**Immunotherapy with HER-2 and VEGF peptide mimics plus metronomic paclitaxel causes superior antineoplastic effects in transplantable and transgenic mouse models of human breast cancer**

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**Abbreviations:** HER-2, human epidermal growth factor receptor 2; hmAb, humanized monoclonal antibodies; PyMT, polyclonal middle T oncoprotein; VEGF, vascular endothelium growth factor

HER-2 and the vascular endothelial factor receptor (VEGF) represent validated targets for the therapy of multiple tumor types and inhibitors of these receptors have gained increasing importance in the clinic. In this context, novel bioactive agents associated with better therapeutic outcomes and improved safety profile are urgently required. Specifically engineered HER-2- and VEGF-derived peptides in combination with low-dose chemotherapy might provide a substantial impact on tumor metastasis and cancer progression. We tested the antitumor effects of HER-2 and VEGF peptide mimics in combination with metronomic paclitaxel in both PyMT and Balb/c murine model challenged with TUBO cells. The combination of low-dose paclitaxel and HER-2 or VEGF peptide mimics had greater inhibitory effects than either agent alone. Peptide treatment caused virtually no cardiotoxic effects, while paclitaxel and the anti-HER-2 antibody trastuzumab (Herceptin), exerted consistent cardiotoxicity. The combination regimen also promoted significant reductions in tumor burden and prolonged survival rates in both transgenic and transplantable tumor models. Tumor weights were significantly reduced in mice treated with HER-2 peptides alone, and even more in animals that received HER-2 peptide with low-dose paclitaxel, which alone had no significant effects on tumor growth in the transgenic model. Specifically engineered native peptide sequences from HER-2 and VEGF used in combination with metronomic paclitaxel demonstrate enhanced anticancer efficacy and an encouraging safety profile. This novel approach to targeted therapy may offer new avenues for the treatment of breast cancer and other solid tumors that overexpress HER-2 and VEGF.

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**Introduction**

ERBB2 (best known as HER-2/neu) is an oncoprotein that is overexpressed in approximately 20–30% cases of breast cancers and is associated with increased aggressiveness and poor clinical outcome.1 HER-2 is a well-established target for immunotherapy and many different anti-HER-2 strategies have been tested, including several humanized monoclonal antibodies (such as trastuzumab and pertuzumab) and small molecule tyrosine kinase inhibitor (like lapatinib). Pertuzumab has been shown to bind the extracellular domain II of HER-2, thereby interrupting dimerization via a mechanism that differs from that of trastuzumab.2

Most solid tumors cannot grow beyond a size of few millimeters without undergoing the so-called “angiogenic switch,” allowing for neovascularization and the consequent supply of nutrients and oxygen in sufficient amounts.3 Thus, angiogenesis inhibition offers an attractive therapeutic strategy for cancer therapy. The pro-angiogenic factor best known today is the vascular endothelial growth factor (VEGF),4 its overexpression being reported in many different types of cancers. HER-2 upregulation is accompanied by increased expression of VEGF, at both the RNA and protein level in a large panel of cancer cells.5 As VEGF and its receptors are profoundly implicated in different forms of cancer, anti-VEGF antibodies have been developed for use in the clinic,
combination treatments with low-dose chemotherapy and antiangiogenic/antitumor agents have generated interest in that they are supposed to result in reduced toxicity and prime targeted antitumor activity. Antiangiogenic agents cause the normalization of tumor vasculature, thereby increasing the accessibility of drugs to the tumor. Many studies have shown greater response rates with the use of a combination approach involving angiogenesis inhibitors in many preclinical settings. Paclitaxel is one of the most widely used chemotherapeutic agents for the treatment of various types of solid tumors. Paclitaxel exerts anticancer effects mainly by inhibiting mitosis and hence causing the apoptotic demise of tumor cells. Extensive studies have been performed with paclitaxel alone or in combination with other anticancer agents, in different types of tumors. Most of these studies showed that combining paclitaxel with other anticancer agents improves response rates. Due to its usage in many types of cancers and its superior antitumor effects, we wanted to gauge the effects of low-dose paclitaxel in combination with HER-2 and/or VEGF peptide mimics, in a transgenic mouse model of human breast cancer.

