ANGIOTENSIN-(1-7): A PEPTIDE HORMONE INHIBITOR FOR THE TREATMENT OF BREAST CANCER TARGETING THE TUMOR MICROENVIRONMENT

BY

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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>[D-Ala$^7$]-Ang-(1-7)</td>
<td>[D-alanine$^7$]-angiotensin-(1-7)</td>
</tr>
<tr>
<td>μg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin converting enzyme 2</td>
</tr>
<tr>
<td>AIIs</td>
<td>Aromatase inhibitors</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>Angiotensin-(1-7)</td>
</tr>
<tr>
<td>Ang-(1-5)</td>
<td>Angiotensin-(1-5)</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blockers</td>
</tr>
<tr>
<td>ATAC</td>
<td>Anastrozole and Tamoxifen alone or in combination study</td>
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<tr>
<td>AT$_{(1-7)}$R</td>
<td>Angiotensin type (1-7) receptor</td>
</tr>
<tr>
<td>AT$_1$R</td>
<td>Angiotensin type I receptor</td>
</tr>
<tr>
<td>AT$_2$R</td>
<td>Angiotensin type II receptor</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CCCWFU</td>
<td>Comprehensive Cancer Center of Wake Forest University</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Ductal Carcinoma <em>In Situ</em></td>
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<td>DOCA</td>
<td>Deoxycorticosterone acetate</td>
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<td>DUSP1</td>
<td>Dual specificity phosphatase 1</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median effective concentration</td>
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<td>Extracellular Matrix</td>
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<td>Epidermal Growth Factor</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>EP</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt; receptors</td>
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<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
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<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FBS</td>
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<td>FGF-1</td>
<td>Fibroblast growth factor-1</td>
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<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>GFR</td>
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<td>GPR30</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>H</td>
<td>Hour</td>
</tr>
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<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>IL-13</td>
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<td>IGF</td>
<td>Insulin like growth factor</td>
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<td>IGF-1R</td>
<td>Insulin like growth factor type 1 receptor</td>
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<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
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<td>KIM</td>
<td>Kinase interaction motif</td>
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<tr>
<td>Kg</td>
<td>Kilogram</td>
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<tr>
<td>LCIS</td>
<td>Lobular Carcinoma In Situ</td>
</tr>
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<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<td>MAPKK</td>
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<tr>
<td>MAPKKK</td>
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<tr>
<td>MKP</td>
<td>Mitogen-activated protein kinase phosphatase</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Nuclear factor-κβ</td>
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<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<td>Non-small cell lung cancer</td>
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<tr>
<td>PARP</td>
<td>poly ADP-ribose polymerase</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<td>PGI₂</td>
<td>Prostacyclin</td>
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<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PGIS</td>
<td>Prostaglandin I synthase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatase inhibitors</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PlGF</td>
<td>Placental Growth Factor</td>
</tr>
<tr>
<td>POP</td>
<td>Prolyl oligopeptidase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RDU</td>
<td>Relative density units</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor node metastases</td>
</tr>
<tr>
<td>TOP</td>
<td>Thimet oligopeptidase</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
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</table>
ABSTRACT

Cook, Katherine Loree

ANGIOTENSIN-(1-7): A PEPTIDE HORMONE INHIBITOR FOR THE TREATMENT OF BREAST CANCER TARGETING THE TUMOR MICROENVIRONMENT

Dissertation under the direction of E. Ann Tallant, Ph.D., Professor and Patricia E. Gallagher Ph.D., Associate Professor

In 2009, 192,000 new cases of breast cancer were diagnosed in the United States and over 40,000 American women died from breast cancer, emphasizing the severity of the breast cancer epidemic. Recent advances in molecular profiling of breast tumors have identified three clinically-relevant subcategories of breast cancer: estrogen receptor (ER) positive, human epidermal growth factor receptor 2 (HER2) amplified, and triple negative (lacking the ER, the progesterone receptor (PR), and with normal expression of HER2) breast cancers. ER positive breast cancer is the most prevalent type of breast cancer accounting for over 70% of breast cancer cases while HER2-amplified breast cancer comprises an additional 20-25% of breast cancer cases. Drugs targeting estrogen biosynthesis, estrogen signaling, or HER2 amplification were developed and used for the treatment of breast cancer; however, drug resistance and adverse side effects have limited their use, emphasizing the need for new targeted drugs to treat breast cancer. The purpose of the studies described in this dissertation was to determine whether angiotensin-(1-7) [Ang-(1-7)], an endogenous seven amino-acid peptide hormone of the renin-angiotensin system, inhibits the growth of HER2-amplified and ER-positive orthotopic breast tumors and to identify the molecular mechanisms for the Ang-(1-7)-mediated reduction in tumor growth.
BT-474, which express the ER receptor and over-express HER2, and ZR-75-1 ER positive cells were injected into a mammary fat pad of athymic mice. Tumors were allowed to grow to a uniform size, 200 mm$^3$ and 100 mm$^3$ for BT-474 and ZR-75-1, respectively, followed by treatment with Ang-(1-7) or saline for 18 days, to determine the effect of the drug on tumor growth. Ang-(1-7) administration reduced BT-474 and ZR-75-1 tumor volume by 68.2% and 77.0%, and tumor weight by 38.9% and 50%, respectively, indicating that the heptapeptide inhibits breast tumor growth. The Ang-(1-7)-mediated reduction in tumor growth was associated with a significant decrease in interstitial fibrosis, from 4.9 ± 1.0%/field to 1.2 ± 0.2%/field in BT474 tumors and from 23.3 ± 2.4%/field to 8.3 ± 0.8%/field in ZR-75-1 tumors, and a 66% reduction in collagen I deposition. Treatment with Ang-(1-7) also decreased perivascular fibrosis in BT-474 tumors, from 49.3 ± 3.2% fibrosis/vessel compared to 13.4 ± 2.2% fibrosis/vessel, demonstrating that the heptapeptide reduced both intratumoral and perivascular fibrosis. These results demonstrate that Ang-(1-7) inhibits both the growth and the tumor-associated fibrosis of HER2-amplified and ER positive breast cancer.

