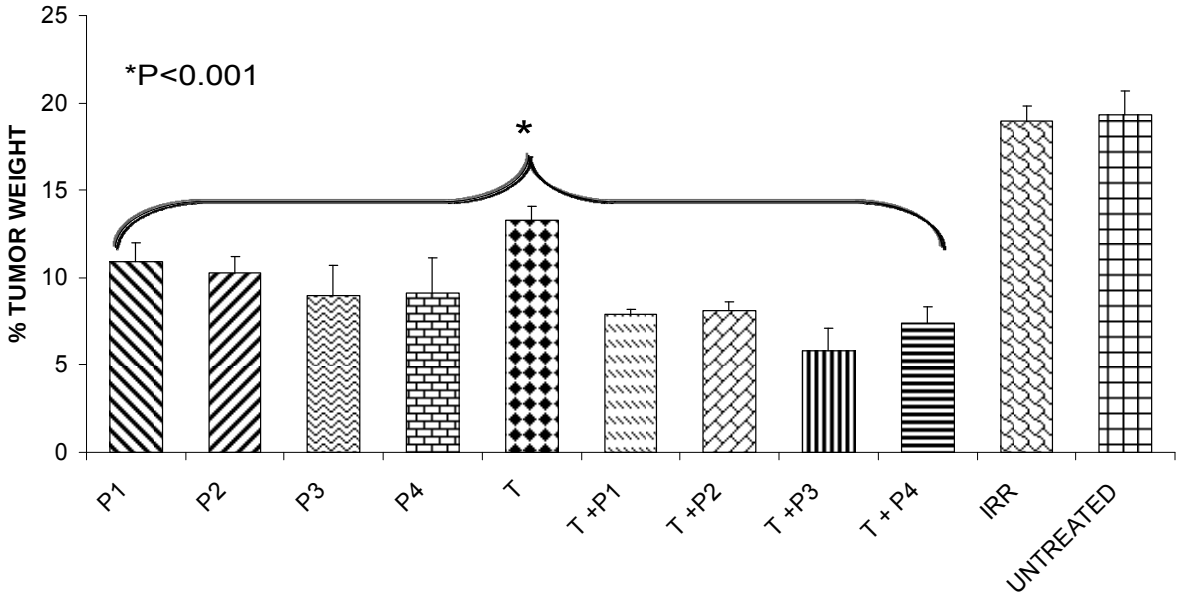


Figure 4.6 Effects of treatment with low dose paclitaxel (T) and peptide mimics on percentage tumor weight in the PyMT transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with equal concentrations (300µg) of HER-2 and VEGF peptide mimics, or in combination with low dose paclitaxel. Mice were treated intravenously weekly from week four to week eleven. Results showed a significant reduction in tumor weight in the treated groups and the effect was equally evident in the case of treatment with paclitaxel and the peptides (*P<0.001). There was a far more reduction in percentage tumor weight (<10%) in the groups treated with both paclitaxel and the peptide mimics. Error bars represent standard deviations from the mean