In order to develop a safe, efficient and poorly toxic anticancer strategy, we are extending our overall approach and combining specially engineered peptide VEGF and HER-2 peptide mimics with a low-dose paclitaxel regimen. The rationale for inclusion of chemotherapeutics is further supported by reports in the literature indicating an enhanced efficacy and better therapeutic outcome of this type of approach. Our in vivo results show that the combination of HER-2 and VEGF peptide mimics with low-dose paclitaxel inhibits angiogenesis, causes tumor shrinkage, and produces better response rates than either single agents alone, in both transplantable and transgenic mouse model. Additionally, we show that such peptide therapeutics combined with metro- nomic paclitaxel are safe, exhibiting no cardiotoxic effects. The most dramatic antitumor effect is observed when paclitaxel is combined with either the HER-2 or VEGF mimics.

Results

Selection, design, synthesis and characterization of peptides. The VEGF peptide mimic residues, corresponding to aa 102–122 (numbered as 76–96 in the crystal structure), correspond to the overlapping binding sites on VEGF for VEGFR2 and avastin. Many FDA-approved humanized monoclonal antibodies that target HER-2 and VEGF have been associated with undesirable toxic profiles. Thus, novel targeted therapies that would to improve clinical outcome at the cost of limited toxicity are urgently required.

The main focus of our laboratory has been to develop HER-2-derived peptide vaccines that stimulate the immune system to produce high affinity antibodies exerting antitumor effects. Previously identified and designed B-cell epitopes from the HER-2 protein have successfully been translated into the clinic as candidate vaccines, combined as a chimeric construct with a “promiscuous” T-cell epitope. More recently, rather than harnessing the immune system to elicit native-like antitumor antibodies upon vaccination, we have embarked on a different, but related, strategy of interrupting ligand:receptor activation by engineered peptide mimics devoid of a T cell-stimulating moiety. We have validated this hypothesis by successfully demonstrating that VEGF peptide mimics with specific modifications are effective both in vitro and in vivo to block the VEGF:VEGFR2 pathway, thereby inhibiting angiogenesis. Similarly, the combination of a HER-2 and a VEGF peptide mimic has been shown to provide enhanced antineoplastic effects in a transplantable BALB/c tumor model. To further refine our immunotherapeutic strategies, we recently completed a combination study in which we immunized mice with the MVF-HER-2 (266–296) peptide vaccine, followed by the administration (on a weekly schedule) of VEGF peptide mimics, resulting in enhanced tumor growth prevention in transplantable tumor models.

One of the greatest challenges in anticancer immunotherapy today is to minimize toxicity and maximize efficacy. Thus, including bevacizumab. Many FDA-approved humanized monoclonal antibodies that target HER-2 and VEGF have been associated with undesirable toxic profiles. Thus, novel targeted therapies that would to improve clinical outcome at the cost of limited toxicity are urgently required.

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in reverse sequence 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.19 Antitumor effects of HER-2 peptide mimics are comparable to that of paclitaxel and trastuzumab.

The antitumor efficacy of the HER-2 peptide mimics was compared with that of paclitaxel, by treating female FVB/n mice (which are positive for the PyMT transgene) with 300 μg of the peptides, trastuzumab or paclitaxel. We found that both the L- and D-amino acid version of the HER-2 peptide mimics are able to inhibit tumor growth, with an efficacy that is comparable to that of paclitaxel and trastuzumab (Fig. 2A).

Since in this model mice develop multiple tumors, we measured only the size of the largest one (Fig. 2A) and we determined the percentage tumor weights (all tumors) after individual treatments with paclitaxel, trastuzumab and the peptide mimics. As shown in Figure 2A, both the L- and D-amino acid HER-2 peptide mimics were able to significantly inhibit tumor growth compared to the untreated control (p < 0.005).