Fibroblasts were isolated from ZR-75-1 orthotopic breast tumors, to identify the molecular mechanisms for the anti-fibrotic response to Ang-(1-7) in breast cancer. Ang-(1-7) markedly attenuated the growth of tumor-associated fibroblasts, in association with a 36% reduction in the extracellular matrix protein fibronectin and a 44% decrease in active transforming growth factor-β (TGF-β), which plays a major role in the production of extracellular matrix proteins. The heptapeptide significantly increased the mitogen-activated protein (MAP) kinase phosphatase DUSP1 in tumor-associated fibroblasts, by 252 ± 29% compared with untreated cells, with an associated 50% decrease in activities
of the MAP kinases ERK1 and ERK2 [1.13 ± 0.23 relative density units (RDU) in untreated cells compared 0.60 ± 0.06 RDU in Ang-(1-7)-treated cells for pERK1, and 2.27 ± 0.56 RDU in untreated cells compared to 0.84 ± 0.08 RDU in Ang-(1-7)-treated cells for pERK2; n = 3-4; p < 0.05].  These results suggest that Ang-(1-7) increases DUSP1 and reduces MAP kinase activities to inhibit the proliferation of tumor-associated fibroblasts and the production of extracellular matrix proteins.

Ang-(1-7) significantly reduced cell proliferation in ZR-75-1 breast tumors, with a 61% reduction in Ki67 immunoreactivity.  Moreover, Ang-(1-7) treatment markedly decreased ERK1/2 MAP kinase activities [for pERK1 from 0.51 ± 0.13 RDU in tumors from mice treated with saline to 0.16 ± 0.06 RDU in tumors treated with Ang-(1-7) and for pERK2 from 1.17 ± 0.33 RDU following treatment with saline to 0.30 ± 0.07 RDU following Ang-(1-7) treatment, p < 0.05; n = 5-6].  The decrease in phospho-ERK activities was accompanied by an increase in the MAP kinase phosphatase DUSP1 [from 0.23 ± 0.03 RDU in tumors from mice treated with saline to 0.51 ± 0.06 RDU in mice treated with Ang-(1-7), p < 0.05; n = 5-6].  Ang-(1-7) treatment of ZR-75-1 cells also increased DUSP1, by 2-fold compared to the amount of the immunoreactive protein in untreated cells, in agreement with a reduction in serum-stimulated ERK1/2 activities in cells treated with the heptapeptide, suggesting that Ang-(1-7) up-regulation of DUSP1 reduced phospho-ERK1/2.  DUSP1 was reduced in ZR-75-1 cells, by transfection with siRNA to DUSP1, decreasing immunoreactivity by 60%.  The reduction in DUSP1 prevented the Ang-(1-7)-mediated decrease in phospho-ERK1/ERK2, demonstrating that the heptapeptide up-regulates the MAP kinase phosphatase DUSP1 to reduce phospho-ERK1/ERK2 and subsequent effects of ERK signaling in ER positive breast cancer cells.
Ang-(1-7) administration also decreased cyclooxygenase 2 (COX-2) and prostaglandin E synthase (PGES) in ZR-75-1 orthotopic tumors, with no effect on prostacyclin synthase (PGIS), suggesting that the heptapeptide alters the ratio of proliferative to anti-proliferative prostaglandins. COX-2 and PGES were also reduced in ZR-75-1 cells treated with the heptapeptide. Co-treatment of ZR-75-1 cells with the serine/threonine phosphatase inhibitor okadaic acid and the tyrosine phosphatase inhibitor sodium vanadate prevented the Ang-(1-7)-mediated down-regulation of PGES, suggesting Ang-(1-7) up-regulates a phosphatase to reduce PGES expression. Moreover, transfection of ZR-75-1 cells with siRNA to DUSP1 ablated the Ang-(1-7)-mediated reduction in PGES, demonstrating that Ang-(1-7) up-regulates the MAP kinase phosphatase DUSP1 to reduce ERK1/2 MAP kinase activities and PGES.

A recently completed Phase I clinical trial on the use of Ang-(1-7) for the treatment of solid tumors demonstrated clinical benefit in 4 of 15 evaluable cancer patients. Since Ang-(1-7) reduced proliferation, inflammation and fibrosis in ZR-75-1 ER-positive and BT-474 HER2-amplified breast cancer cells and tumors, these results suggest that Ang-(1-7) may be an effective, first-in-class compound for the treatment of breast cancer by reducing tumor growth and targeting the reactive tumor microenvironment.
Chapter I: Introduction

The Renin-Angiotensin System and Angiotensin-(1-7)

The renin-angiotensin system (RAS) plays a central role in the control of blood pressure homeostasis, naturesis, and cardiovascular function (1). As shown in Figure 1, the RAS pathway is activated when angiotensinogen, the inactive precursor protein, is cleaved by the enzyme renin to form the decapeptide angiotensin I (Ang I). The pathway diverges in the catabolism of Ang I. Ang I can be processed by angiotensin-converting enzyme (ACE) to form the active eight amino acid peptide, angiotensin II (Ang II), or Ang I can be converted by neprilysin, prolyl-oligopeptidase (POP), or thimet oligopeptidase (TOP) to angiotensin-(1-7) [Ang-(1-7)]. ACE catabolizes the active heptapeptide Ang-(1-7) to the inactive degradation product Ang-(1-5) (2). The two arms of the pathway converge through the enzymatic actions of angiotensin-converting enzyme 2 (ACE2) that cleaves one amino acid from Ang II to form Ang-(1-7). The dissimilar carboxyl-terminal residues of Ang II and Ang-(1-7) lead to different receptor binding and diverse physiological activities (3).