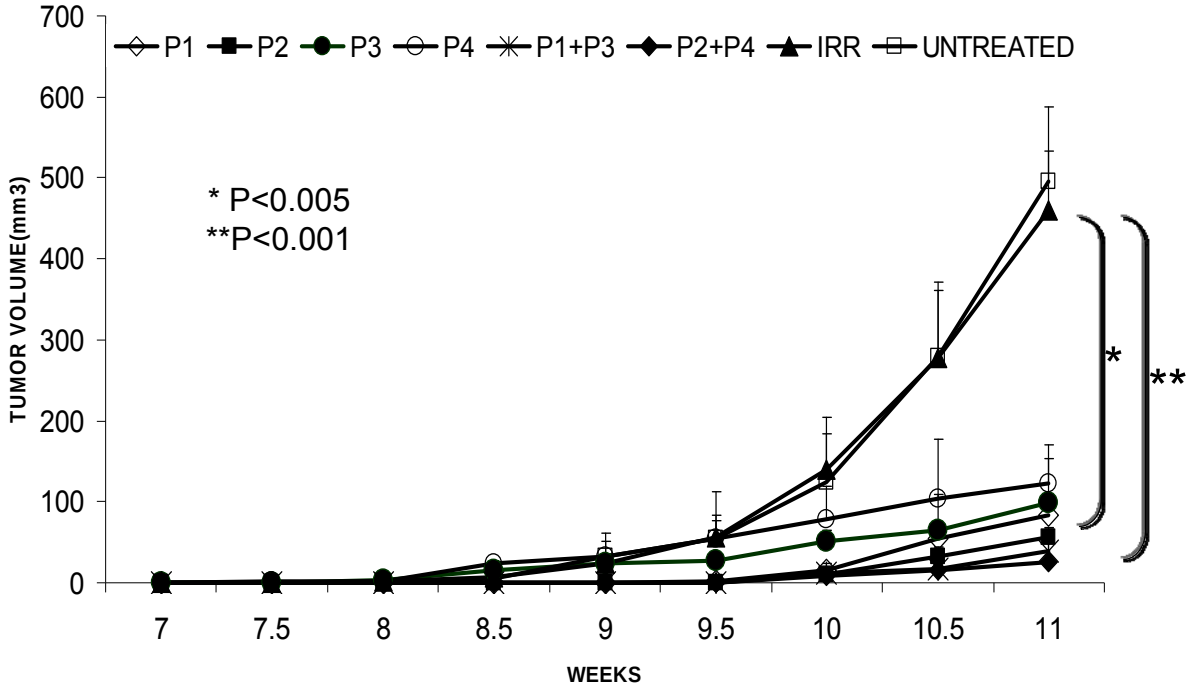


Figure 4.7 Effects of Combination treatments with HER-2 and VEGF peptide mimics in a transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with HER-2 or VEGF peptide mimics (300µg) or a combination of both. Mice were treated intravenously weekly from week four to week eleven. Tumor sizes were measured twice weekly using vernier calipers. Results showed a significant delay in tumor growth and development in the groups treated with HER-2 peptides alone (*P<0.005) and an even better response was observed when these peptides were combined with VEGF peptide mimics (**P<0.001) when compared to untreated. Error bars represent standard deviations from the mean.

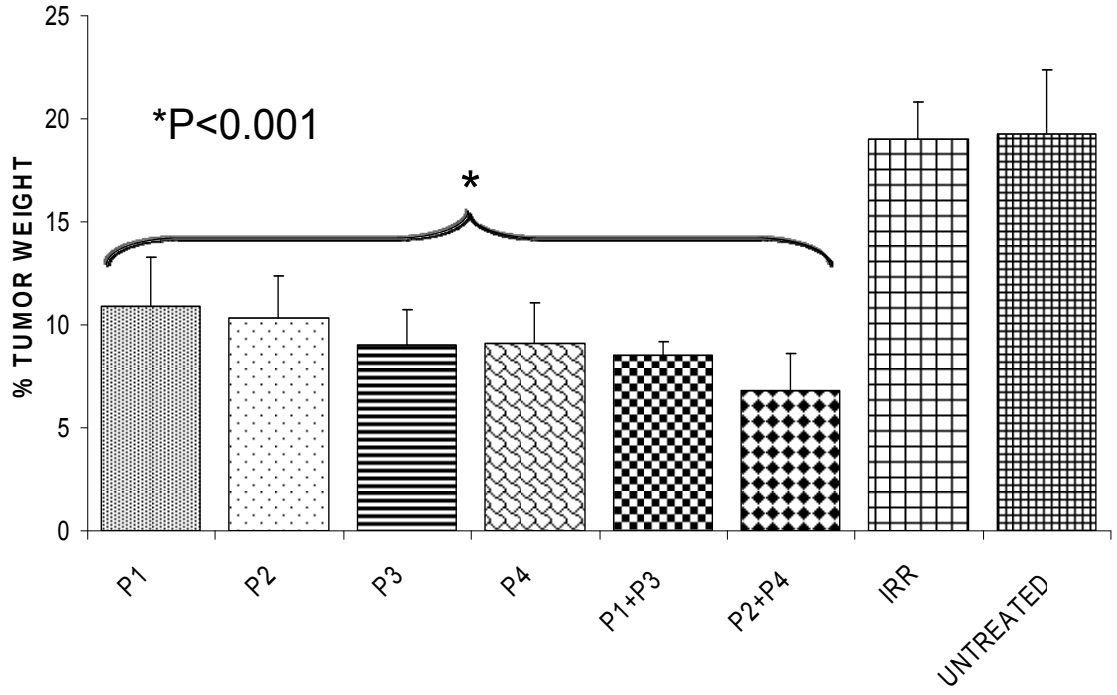


Figure 4.8 Effects of combination treatment with HER-2 and VEGF peptide mimics on percentage tumor weight in the PyMT transgenic mouse model. Female Fvb/n mice (n=5) that are heterozygous positive for the PyMT transgene at the age of 4 weeks were treated with equal concentrations (300μg) of HER-2 and VEGF peptide mimics, or combination of both. Mice were treated intravenously weekly from week four to week eleven. Results showed a significant reduction in tumor weight in the treated groups and the effect was equally evident in the case of treatment with the peptides (*P<0.001). There was a far more reduction in percentage tumor weight (<10%) in the groups treated with both peptide mimics. Error bars represent standard deviations from the mean.