**Figure 2.** Antitumor effects of HER-2 peptide mimics are comparable to that of paclitaxel and trastuzumab. Female Fvbn mice (n = 5) were treated intravenously with HER-2 peptide mimics, paclitaxel or trastuzumab. Mice were treated weekly from week 4 to 10 and tumor volume was measured twice weekly. All mice were sacrificed at week 11 and tumors extracted and weighed. All treatments significantly inhibited tumor growth with a p value of < 0.001 (A) and the effect was also evident on normalized tumor weight (B). Error bars represent standard deviations from the mean.

VEGF peptide mimics are shown in Figure 1. Briefly, the strategy to create a conformational peptide consisting of an anti-parallel β sheet is described elsewhere,9 where the sequence was modified in a way that the resulting peptide non-cyclized (NC) peptide VEGF-P3 adopted a conformation very similar to the native structure. It also required two artificial cysteines to be introduced between Gln79 and Gly92, and between Ile80 and Glu93. After synthesis and purification of NC VEGF-P3, disulfide bonds were formed by oxidation, enabling the formation of the twisted anti-parallel β-sheet structure in the cyclized (CYC) version of the peptide.17 The rationale behind retro-inverso (RI) peptidomics is that they should present comparable biological activity but higher bioavailability than their natural counterparts. The RI peptide analog VEGF-RI-P4 was synthesized using D-amino acids in reverse sequence 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.19

**Antitumor effects of HER-2 peptide mimics are comparable to that of paclitaxel and trastuzumab.** The antitumor efficacy of the HER-2 peptide mimics was compared with that of paclitaxel, by treating female FVB/n mice (which are positive for the PyMT transgene) with 300 μg of the peptides, trastuzumab or paclitaxel. We found that both the L- and D-amino acid version of the HER-2 peptide mimics are able to inhibit tumor growth, with an efficacy that is comparable to that of paclitaxel and trastuzumab (Fig. 2A). Since in this model mice develop multiple tumors, we measured only the size of the largest one (Fig. 2A) and we determined the percentage tumor weights (all tumors) after individual treatments with paclitaxel, trastuzumab and the peptide mimics. As shown in Figure 2A, both the L- and D-amino acid HER-2 peptide mimics were able to significantly inhibit tumor growth compared to the untreated control (p < 0.005).
Our results show that the treatment with individual HER-2 peptide mimics (P1 and P2) produced a significant reduction in tumor growth (p < 0.005). When the HER-2 peptide mimic was combined with low-dose paclitaxel, antitumor effect were further ameliorated (p < 0.001) (Fig. 4A). On the other hand, VEGF peptide mimics (P3 and P4) alone were able to inhibit tumor growth but the effects were not statistically significant (*p < 0.195). However, when combined with low-dose paclitaxel, an improved antitumor effect was observed (p < 0.001) (Fig. 4B). These results show that low-dose paclitaxel and VEGF peptide mimics alone had no significant effect on tumor growth, while significant anticancer activity was observed when the peptide mimics were combined with paclitaxel.

We were also interested in examining the antitumor effects of the combination of HER-2 and VEGF peptide mimics. As shown in Figure 4, treatment with individual HER-2 peptides (P1 and P2) had significant effects on tumor growth (*p < 0.005) while the VEGF peptide mimics (P3 and P4) showed no significant activity, though there was a delay in tumor development when compared to no therapy. The combination of HER-2 and VEGF peptide mimics (P1 + P3 and P2 + P4) produced greater antitumor effects as compared with individual HER-2 or VEGF peptide mimics (**p < 0.001). There was also a significant delay in the onset of tumor development in response to the combination therapy (around week 10) as compared with either monotherapies (around week 8.5). Next, we looked at the overall effects of the combination treatment on the tumor burden, by measuring tumor weight. Mice receiving both HER-2 and VEGF peptides showed tumor weight reduced to less than 10% of the control value, while mice treated with either peptide alone (Fig. 2B). Our results show that HER-2 peptides (P1 and P2) are able to cause a significant reduction in tumor weight (*p < 0.001) when compared with irrelevant peptides (Fig. 2B) or to no therapy. The administration of trastuzumab and paclitaxel reduced tumor weight comparably to HER peptide mimics.