The biological activity of Ang II is mediated by two distinct G protein-coupled receptors; angiotensin type 1 and angiotensin type 2 receptors (AT1R and AT2R). The majority of Ang II activities such as vasoconstriction, stimulation of growth, and the promotion of fibrosis are mediated by AT1R activation. The formation and physiological properties of Ang II are well established and widely studied due to the role of Ang II in hypertension and cardiovascular control. ACE inhibitors that reduce circulating levels of
Figure 1: The renin-angiotensin system. The inactive precursor protein, angiotensinogen, is converted by the enzyme renin to the decapeptide angiotensin I (Ang I). Ang I is further processed by angiotensin converting enzyme (ACE) into angiotensin II (Ang II). Ang I can also be converted through the action of neprilysin (NEP), prolyl-oligopeptidase (POP), and thimet-oligopeptidase (TOP) into the seven amino acid peptide, angiotensin-(1-7) (Ang-(1-7)). The two arms of the pathway converge by the proteolytic cleavage of Ang II by angiotensin converting enzyme 2 (ACE2) into Ang-(1-7). Ang II biological activities are mediated through the angiotensin II type I or type 2 receptor (AT₁R or AT₂R). Ang-(1-7) binds to a unique G-protein coupled receptor encoded by the mas gene (AT₁(1-7)R or mas) to exert its biological properties.
Angiotensinogen

Renin

Ang I

ACE

Ang II

ACE2

Ang-(1-7)

Neprilysin POP, TOP

AT\(_1\)R

AT\(_2\)R

Vasoconstriction
Proliferative
Pro-Fibrotic

PE

AT\((1-7)\)R

Vasodilation
Anti-Proliferative
Anti-Fibrotic
Ang II and angiotensin receptor blockers (ARB) that prevent binding of the peptide were developed to treat high blood pressure, highlighting the important role of the RAS in cardiovascular disease.

Ang-(1-7) is a seven amino acid circulating hormone of the RAS. Ang-(1-7) was originally thought to be an inactive degradation product of the RAS. However, studies showed that the vasodilatory, anti-proliferative, and anti-fibrotic properties of Ang-(1-7) counteract many of the actions of Ang II (4). Further evidence of the biological activity of the heptapeptide resulted from the discovery of a unique Ang-(1-7) receptor. Ang-(1-7) exerts its biological activity through the G protein-coupled Ang-(1-7) receptor, encoded by the mas gene. Santos et al. showed that Ang-(1-7) binds to the mas receptor with high affinity. Moreover, $^{125}$I-Ang-(1-7) binding and the functional response to the heptapeptide were lost in mas knockout mice, demonstrating that mas functions as an Ang-(1-7) receptor (5). Activation of the mas receptor by Ang-(1-7) elicits downstream G-coupled receptor signaling and results in an increase in nitric oxide (NO) (6). The release of NO by Ang-(1-7) is involved in many of the physiological effects of the heptapeptide, such as vasodilation, growth, and angiogenesis.

Ang-(1-7) also plays a role in homeostatic maintenance of various organ systems and the response to stress. For example, studies in the heart show that coronary ligation results in an increase in Ang-(1-7) levels (7). In addition, Ang-(1-7) improves cardiac function in response to ischemia/reperfusion and protects against ischemic-induced cardiac arrhythmia in isolated rat hearts (8-10). The heptapeptide was also cardioprotective; chronic Ang-(1-7) treatment reduced the development of heart failure in response to coronary artery ligation in rats (11). In other major organs such as the
kidney, Ang-(1-7) produced natriuretic and diuretic effects with corresponding increases in glomerular filtration rate (GFR) (12, 13). In studies conducted with [mRen2]27 transgenic rats and salt-depleted Sprague-Dawley transgenic rats, intrarenal Ang-(1-7) blockade caused a decrease in GFR, renal plasma flow, and sodium excretion (14). Expression of Ang-(1-7) was found in brain regions, including the hypothalamus, medulla oblongata, and amygdale (15). The main actions of Ang-(1-7) on the brain are the regulation of baroreceptor reflex and neural control of the homeostatic blood pressure. Ang-(1-7) augmented the gain of the baroreflex control of heart rate (16).

Perhaps the most well known physiological effect of Ang-(1-7) are those exerted in the vasculature; Ang-(1-7) produced endothelium-dependent dilation of the coronary arteries of pigs and dogs, which was mediated by NO and bradykinin (17, 18). Moreover, ACE inhibition resulted in vasodilation mediated in part through an increase in Ang-(1-7) (19). The regulation of vasodilation in rats by Ang-(1-7) was inhibited by administration of indomethacin, a non-steroidal anti-inflammatory drug (NSAID), suggesting Ang-(1-7) modulation of prostaglandins during vasodilation (20). These studies highlight the effect of Ang-(1-7) in the homeostatic regulation of various organ systems, but also demonstrate that pharmacological manipulation of the RAS may be beneficial in the treatment of many diseases.

**Ang-(1-7) Anti-proliferative Properties**

Freeman et al. were the first to demonstrate that the heptapeptide had anti-proliferative properties. Treatment of vascular smooth muscle cells (VSMCs) with Ang-(1-7) decreased mitogen-stimulated vascular growth in a dose-dependent manner with an
EC50 in the nanomolar range and a maximal effect at the 1 µM (21). The reduction in DNA synthesis by Ang-(1-7) was completely blocked by pretreatment with [D-Ala7]-Ang-(1-7), a receptor antagonist for the angiotensin-(1-7) receptor (AT(1-7)R), indicating that the anti-proliferative properties of the heptapeptide were mediated through a specific AT(1-7)R (1). The molecular mechanisms of growth reduction by the heptapeptide included stimulation of prostacyclin production and an increase in cAMP to activate the cAMP-dependent protein kinase (22).