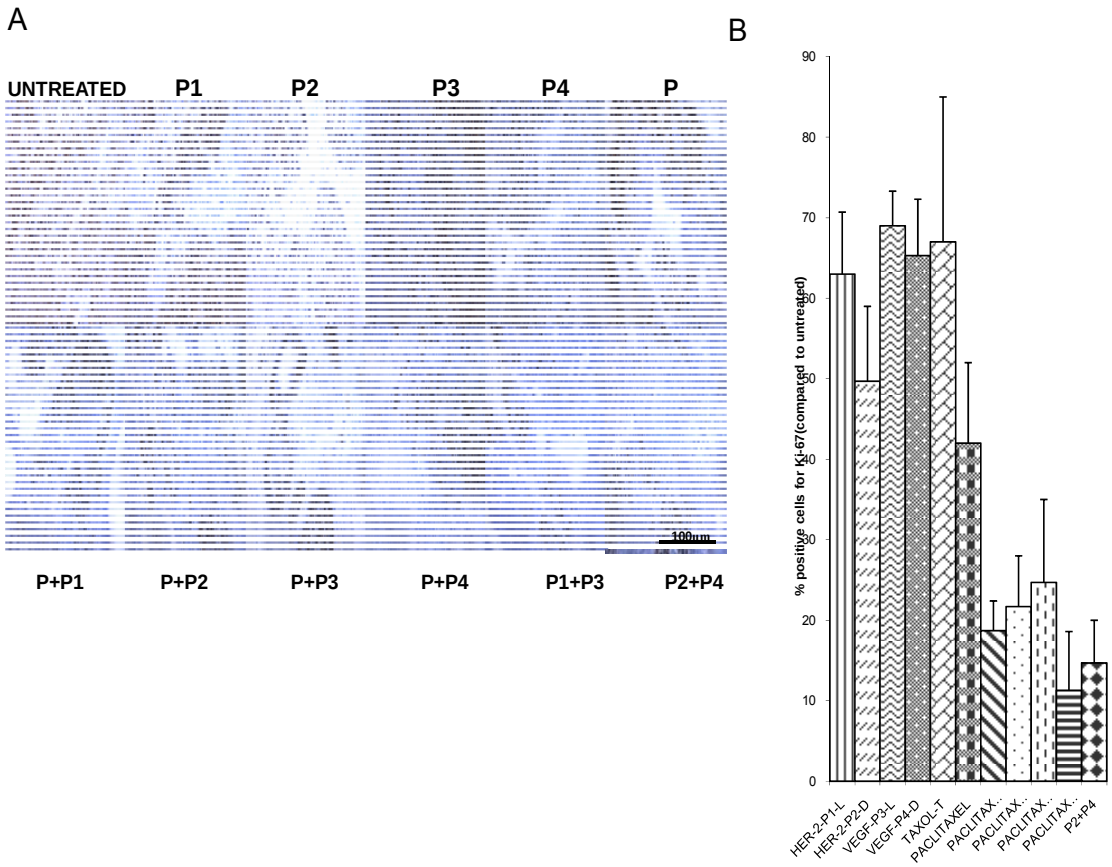


Figure 4.9: Quantification of the number of actively dividing cells in tumor sections from the transgenic PyMT mouse model using Ki-67 staining. (A) Tissue sections show the amount of positive cells. The stain is specific for cells that are actively dividing. (B) Quantification of the staining using Image J software. Data represent mean values and error bars represent standard deviations from the mean.

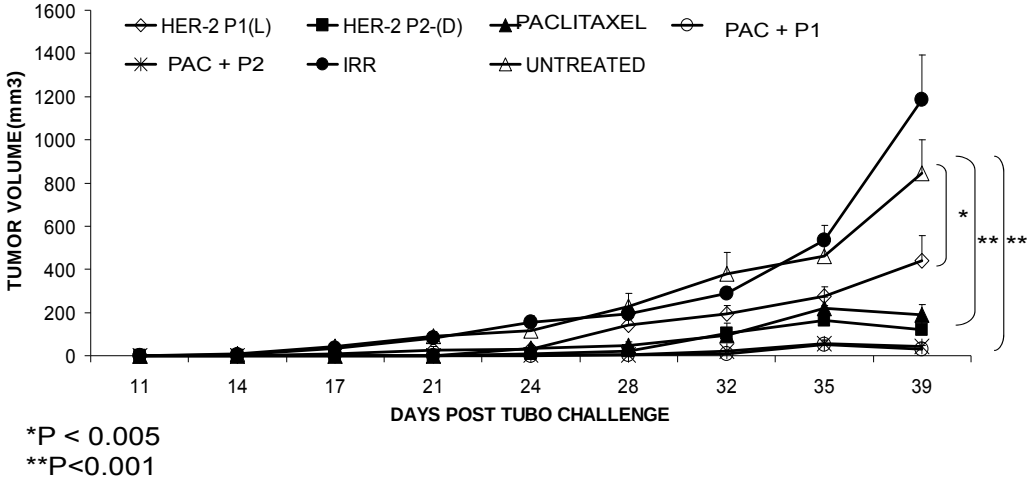


Figure 4.10 Inhibition of tumor growth and development in a transplantable mouse model using low dose paclitaxel and HER-2 peptide mimics. Wild type BALB/c mice (n=5) at the age of 5-7 weeks were challenged with TUBO cells that were derived from BALB-*neu*T mice which are transgenic for the HER-2/*neu* oncogene. Mice were treated intravenously with paclitaxel, HER-2 peptides, VEGF peptides or a combination of paclitaxel and the peptides. Results showed a delay in tumor growth and development in the treated groups and the effect was more evident in the case of combination treatment with taxol and the peptides (**P<0.001). Tumor measurement was performed using calipers and error bars represent standard deviations from the mean.