**Cardiotoxic effects of HER-2 peptide treatment in comparison with trastuzumab and taxol.** One of the major drawbacks of current chemotherapeutic regimens is cardiotoxicity. Trastuzumab has been shown to cause cardiotoxic effects in a proportion of cancer patients.20,21 Along similar lines, studies have demonstrated the need to optimize the treatment regimen for paclitaxel due to numerous side effects as a result of its limited specificity. We therefore investigated the cardiotoxic effects of our peptide mimics and compared them with those of trastuzumab and paclitaxel. We measured the levels of cardiac troponin I in the serum, as collected by retro-orbital bleeding. Our findings indicate that trastuzumab and paclitaxel cause a significant increase in serum levels of cardiac troponin I (*p < 0.001) while HER-2 peptide mimics do not (*p = 0.25), as compared with no therapy (Fig. 3).

**Combination treatment with low-dose paclitaxel and HER-2 or VEGF peptide mimics exerts superior antitumor effects in a transgenic mouse model.** After confirming the cardiotoxic effects of paclitaxel and trastuzumab at the usual dose of 300 μg/mouse, we reduced the amount to a non-toxic dose of 60 μg/mouse (personal communication from Dr. Ginoula Clement). We hypothesized that combination regimens employing low-dose paclitaxel and peptide mimics would yield synergistic/additive antineoplastic effects in vivo in the absence of significant cardiotoxicity. In order to test this hypothesis, we used 60 μg of paclitaxel in combination with 300 μg of HER-2 or VEGF peptide mimics in the PyMT transgenic mouse model of human breast cancer.22 Our results show that the treatment with individual HER-2 peptide mimics (P1 and P2) produced a significant reduction in tumor growth (p < 0.005). When the HER-2 peptide mimic was combined with low-dose paclitaxel, antitumor effect were further ameliorated (p < 0.001) (Fig. 4A) pointing to an additive interaction. On the other hand, VEGF peptide mimics (P3 and P4) alone were able to inhibit tumor growth but the effects were not statistically significant (*p < 0.195). However, when combined with low-dose paclitaxel, an improved antitumor effect was observed (p < 0.001) (Fig. 4B). These results show that low-dose paclitaxel and VEGF peptide mimics alone had no significant effect on tumor growth, while significant anticancer activity was observed when the peptide mimics were combined with paclitaxel. We were also interested in examining the antitumor effects of the combination of HER-2 and VEGF peptide mimics. As shown in Figure 4, treatment with individual HER-2 peptides (P1 and P2) had significant effects on tumor growth (*p < 0.005) while the VEGF peptide mimics (P3 and P4) showed no significant activity, though there was a delay in tumor development when compared to no therapy. The combination of HER-2 and VEGF peptide mimics (P1 + P3 and P2 + P4) produced greater antitumor effects as compared with individual HER-2 or VEGF peptide mimics (**p < 0.001). There was also a significant delay in the onset of tumor development in response to the combination therapy (around week 10) as compared with either monotherapies (around week 8.5). Next, we looked at the overall effects of the combination treatment on the tumor burden, by measuring tumor weight. Mice receiving both HER-2 and VEGF peptides showed tumor weight reduced to less than 10% of the control value, while mice treated with either peptide alone...
exhibited tumor weight greater than 10% of the control value in the cases. The best result was obtained with the R1 HER-2 peptide and VEGF peptide mimics.