The anti-proliferative properties of Ang-(1-7) were investigated in vivo in a model of vascular injury where the rat left carotid artery was denuded using a balloon catheter. Ang-(1-7) infusion increased the circulating levels of the heptapeptide three-fold, which attenuated neointimal formation in the carotid artery following injury (23). Administration of the heptapeptide had no apparent effects on the underlying media layer, indicating that the anti-growth properties of Ang-(1-7) only affected proliferating cells (23). In support of these findings, treatment of rats with Ang-(1-7) following the implantation of stents into the aortas significantly reduced neointimal thickness and the percentage of stenosis when compared to saline-treated animals, demonstrating the anti-proliferative properties of the hormone (24).

The heptapeptide decreased mitogen-stimulated protein synthesis in cardiac myocytes, an effect that was blocked by pre-treating with the AT(1-7)R antagonist [D-Ala7]-Ang-(1-7), suggesting that the anti-growth properties of Ang-(1-7) in cardiac myocytes are mediated by a specific AT(1-7) R (25). This is in agreement with studies by
Loot et al., demonstrating that Ang-(1-7) reduced myocyte cross-sectional area following treatment with the heptapeptide for 8 weeks (11). Moreover, Ang-(1-7) significantly reduced DNA synthesis in isolated cardiac fibroblasts, illustrating the anti-proliferative properties of Ang-(1-7) in multiple cell types (22, 25).

**Anti-fibrotic Effects of Ang-(1-7)**

Numerous studies demonstrated the role of Ang-(1-7) as an anti-fibrotic agent in the treatment of hypertension-induced cardiac fibrosis. Ang-(1-7) infusion prevented cardiac fibrosis in the deoxycorticosterone acetate (DOCA)-salt-induced model of hypertension in male Sprague-Dawley rats (26). Infusion with the heptapeptide resulted in a significant decrease in left ventricular wall fibrosis and reduced perivascular fibrosis in DOCA-treated rats, suggesting that Ang-(1-7) counteracts cardiac fibrosis induced by chronic increases in blood pressure (26). In isolated cardiac fibroblasts, Ang-(1-7), through binding to a specific non-angiotensin type 1 receptor or non-angiotensin type 2 receptor (non-AT₁R or non-AT₂R), receptor reduced collagen formation as well as transforming growth factor-β (TGF-β), endothelin-1, and leukemia inhibitory factor, demonstrating possible molecular mechanisms of the Ang-(1-7)-mediated inhibition of cardiac fibrosis (27). Santos et al. used a mas-deficient knockout mouse to further elucidate the anti-fibrotic effects of Ang-(1-7). Mas receptor knockout mice have impaired cardiac function with increased deposition of collagen I, collagen III, and fibronectin, suggesting that the loss of mas increases cardiac fibrosis (28). In the mouse kidney, mas receptor deletion resulted in a marked increase in the deposition of collagen III, collagen IV, and fibronectin, which are all markers of fibrosis (29). Furthermore,
studies by our group showed that Ang-(1-7) infusion reduces cardiac fibrosis in an Ang II-stimulated model of hypertension. Taken together, these studies clearly indicate that the heptapeptide is an anti-fibrotic agent (30).

**Implications of the RAS in Cancer**

As the most extensively characterized peptide in the RAS, the involvement of Ang II in various diseases has been studied. Dysregulated RAS components such as decreased ACE expression, elevated Ang II receptors, and increased pro-renin are found in different types of cancer, including breast cancer, suggesting a role for the RAS in cancer initiation and progression (31, 32). Moreover, studies investigating Ang II signaling in cancer cells indicate that the octapeptide stimulates breast epithelial proliferation by activation of protein kinase C (PKC) and the phosphorylation and activation of the MAP kinases ERK1/2 (33, 34). Preclinical studies investigated whether AT1 receptor blockers or ACE inhibitors, which either block Ang II binding or reduce plasma Ang II, might be used in the treatment of cancer. The AT1 receptor blockers candesartan or losartan were effective in reducing tumor growth in fibrosarcoma, metastatic lung cancer, ovarian cancer cells, prostate cancer cells, and C6 rat glioma (35-38). Administration of the ACE inhibitor perindopril in a model of head and neck squamous cell carcinoma reduced tumor development in a model of head and neck squamous cell carcinoma through a reduction in angiogenesis and inhibition of VEGF expression (39, 40). The results of these studies indicate that RAS components play an important role in the regulation of carcinogenesis and drugs such as ACE inhibitors and ARBs may prove beneficial in the treatment of cancer. Further evidence for the role of
the RAS in the development of cancer resulted from a retrospective epidemiological study comparing the relative cancer risk of 5207 patients administered different anti-hypertensive therapies. A significant reduction in the incidence of cancer in patients who were treated with ACE inhibitors was observed with the highest reduced risk in lung and gender-specific cancers (41). Treatment with ACE inhibitors increases circulating Ang-(1-7), by blocking the ACE-catalyzed degradation of Ang-(1-7) to Ang-(1-5) (42). This evidence, coupled with the anti-proliferative properties of Ang-(1-7) in actively growing VSMCs, cardiac fibroblasts, and cardiac myocytes, led to the investigation of Ang-(1-7) as a potential chemotherapeutic agent for the treatment of cancer (21, 22, 25).

The Effects of Ang-(1-7) in Cancer

A direct role of Ang-(1-7) in inhibiting cancer growth was first investigated by Gallagher and Tallant in vitro in three lung adenocarcinoma cell lines (43). Ang-(1-7) significantly reduced the time-dependent growth of SK-LU-1, A549 and SK-MES-1 lung cancer cells. Administration of the heptapeptide inhibited lung cancer cell DNA synthesis with EC_{50}s in the sub-nanomolar range. Moreover, blockade of the AT_{1,7} receptor by the specific antagonist [D-Ala^7]-Ang-(1-7) reversed the inhibitory effect on DNA synthesis observed with Ang-(1-7) treatment. The three lung cancer cell lines expressed mas mRNA and protein, suggesting that mas activation mediates the anti-proliferative response to Ang-(1-7). Treatment with other angiotensin peptides and receptor antagonists to the AT_1 or AT_2 receptors did not reduce DNA synthesis in lung cancer cells, demonstrating the specificity of the response to Ang-(1-7). Ang-(1-7) significantly reduced the phosphorylation of mitogen-activated protein (MAP) kinases, a
cell signaling enzyme involved in cell growth and proliferation, suggesting that the reduction in cell growth by the heptapeptide may be in part due to the decrease in MAP kinase activities.