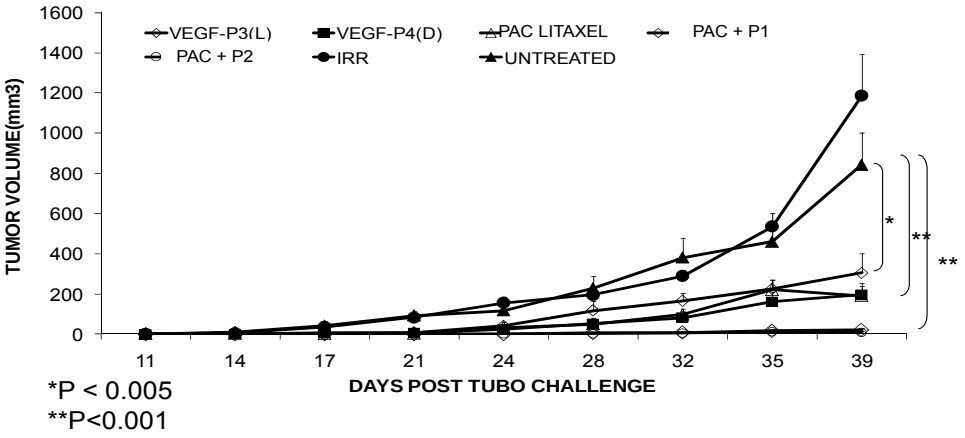


Figure 4.11 Inhibition of tumor growth and development in a transplantable mouse model using low dose paclitaxel and VEGF peptide mimics. Wild type BALB/c mice (n=5) at the age of 5-7 weeks were challenged with TUBO cells that were derived from BALB-*neu*T mice which are transgenic for the HER-2/neu oncogen. Mice were treated intravenously with Taxol, HER-2 peptides, VEGF peptides or a combination of taxol and the peptides. Results showed a delay in tumor growth and development in the treated groups and the effect was more evident in the case of combination treatment with taxol and the peptides (**P<0.001) as compared to peptides alone (*<0.005. Tumor measurement was performed using calipers and error bars represent standard deviations from the mean.

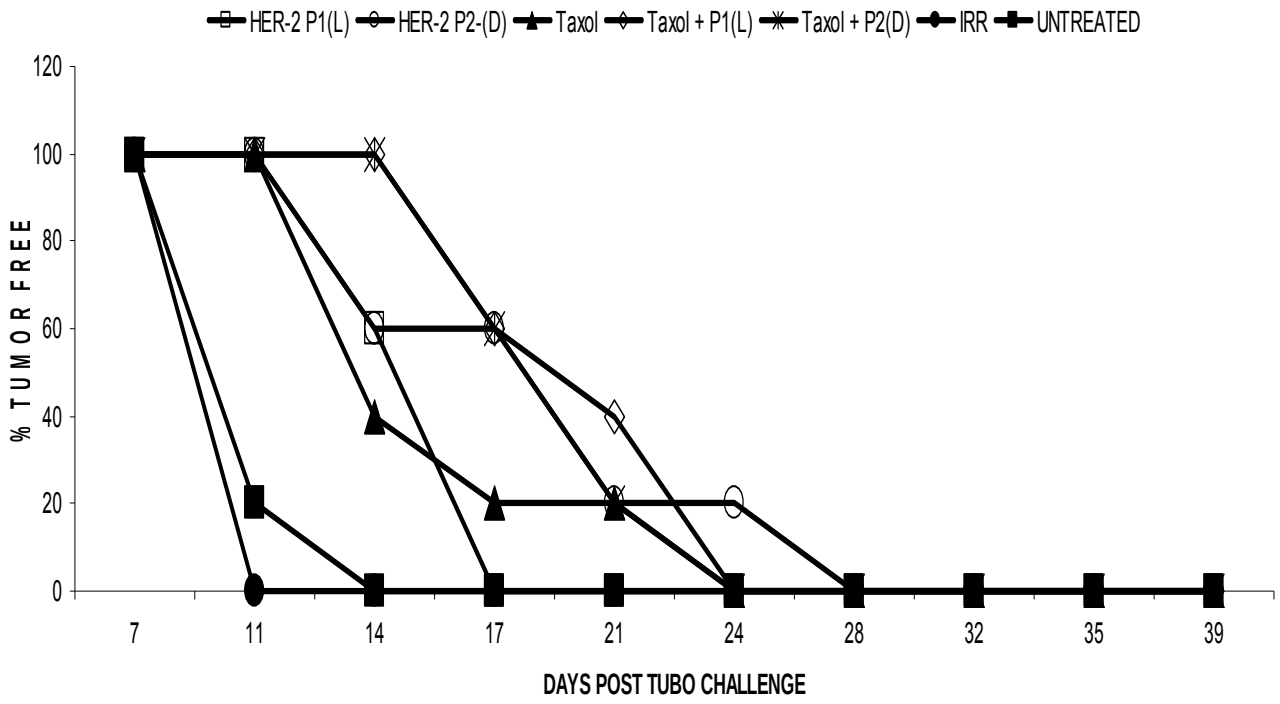


Figure 4.12 Effects of HER-2 peptides and paclitaxel (T) on the tumor free survival rate in a transplantable mouse model. Mice treated with peptides and paclitaxel showed an increase in survival rate. HER-2 peptide mimics in combination with low dose paclitaxel . Mice were treated weekly intravenously with either 100μg of peptide or 20ug of paclitaxel or a combination of both. Error bars represents standard deviations from the mean.