Antitumor effects of peptide mimics and paclitaxel in a transplantable mouse model. We evaluated the antitumor and antiangiogenic activities of the peptides alone or in combination with paclitaxel in a transplantable tumor model. To this aim, we used wild-type Balb/c mice that were challenged with TUBO cells, very aggressive tumor cells established from the Balb-neuT transgenic mice.23 Peptides alone were able to cause a delay in the onset of tumor development (*p < 0.001) (Fig. 5A and B), a delay that was increased when peptides were combined with paclitaxel. The degree of inhibition observed with the combination treatment was greater than that observed with either peptide or paclitaxel alone. We also examined the effects of these treatments on tumor weight, finding that single treatments (peptides or paclitaxel) caused a significant reduction of tumor burden (*p < 0.005), which was largely exacerbated in the case of the combination regimen (**p < 0.001) (Fig. 6). Mice treated with the combination of HER-2 and VEGF peptides or paclitaxel and either peptide showed a greater reduction in size (Fig. 7).

Combination treatment decreases the number of actively dividing cells in both transplanteable and transgenic mouse model of breast cancer. Paclitaxel mainly operates as a mitotic inhibitor.24,25 In order to clarify the antitumor mechanisms elicited by the HER-2 and VEGF peptide mimics and compare them with those of paclitaxel, we examined the effects of the different treatments on the number of actively dividing cells. Figures 8 and 9 show the amount of dividing cells in transgenic and transplantable models respectively, using Ki-67 staining. The number of cells greatly decreased in the case of single agent treatment, a decrease that was largely exacerbated by the combination regimen (p < 0.0001).

Combination treatment significantly decreases the microvascular density. To further clarify the mechanisms of action of HER-2 and VEGF peptide mimics, we examined vessel density in tumors using an anti-CD31 antibody. The number of microvessels positive for anti-CD31 staining in the tumors resected from mice treated with the peptides alone or in combination with paclitaxel was lower than that observed in control untreated tumors, in both models used in this study (p < 0.001) (Figs. 10 and 11).

Discussion

A plethora of anticancer agents have been developed to target tyrosine kinase receptors (RTKs), including antibodies against RTKs or their ligands and small molecule inhibitors that target RK intracellular kinase domain.26-28 Several FDA-approved therapies targeting both HER-2 (e.g., trastuzumab) and VEGF (e.g., bevacizumab) promoted significant toxic effects including cardiac dysfunction and congestive heart failure.6,7,29 Moreover, many patients treated with these drugs undergo disease progression due to development of resistance. The clinical application of humanized monoclonal antibodies is generally limited by a great number of concerns including the frequency of treatments, their costs, the limited duration of their effects, an undesirable immunogenicity, the development of acquired resistance, and substantial risks of cardiotoxic episodes.30 Similarly, small molecule RTK inhibitors such as sunitinib, which have entered clinical trials alone or in combination with radiotherapy or conventional chemotherapy, are overshadowed by problems of efficacy, development of resistance and undesirable safety profiles, which altogether impede their clinical progress.7 Despite the relative success of these drugs, there is an unmet need for novel molecular cancer therapeutics. Innovative therapies that target molecular pathways aberrantly activated only in cancer cells (thus exerting potent antineoplastic effects but low toxicity) are urgently needed.

The development of peptides that specifically block receptor-ligand interactions, thanks to structure-based design, constitute a promising avenue for the development of highly targeted anticancer therapeutics. The main objective of this strategy is to preserve the conformational integrity of the bioactive surface while retaining sufficient flexibility to cooperatively bind a given receptor. Such peptide mimics are water soluble, usually
released in the serum upon treatment (Fig. 3). The HER-2 peptide was associated with the lowest toxicity profile.

We postulated that combining low-dose paclitaxel with peptide mimics may yield additive and or synergistic effects in vivo with little or no cardiotoxicity. Many studies have also shown that low doses of chemotherapy are relatively non-toxic and yields greater antitumor effects when combined with other anticancer interventions, such as radiation therapy. Results obtained with the combination of low-dose paclitaxel with either HER-2 or VEGF peptide mimics point to increase antitumor effects in vivo as compared with single treatments (Fig. 4A and B). Low doses of paclitaxel alone or in combination with peptide mimics did not increase cardiotoxicity (results not shown). These results illustrate the validity of using minimal doses of chemotherapy in combination with other anticancer agents.