The effect of Ang-(1-7) on lung cancer in vivo was first examined in A549 lung cancer xenografts in athymic mice. Infusion of Ang-(1-7) for 28 days significantly reduced the growth of human lung tumor xenografts when compared to the saline-treated counterparts (44). Furthermore, the heptapeptide markedly reduced the proliferation of human lung cancer cells as measured by decreased Ki67 immunoreactivity, indicating that a reduction in cell proliferation led to an inhibition of tumor growth. Menon et al. showed that Ang-(1-7) significantly reduced cyclooxygenase 2 (COX-2) mRNA and protein in treated A549 tumors suggesting that Ang-(1-7) inhibited lung cancer xenograft growth through a decrease in COX-2 and the production of proinflammatory prostaglandins.

Ang-(1-7) is also a potent anti-angiogenic agent in non-small cell lung adenocarcinoma (45). The administration of Ang-(1-7) to athymic mice bearing A549 human lung cancer xenografts for 30 days resulted in a reduction in tumor growth; histological assessment of tissue sections from mice treated with Ang-(1-7) or saline showed that tumors from animals treated with Ang-(1-7) had reduced vessel density, suggesting that the heptapeptide inhibited angiogenesis to reduce tumor growth. The reduction in vessel density was associated with a decrease in vascular endothelial growth factor (VEGF) expression in tumor extracts. Moreover, the reduction in VEGF by Ang-
(1-7) was mediated by the AT\textsubscript{(1-7)} receptor in human A549 lung cancer cells. Ang-(1-7) reduced human endothelial cell tubule formation as well as blood vessel branching in the \textit{in vivo} chick chorionic allantoic membrane, as shown in Figure 2. The reduction in tubule formation and branch point formation by Ang-(1-7) was reversed by co-treatment with a specific receptor antagonist, demonstrating that the anti-angiogenic effect of the heptapeptide is mediated through the activation of an AT\textsubscript{(1-7)} receptor.

A Phase I clinical trial of Ang-(1-7) as a single agent chemotherapeutic for the treatment of solid tumors was recently completed in the Wake Forest University Comprehensive Cancer Center (46). Adverse events reported in the study that may be linked to Ang-(1-7) include 10 Grade 2 toxicities with the most common complaint being musculoskeletal pain and fatigue, 2 Grade 3 toxicities of cranial neuropathy and thrombosis, that may be possibly linked to therapy was reported, and 1 Grade 4 toxicity consisting of cerebrovascular ischemia, suggesting limited side effects when administered to patients. Of the 18 patients enrolled, 15 were evaluable for clinical response with 3 patients showing stable disease for over 3 months and one patient for over 10 months. Moreover, the stable disease observed with Ang-(1-7) treatment was associated with a significant reduction in the pro-angiogenic factor, placental growth factor (PIGF), supporting the pre-clinical evidence that the heptapeptide is an anti-angiogenic agent. This result suggests that Ang-(1-7) may be a first-in-class chemotherapeutic agent for the treatment of cancer by targeting a specific AT\textsubscript{(1-7)} receptor, \textit{mas}. The goal of the studies described in this dissertation was to determine whether Ang-(1-7) reduced human breast
Figure 2. Ang-(1-7) inhibits angiogenesis. *In vivo* effects of Ang-(1-7) in the chicken embryo (left panel). Eggs were incubated for 4 days and then treated with media containing albumin (Control) with or without 100 nM Ang-(1-7). Neovascularization was quantified at day 6 by comparing pictures taken at the start and at the end of the treatment (n = 5, * denotes p < 0.05).
Control

Ang-(1-7)

Branch Points

Control Ang-(1-7) Control Ang-(1-7)

Control Ang-(1-7) Control Ang-(1-7)
Breast Cancer

The American Cancer Society estimated that almost 1.5 million new cases of cancer cases was diagnosed last year and that over half a million people in the United States died from cancer, making cancer the leading cause of death in Americans (both male and female) under the age of 85 (47). The same study estimated 192,000 new cases of breast cancer in the United States, which represents the most common type of diagnosed cancer among women (47, 48). Furthermore, breast cancer is the second highest killer of all cancer types in women, second only to lung and bronchial cancer, with more than 40,000 reported deaths in women in the United States last year (47). It is estimated that over 1.15 million new cases of breast cancer were diagnosed worldwide last year and resulted in over 411,000 deaths in women, making breast cancer the leading cause of cancer mortality in women worldwide (49).

Breast cancer is a complex disease with diverse clinical and pathological characteristics. The diversity in breast cancer led to the development of a classification system that separates breast cancers based upon location, size, grade, node involvement, metastases and genetic molecular expression profiling of the receptor status. Classification of breast tumors based upon the initial location of the neoplasm is used to determine whether the cancerous cells are contained in the ducts of the breast, known as ductal carcinoma in situ (DCIS), or in the lactiferous ducts, referred as lobular carcinoma in situ (LCIS). Inflammatory breast cancer is a rare type of tumor that results in the
formation of plaques that block lymph nodes, causing breast swelling and inflammation. The most common classification of breast cancer is the tumor, node, and metastases method of classification (TNM), which was created by the American Joint Commission on Cancer (50). TNM status is based upon tumor size as well as invasiveness and ranges from 0 to 4, where T refers to the primary tumor. The TNM grade is an important measure that predicts survival of breast cancer patients (51). A T0 grade shows no evidence of a primary tumor, while a tumor up to 2 cm in size is classified as T1. A T2 tumor is larger than 2 cm, but less than 5 cm in size and a T3 tumor is greater than 5 cm in size. T4 denotes a tumor of any size that has a direct extension to the chest wall or skin. A Tis tumor has not invaded the surrounding healthy tissue but is located in the ducts. This classification includes DCIS or LCIS. The node status is graded on a scale from N0 to N3, where an N0 tumor has no regional lymph node metastasis, N1 has metastasis to movable ipsilateral axillary lymph nodes, N2 has metastasis to ipsilateral axillary lymph nodes fixed to each other or to other structures, and N3 has metastasis to ipsilateral internal mammary lymph nodes. M0 denotes no distant metastasis, whereas M1 signifies the presence of distant metastasis (52).