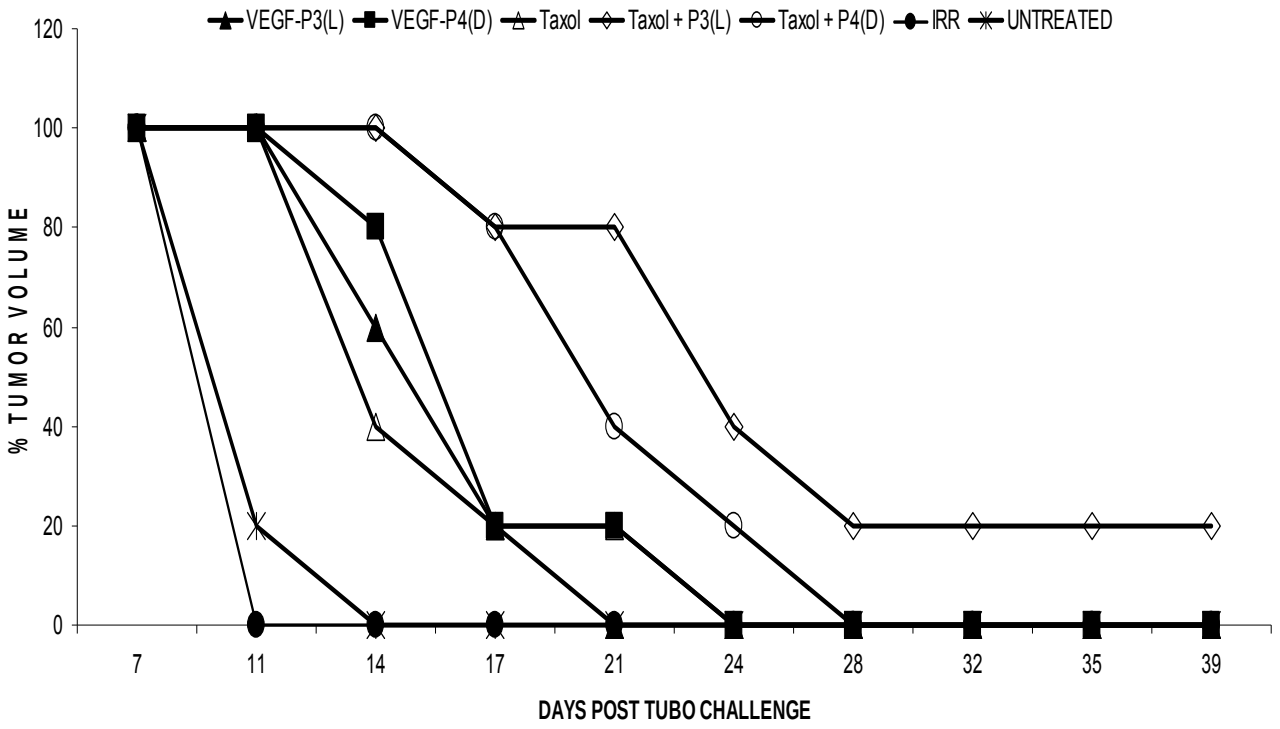
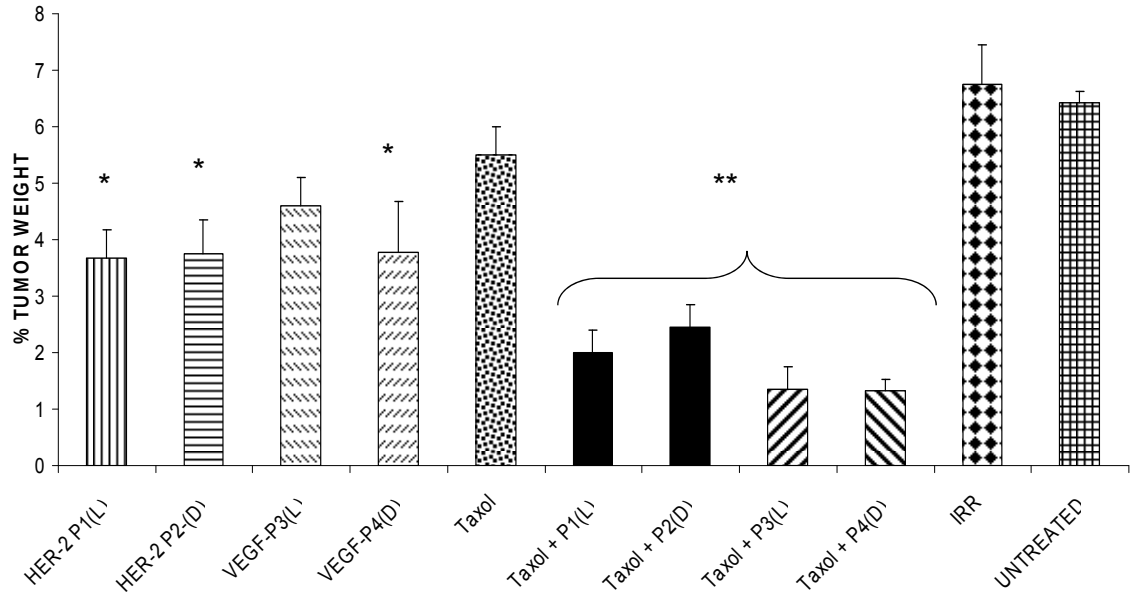


Figure 4.13 Effects of VEGF peptides and paclitaxel (T) on the tumor free survival rate in a transplantable mouse model. Mice treated with peptides and paclitaxel showed an increase in survival rate. HER-2 peptide mimics in combination with low dose paclitaxel. Mice were treated weekly intravenously with either 100µg of peptide or 20µg of paclitaxel or a combination of both. Error bars represents standard deviations from the mean.