In the present study, we have focused our efforts on the combination of HER-2 and VEGF peptide mimics accompanied by low doses of chemotherapy. First, we evaluated the antitumor effects of HER-2 peptides (P1 and P2) in comparison to that of paclitaxel and trastuzumab. We found that these treatments exert comparable antitumor effects (Fig. 2A and B). However, both paclitaxel and trastuzumab are associated with elevated cardiotoxicity, as demonstrated by the amounts of cardiac troponin I released in the serum upon treatment (Fig. 3). The HER-2 peptide was associated with the lowest toxicity profile.

Figure 5. Antitumor effects of combination treatment with HER-2 and VEGF peptide mimics in a transgenic mouse model. Combination treatment with both HER-2 and VEGF peptide mimics (P1 + P3 and P2 + P4) cause a greater inhibition of tumor growth (**p < 0.001) while treatment with HER-2 peptides alone was associated with a less significant effect (*p < 0.005) when compared with no therapy (A). All treatments caused a significant reduction in percentage tumor weight (*p < 0.001) as shown (B). Error bars represent standard deviation of the mean.

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Figure 6. For figure legend, see page 1011.
observed when paclitaxel was combined with the VEGF, rather than with HER-2, peptide mimics. The D-amino VEGF peptide in combination with paclitaxel showed the greatest antitumor effects, with 20% tumor-free animals at the end of the experiment (Figs. 6 and 7). Tumor growth was relatively slower in mice subjected to the combination treatment than in mice receiving peptide mimics as single agents (Fig. 6). Tumors extracted from mice treated receiving the combination therapy also showed decreased vascularization (Fig. 7). Thus, our data indicate that the combination of paclitaxel and HER-2 or VEGF peptide mimics treatment causes a consistent decrease in tumor burden.

The effect of such a combination treatment in a transplantable tumor model was similar to that observed in the transgenic model, further validating our strategy. Low-dose paclitaxel in combination with HER-2 or VEGF peptide mimics caused a greater inhibition of tumor growth and development than either of these interventions alone (Figs. 6 and 7). Immunohistochemical studies showed a consistent reduction in the amount of actively dividing cells and microvascular density in mice receiving the combination treatment as compared with animals receiving monotherapy, in both transgenic and transplantable models (Figs. 8 and 9). These results illustrate that minimal doses of paclitaxel in combination with HER-2 and VEGF peptide mimics additively inhibits cancer cell growth. Similar additive effects were evident on the inhibition of microvessel density, as determined by immunohistochemistry upon staining with an anti-CD31 antibody. Tumor growth and metastasis is highly dependent on increased microvascular density (for the supply of oxygen and nutrient) and the reduction of blood vessels is very important for antitumor responses. In both the transgenic and transplantable models, microvascular density was greatly decreased when the combination treatment was used (Figs. 10 and 11), indicating that the inhibitors, when used in combination, were able to prevent tumor growth by affecting vascularization.

In conclusion, our results illustrate that HER-2 and VEGF peptide mimics exert potent antitumor effects when combined with both morphological characteristics and biochemical phenotype. Tumor growth and development in this model results from the overexpression of HER-2 and VEGF, eventually promoting metastasis. In the other model, we used TUBO cells, which overexpress HER-2/neu. Tumor growth and metastasis in this model also depends on the elevated expression of HER-2 and VEGF. Therefore, both these models are adequate to test the validity and the efficacy of the HER-2 and VEGF peptide mimics.