A more recent method of breast cancer classification involves molecular profiling of the receptor status. This method determines the presence of estrogen receptor (ER) and progesterone receptor (PR) as well as amplification of the human epidermal growth factor 2 (HER2) receptor. This advancement in technology allows the division of breast cancer cases into four basic subcategories (53-55).
1. Luminal: Gene expression of the luminal breast cancer subtype is representative of the luminal epithelial component of the breast. The molecular profile of luminal breast cancer is characterized by expression of glandular cytokeratins 8/18, ER, and ER activation gene products including the cell cycle regulator, cyclin D1. Luminal breast cancer is further divided into two separate types: luminal A and luminal B. While both luminal A and luminal B breast tumors express estrogen receptors, there are defining characteristics that differentiate the two groups. Luminal A has higher ER-regulated gene expression and a lower proliferation index than luminal B breast cancer. Luminal B breast cancer may express an elevated level of HER2 in addition to ER and PR expression, and luminal B tumors have a significantly poorer prognosis than luminal A tumors. These tumor types are sensitive to hormonal therapies.

2. HER2 type: The cluster of gene expression profiles of this type of tumor is characterized by the over-expression of ERBB2 (erythroblastic leukemia viral oncogene homolog 2) and is co-localized and amplified with GRB7 and TRAP100 genes. HER2-positive breast cancer over-expresses ERBB2. HER2-amplified tumors are high grade, poorly differentiated tumors, have low or no hormone receptor expression, and are more likely to have axillary lymph node involvement than luminal A tumors. HER2-amplified tumors respond to targeted therapies such as trastuzumab (Herceptin), a monoclonal antibody that blocks the HER2 receptor, or lapatinib (Tykerb), which is a dual tyrosine kinase inhibitor that blocks signaling through HER2.
3. Basal-like: The gene expression signature of this type of tumor is similar to those expressed by cells of the basal layer of mammary ducts and glands. These tumors can be characterized by expression of markers for cytokeratin 5/6 and the epidermal growth factor receptor (EGFR). Basal-like breast cancer includes triple-negative breast cancers, which lacks the ER, do not express the PR, and have normal HER2 expression. Triple negative breast cancer predominately affects young women as well as minority women when compared to their Caucasian counterparts. There are no targeted therapeutic options for these patients and they are limited to cytotoxic chemotherapeutics.

4. Normal breast-like: This type of breast tumor has a profile of genes expressed by adipose tissue such as fatty-acid binding protein 4 and PPARγ and other non-epithelial cell types. These tumors also showed strong expression of basal epithelial genes and low expression of luminal epithelial genes.

As shown in Figure 3, these four groups represent the main subcategories of breast cancer classified by molecular profiling analysis. The bulk of this dissertation work involves investigation into possible chemotherapeutic agents for the treatment of luminal ER positive and HER2-amplified breast cancer. A more detailed pathological and molecular summary of these breast cancer subgroups is described in the following sections.
Figure 3: Molecular profiling of breast tumors has led to three distinct subcategories of breast cancer. Advances in molecular profiling of breast tumors have identified three main sub-categories of breast cancer based on receptor expression. Luminal breast cancers, accounting for 60-70% of all breast cancer cases, express the estrogen receptor (ER) and are sensitive to hormonal therapies such as aromatase inhibitors (AIs) or tamoxifen, but adverse side effects may limit their use in patients. Human epidermal growth factor receptor (HER)-2 over-expressing breast cancer, accounting for 20-25% of all breast cancer cases, are sensitive to HER2 targeting therapies such as Herceptin; however, toxicities limit their use in the clinic. Lastly, basal or triple negative breast cancer, accounting for the remaining 10-15% of breast cancer cases, lack the ER, the progesterone receptor (PR), and have normal expression of HER2, are an aggressive form of breast cancer with currently no targeted therapy options.
Adapted with permission from Sørlie T et al. PNAS 2003;100:8418-8423
**Estrogen Receptor-Positive Breast Cancer**

Luminal breast cancer is characterized by the expression of the ER and comprises 60-70% of all breast cancer cases, making ER-positive breast cancer the most diagnosed form of breast cancer. At least 10% of the tumor cells must stain positive for ER to classify a breast tumor as ER positive. Hormonal therapy is an option for a patient with this type of tumor, while it is harder to predict the response to hormonal therapy for patients when less than 10% of the tumor cells show positive immunoreactivity for the ER (56). ER-positive breast cancer is correlated with a higher survival rate when compared to ER-negative breast cancer; however, ER-positive breast cancer is more likely to reoccur than ER-negative breast cancer. ER signaling plays an important role in tumorigenesis, proliferation, and cell survival, which is described in the following section (53, 57, 58).