*P <0.005
**P <0.001

Figure 4.14 Effects of combination treatment on percentage tumor weight. Tumors from mice treated with single or combination agents were excised and weighed. Mice that were treated with both peptides and paclitaxel (T) showed a P<0.001 as compared to the control while groups treated with either peptides alone or paclitaxel alone showed a significant difference with P<0.005. Error bars represent standard deviations of the mean.

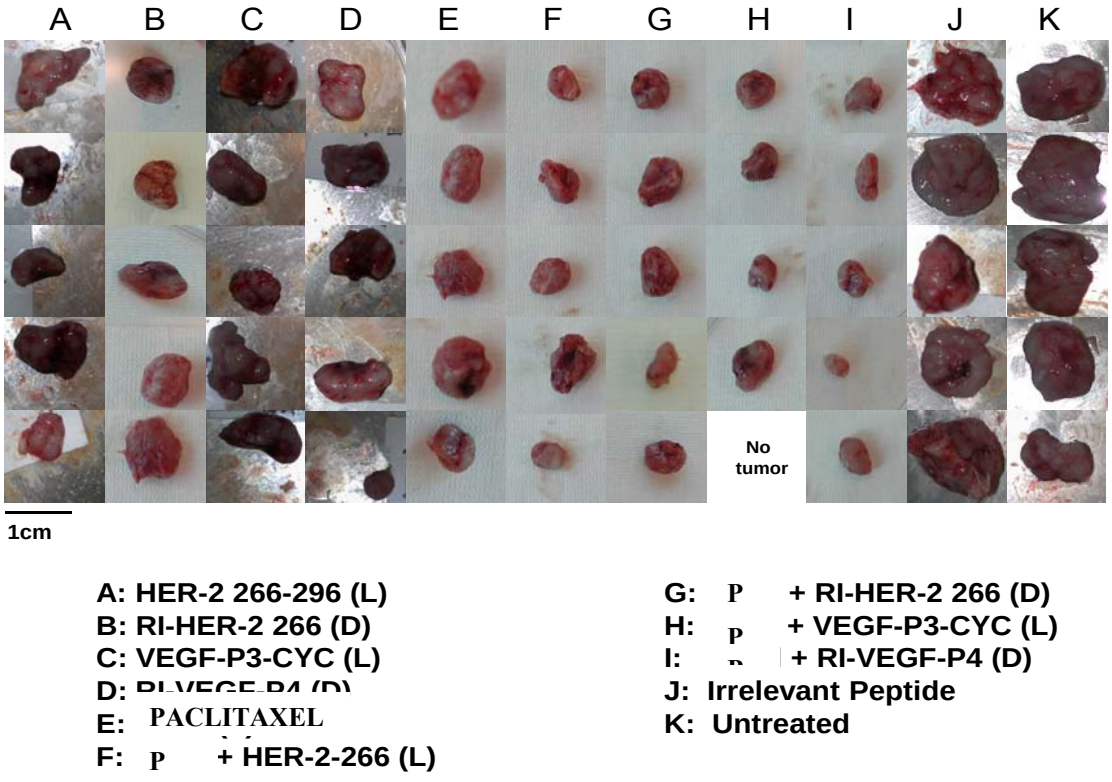


Figure 4.15 Photos of tumors from mice in the different treatment groups. Tumors were removed at the end of treatment and pictures taken using a camera. Photos show a great reduction in size which is most evident in the case of combination treatment.