In order to assess any side effects due to treatment, we examined cardiotoxic effects as induced by the HER-2 peptides (in comparison with paclitaxel and trastuzumab) by measuring serum levels of cardiac troponin I after treatment. The administration of paclitaxel or trastuzumab, but not that of HER peptide mimics, induced significant cardiotoxicity. We next evaluated the effects of individual HER-2 and VEGF peptide mimics vs. combination regimens based on equivalent peptide amounts and we obtained significant inhibition of tumor growth in vivo (*p < 0.001) as compared with individual treatment with HER-2 peptides (*p < 0.005) (Fig. 5A). We also compared peptide mimics, individually taken, with paclitaxel and our results were similar in all cases (*p < 0.01) (Fig. 5B). Of note, the combination of HER-2 and VEGF peptide mimics exerts no significant cardiotoxicity. Actually, the best results were
with low-dose paclitaxel. The advantages of our strategies are that these peptides are generally safe, and this safety profile remains unchanged even when peptides are combine with low-dose paclitaxel. In the case of combining HER-2 with VEGF peptide mimics, d-amino acid peptides had better efficacy than their l-amino acid counterparts. These results are consistent with our hypothesis that that the d-amino acid derivatives should be more effective than their l-counterparts due to their greater stability in vivo. We conclude that combining peptide mimics with low-dose chemotherapy may offer a beneficial alternative to standard regimens in the clinical practice. Combination treatments targeting angiogenesis and metronomic chemotherapy may result in better patient survival and little toxicity. There is an urgent need for safe combination approaches that enhance antitumor immunity by targeting tumor-associated antigens and molecules involved in tumor angiogenesis. To circumvent the development of resistance in targeted therapy, strategies aimed at combining inhibition of compensatory pathways may offer substantial advantages in the future. Our work in that respect is aimed at evaluating the combinatorial inhibition of HER-1, HER-3 and the insulin growth factor receptor (IGFR) for the therapy of intractable cancers.

**Materials and Methods**

**Drugs.** Paclitaxel was purchased from the Ohio State University Pharmacy and trastuzumab was a kind gift from Dr. William Carson.

**Synthesis and characterization of conformational peptides.** Peptide synthesis was performed on a Milligen/Biossearch 9600 peptide solid phase synthesizer (Bedford, MA) using Fmoc/r-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/g) (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, and in the VEGF peptides two cysteines were inserted between amino acid Gln79 and 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. Intramolecular disulphide bonds were formed using iodine oxidation as described and disulphide bridge formation was further confirmed by maleimide-PEO2-biotin reaction and subsequent analysis using electrospray ionization mass spectroscopy. All fractions were analyzed on analytical RP-HPLC and characterized by MALDI (Matrix Assisted Laser Desorption Ionization mass spectroscopy) at CCIC (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, Ohio). RP-HPLC fractions showing same mass spectrum peak were pooled together and lyophilized. RP-HPLC pure peptides MVF-VEGF-P3, VEGF-P3 containing two Cys residues in each peptide were cyclized using acetic acid-iodine method, further purified on RP-HPLC and characterized by mass spectroscopy using established protocol as reported earlier. Amino acid sequences and molecular weight of all peptides and their molecular weights are shown in Figure 1.

Cardiototoxicity studies. Groups of female mice (n = 5) that are heterozygous for the PyMT transgene were treated intravenously with 300 μg of either trastuzumab, paclitaxel, HER-2 α-amino acid peptide or the HER-2 β-amino acid peptide. Treatment was started from week 4 till week 11 and tumor sizes were measured twice weekly using calipers and tumor...
In the combination studies groups of tumor bearing mice (n = 5) received a combination treatment of 60 μg of paclitaxel + 300 μg 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 × width²/2.

In vivo antitumor studies in 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 × 10⁷ TUBO cells. On the day of TUBO challenge, the mice were intravenously treated with 100 μg of either HER-2 or VEGF peptides or 20 μg of paclitaxel. In the combination studies groups of tumor bearing mice

**Figure 10.** Combination treatment decreases microvascular density in tumors in transgenic mouse model. Evaluation of vessel density in tumor sections. (A) Vascular staining using anti-CD31 antibody. (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.
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. For immunohistochemical analysis, a non-parametric Kruskal-Wallis test was used because of the small sample size and lack of normality in the distribution. The p values were adjusted using the Holm’s procedure to conserve the overall Type I error rate at 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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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. For immunohistochemical analysis, a non-parametric Kruskal-Wallis test was used because of the small sample size and lack of normality in the distribution. The p values were adjusted using the Holm’s procedure to conserve the overall Type I error rate at 0.05.

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