**Estrogen Signaling in ER-Positive Breast Cancer**

There are four main mechanisms by which estrogen signaling occurs in cancer cells. The classical mechanism of estrogen signaling is mediated by the binding of estrogen or 17β-estradiol to nuclear steroid receptors, either ER-α or ER-β, resulting in growth, proliferation, development, and regulation of homeostasis in different tissue types and organ systems. The binding of estrogen to its receptor results in the transposition of the receptor into the nucleus where it binds to the estrogen response element (ERE) and activates the transcription of estrogen responsive genes (58). The classical estrogen receptors, ER-α and ER-β, are encoded by different genes and have distinct expression patterns that depend on tissue type. ER-α is predominately expressed...
in the uterus, liver, kidney, and heart whereas ER-β is primarily found in the ovary, lung, bladder, prostate, gastrointestinal tract, and nervous system. However, ER-α and ER-β are co-expressed in the mammary gland, thyroid gland, bone, and adrenal glands (57). The widespread expression of estrogen receptor highlights the essential role of estrogen signaling in many cellular processes. Moreover, estrogen-regulated genes, such as cyclin D1, PR, cathepsin D, GATA-3, and c-myc, are important for cell survival and proliferation (59). Activation of estrogen signaling can also be mediated through a ligand-independent process where the ER is activated by crosstalk with other growth factors, such as epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1). These growth factors phosphorylate and activate the ER, which results in the transcription of ERE-regulated genes. The third pathway of estrogen bioactivity is an ERE-independent signaling mechanism, where the estrogen-bound ER interacts with Fos/Jun to activate AP-1-mediated gene transcription (60). Finally the fourth mechanism of estrogen signaling is known as estrogen’s non-genomic actions. Figure 4 illustrates estrogen signaling and targets of hormonal therapy. The non-genomic effects of estrogen, which occur when estrogen binds to estrogen plasma membrane receptors, trigger an immediate activation of multiple cell signaling pathways. For example, estrogen activates the cAMP/protein kinase A (PKA) pathway in pulmonary smooth muscle cells and PC12 cells through a calcium-dependent mechanism (61, 62). In endometrial cells and liver-derived hepatocytes, the non-genomic effects of estrogen increase DNA synthesis and stimulate cell cycle progression through phospholipase C (PLC)-dependent inositol triphosphate (IP3) production and protein kinase C (PKC)-α activation (63, 64). Furthermore, estrogen rapidly induces the activation of MAP kinase
signaling and the phosphatidylnsitol-3-OH kinase (PI3K)/serine/threonine protein kinase (AKT) pathway in mammary gland-derived MCF-7 breast cancer cells (57, 65, 66). A recently discovered plasma membrane receptor known as G-coupled protein receptor 30 (GPR30) binds estrogen and is responsible for non-genomic signaling upon estrogen binding. Studies in ER-negative breast cancer cells, such as the MDA-MB-231 and SK-BR-3 cell lines, demonstrate the over-expression of GPR30 in breast cancer cells, implicating non-genomic estrogen effects in these types of breast cancer cells (67, 68). A key stimulatory pathway activated through estrogen-mediated GPR30 signaling is the stimulation of EGFR and cAMP, suggesting a growth-promoting effect of estrogen in ER-negative breast cancer cells (67, 69). Moreover, in a study that screened the expression of GPR30 in over 320 human breast tumors, 60% of the tumors expressed GPR30 and co-expression of ER-α and GPR30 was detected in 40% of the cases, suggesting that a subpopulation of breast cancers express GPR30 but not ER-α. Supporting data showed that, out of 122 ER negative tumors, 50% were positive for GPR30 expression, suggesting that ER negative tumors may still respond to estrogen.

Options for the Treatment of Breast Cancer

Strategies for the treatment of breast cancer depends upon numerous factors such as disease stage, receptor status, the number or location of lymph nodes involved, and presence of metastatic disease (52). The initial treatment of breast cancer usually involves breast conservation surgery such as a lumpectomy or partial mastectomy where the primary tumor is removed and axillary nodes are inspected to detect cancerous cells. Other types of surgery include a total mastectomy where the breast with the tumor is
**Figure 4: ER and HER2 signaling and therapeutic targeting.** Estrogen receptor (ER) signaling and human epidermal growth factor receptor-2 (HER2) signaling play vital roles in breast cancer progression, making these signaling pathways attractive targets for drug development. There are four mechanisms of estrogen (E2) signaling. The first is the classical pathway where E2 binds to the ER to activate the estrogen response element (ERE) and regulates gene transcription. The second mechanism of E2 signaling is the ligand-independent activation of the ER, where mitogen-activated protein (MAP) kinase activation phosphorylates and activates the ER, independent of E2 binding. The third mechanism of E2 signaling actions is the ERE-independent pathway, where the E2/ER complex binds to Jun and Fos resulting in the activation of AP-1 mediated transcription. Fourth, in the acute or non-genomic pathway of E2 signaling, E2 activates MAP kinase and phosphoinositol-3-kinase (PI3K), through stimulation of G-protein coupled receptor 30 (GPR30). Aromatase inhibitors (AI) target the production of E2, while tamoxifen prevent the activation of the ER. Trastuzumab, a monoclonal antibody targeting HER2 prevents epidermal growth factor (EGF) activation of EGF receptor signaling.
removed or radical mastectomy where lymph nodes and chest wall muscles are removed together with the cancerous breast (52). Following surgery, radiation therapy may be recommended to eradicate any remaining cancerous cell. Radiation treatment reduces the risk of local reoccurrence and is recommended for patients who undergo breast-conserving surgery. The time line of treatment for breast cancer may also include adjuvant therapies, given with or without radiation, which are often recommended when the patient is at risk for disseminated disease (70). Adjuvant therapy may include the use of systematic or regional therapies that interfere with cancer cell survival and reduce the risk of breast cancer recurrence (71), such as DNA intercalating agents (cyclophosphamide or anthracyclines) or agents that interfere with cell structure such as taxanes. Depending on the receptor status, patients can undergo regimens of targeted therapies; patients whose breast tumors over-express HER2 can be treated with anti-HER2 antibodies and hormonal therapies can be used in patients with hormone (ER positive, PR positive) responsive tumors to prevent recurrence and dissemination of breast cancer cells.