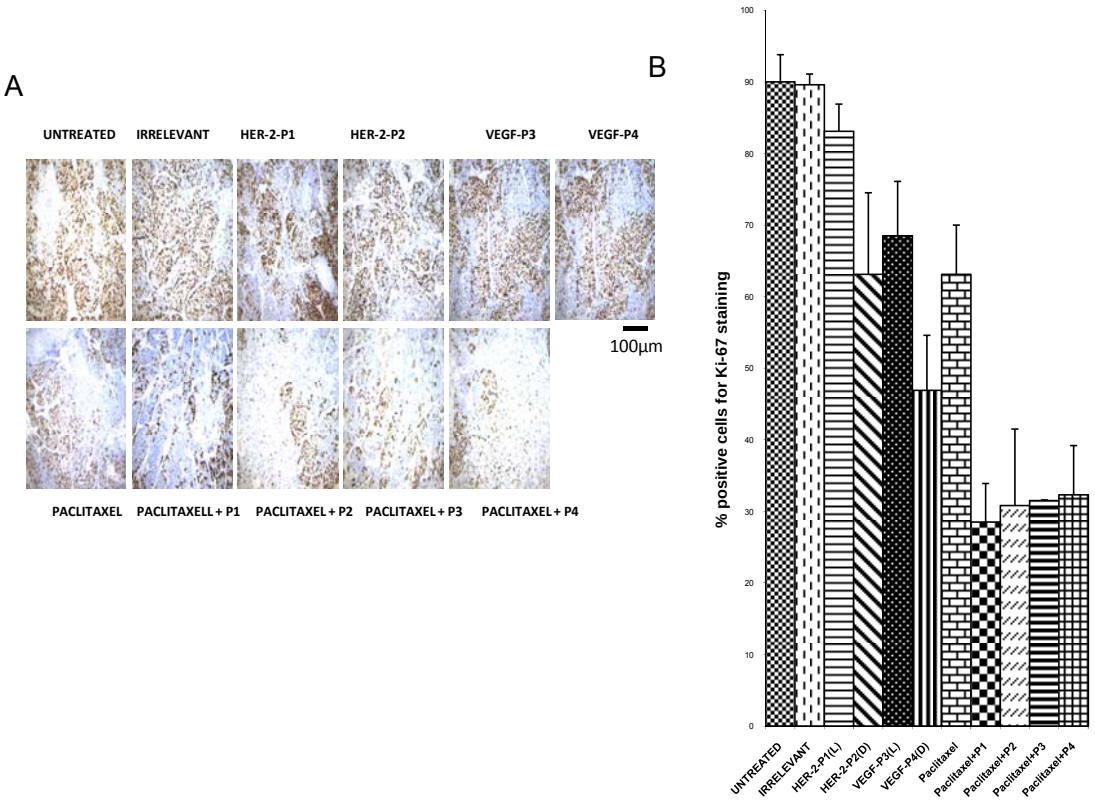


Figure 4.16: Quantification of the number of actively dividing cells in tumor sections from the transplantable model using Ki-67 staining. (A) Tissue sections show the amount of positive cells. The stain is specific for cells that are actively dividing. (B) Quantification of the staining using Image J software. Data represent mean values and error bars represent standard deviations from the mean.

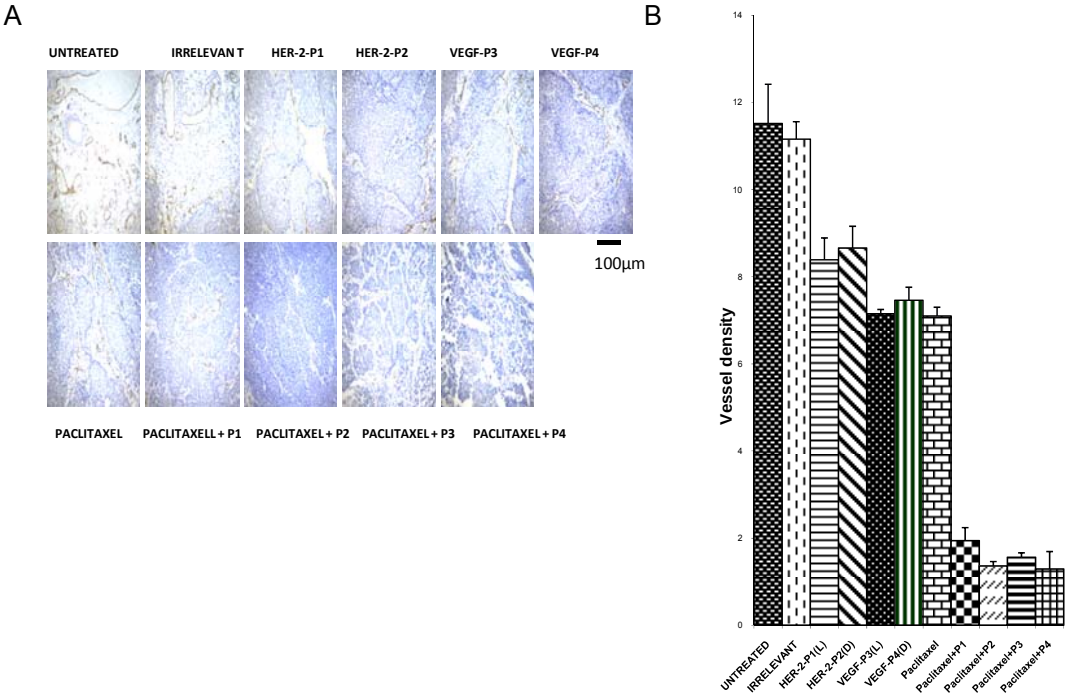


Figure 4.17: Evaluation of vessel density in tumor sections from the transplantable model after treatment. (A) Vascular staining using anti-CD31 antibody and (B) Effects of combination treatment on the tumor vessel density after quantification with the Image J software. Data represents mean values and error bars represents mean standard deviations.

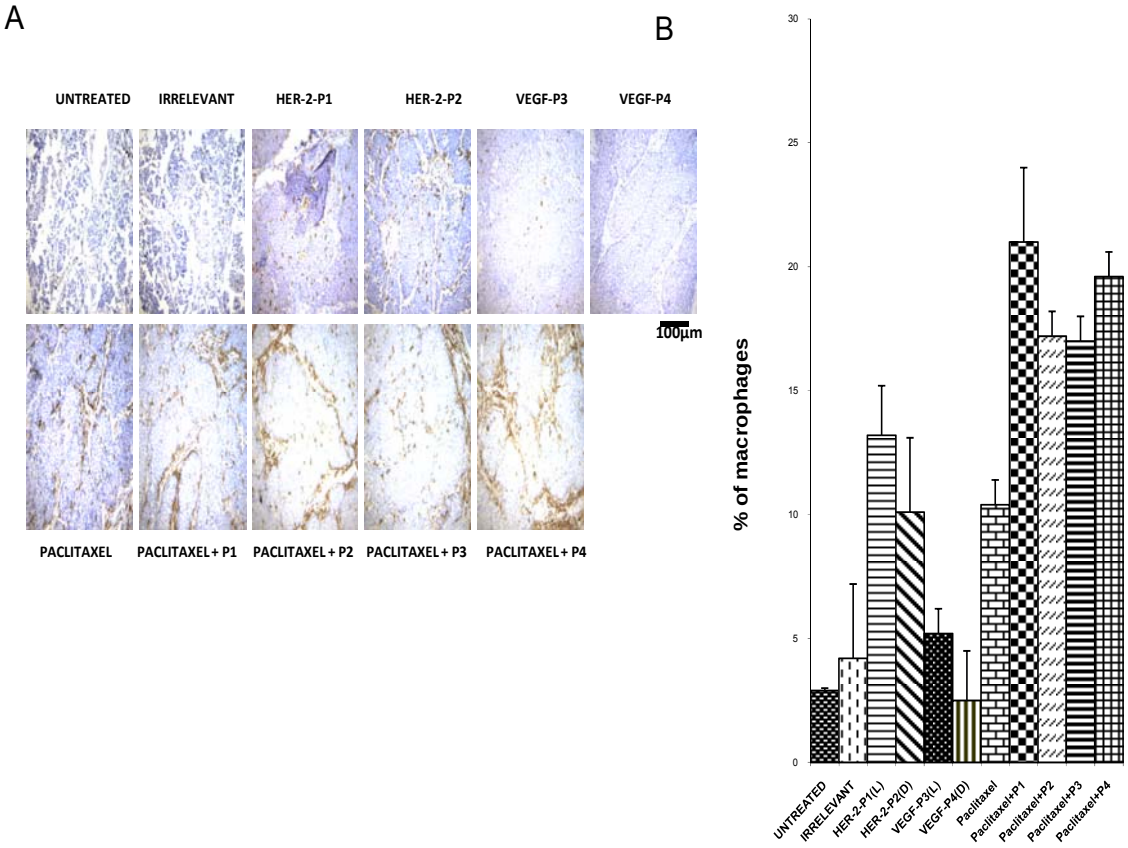


Figure 4.18: Quantification of the amount of macrophages in tumor sections from the transplantable model after treatment. (A) Staining was done using F4/80 staining and (B) amount of positive cells were quantified using the Image J software. Data represents mean values and error bars represents mean standard deviation

CHAPTER 5

SUMMARY AND FUTURE PERSPECTIVES

The main goal of the research presented in this thesis was to evaluate the effects of combination treatment with HER-2 and VEGF peptide mimics and also to show that peptides are relatively safer when compared with most methods of chemotherapy. Chapters 2 and 3 described the beneficial effects of targeting HER-2 and VEGF using novel HER-2 and VEGF B-cell epitopes that are designed in our laboratory taking advantage of the crystal structure of the HER-2 ECD bound to the pertuzumab and VEGF bound to VEGFR2. Chapter 4 focuses more on the toxic effects of chemotherapy with trastuzumab and paclitaxel when compared to our peptides. It also illustrates the use of low dose chemotherapy with other cancer treatment options which will lead to better responses and minimal toxicity. The HER-2 peptide vaccines was designed with the consideration of the structure of HER-2 bound to pertuzumab, a mAb with proven inhibitory properties against HER-2 expressing cancer cells.

The advantage of using peptides over passive immunotherapy with mAbs such as trastuzumab, pertuzumab and bevacizumab is an important aspect of this work. The average half-life of IgG administered intravenously is about 12 days. Trastuzumab for example is administered weekly making treatment with these monoclonal antibodies very expensive. It costs more than $50,000 USD for a patient to be placed on trastuzumab for a year which most average Americans cannot afford. Additionally, serious effects of

cardiotoxicity have been reported in most patients as also illustrated in chapter 4 of this dissertation. There are also reports of undesired immunogenicities and poor penetrating abilities due to their large sizes. Active immunotherapy with peptide vaccines affords the possibility of training the human immune system to take care of these cancer cells by generating a long-lasting antibody response through the production of memory B-cells. This is therefore considered a more cost effective strategy when compared to passive immunotherapy with humanized monoclonal antibodies. We have clearly demonstrated in two different mouse models that combination treatment with our HER-2 and VEGF peptide mimics produced additive anti-tumor effects and the peptides are relatively safer when compared to paclitaxel and trastuzumab. A lot of studies have demonstrated the beneficial effects of using a combination strategy to target cancer cells and *in vitro* studies have demonstrated that a combination of pertuzumab and trastuzumab synergistically inhibit the survival of breast cancer cells [201]. The combination of these peptide mimics could be translated into the clinic to investigate the efficacy of targeting both HER-2 and VEGF in HER-2 positive cancers. Additionally, future studies could be done to investigate possible combination of our peptide mimics with low doses of chemotherapy.

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