**Adjuvant Therapy Targeting Estrogen Signaling**

Adjuvant therapy options for patients with ER-positive breast cancer include the use of targeted endocrine therapies such as selective estrogen receptor modulators (SERM) or aromatase inhibitors (AIs) (70). Figure 4 indicates the mechanism of action of tamoxifen and AIs in estrogen signaling. SERMs, which include tamoxifen and raloxifene (second generation SERM with fewer adverse side effects compared with
tamoxifen), are a group of chemical compounds that structurally resemble estrogens but have anti-estrogenic growth properties. Tamoxifen, the most commonly administered SERM, is referred to as a partial estrogen agonist and a partial estrogen antagonist. Tamoxifen exerts positive estrogenic effects on bone, serum lipid concentrations, and the endometrium, while concurrently having anti-estrogenic activity in the breast (72). The maximum benefits of tamoxifen are reached 5 years after treatment and include a 51% reduction in reoccurrence and a 28% reduction in the number of deaths during years 0-4.

The main mechanism of tamoxifen action occurs through inhibition of the C-terminal activation function-2 (AF-2) activity of the ER with no effect on N-terminal activation function-1 (AF-1) (73). Since the majority of ER signaling in the breast is a result of AF-2 activity resulting in ERE binding and transcription of ER regulated genes, tamoxifen acts as an anti-estrogen in breast epithelium tissue. However, in other tissues, such as the uterus, where the majority of estrogen signaling is a result of AF-1 activation, tamoxifen does not exert its anti-estrogenic effects.

AIs are also used as the first-line therapy in the treatment of postmenopausal women with advanced ER-positive breast cancer (74). AIs were developed to combat the local conversion of androgens to estrogens in the breast tissue. The conversion of androgen to estrogens is catalyzed by the aromatase enzyme and results in a 4- to 6-fold higher level of estrogens in the breast when compared to the serum levels of estrogens in postmenopausal women; the resulting estrogen levels are comparable to the estrogen levels in the breast tissue of premenopausal women (75). Additionally, the local estrogen concentration in breast tissue is higher in malignant carcinomas when compared with
normal breast tissue and over two-thirds of breast carcinomas contain aromatase activity, suggesting that aromatase may be a molecular target for the treatment of ER-positive breast cancer (76). The reversible inhibitor of aromatase, anastrozole, and the SERM, tamoxifen, alone or in combination (ATAC) trial showed that breast cancer patients did not significantly benefit from the combined treatment when compared to tamoxifen alone. However, treatment with anastrozole improved disease-free survival over tamoxifen treatment alone, indicating an improved efficacy of AI over tamoxifen (77). Clinical trials with patients on tamoxifen for 5 years, followed by treatment with the AI letrozole, showed significant survival benefits (78).

**Endocrine Therapy Resistance**

Given the complexity of the ER signaling mechanism, it is understandable that tamoxifen resistance can develop in patients undergoing therapy. Tamoxifen resistance occurs through many different mechanisms including mutations in the ER, ER phosphorylation by other growth factors, posttranslational modifications of the ER, and ER coactivators as mediators of resistance (73). Mutations in the ER receptor in breast cancer are rare; however, a screening of metastatic breast tumors identified three missense mutations which resulted in a mutant form of ER that was constitutively active in the absence of estrogen, in the presence of the ligand, or in the presence of tamoxifen (79). Patients who have this mutation are resistant to endocrine therapies. Resistance to endocrine therapy may also develop through ER phosphorylation by other growth factors. As previously described, growth factors such as EGF activate estrogen signaling in ovariectomized mice in the absence of the estrogen but not in ER knockout animals,
indicating that EGF stimulates estrogen signaling through the ER, which may lead to tamoxifen resistance (80, 81). Other cell signaling pathways, including the MAP kinases ERK1/2, phosphorylate the Ser118 residue of ER within the AF-1 region, which increases ER activity and results in ligand-independent activation of estrogen signaling that may result in drug resistance. Increased ERK1/2 activity was identified in several breast cancer cell lines derived from endocrine-resistant breast cancer. Moreover, increased ERK1/2 activity correlates with poorer quality and shorter duration of endocrine therapies in patients with decreased survival, implicating ERK1/2 activity as a mediator of endocrine therapy resistance (82, 83). A third important signaling pathway implicated in hormonal therapy resistance is the PI3K/Akt pathway. AKT, a protein kinase that is involved in cell survival and proliferation, phosphorylates the Ser167 residue of ER and results in ligand-independent activation of ER (84). Another possible mechanism of tamoxifen resistance could occur through the modulation of co-activator proteins. The nuclear receptor co-activator 3 (NCOA3) is a transcriptional co-activator protein that is involved in acylating histones to facilitate DNA synthesis, specifically ER-dependent transcription. NCOA3, also referred to as “amplified in breast cancer 1” (AIB1), was identified in 4 out of 5 of breast cancer cases and correlated with ER positivity (85). Over-expression of NCOA3 or AIB1 in experimental breast cancer models increased the agonist properties of tamoxifen, suggesting that amplification may contribute to tamoxifen resistance (86, 87). Furthermore, patients with elevated AIB1 levels had poorer disease-free survival, which is indicative of tamoxifen resistance (88).
If breast cancer reoccurs in patients after adjuvant SERM treatment or if adverse side effects inhibit the use of SERMs, patients may be given aromatase inhibitors (AIs). Since AI prevent the synthesis of estrogen, they overcome the tamoxifen resistance of ER positive tumors; however, even though AIs have limited cross-reactivity, tumors may also develop resistance through cellular mechanisms similar to those of SERMs, in particular tyrosine kinase receptor activation and MAP kinase signaling (75).

Based upon the number of mechanisms by which breast tumors can overcome the inhibitory effects of tamoxifen and AIs, it is not surprising that approximately 50% of ER-α-positive breast cancer cases develop endocrine therapy resistance. Thus there is a clear need for the development of new chemotherapeutic drugs for ER-positive breast cancer (89).

**Human Epidermal Growth Factor 2 Receptor (HER2)-Amplified Breast Cancer**

HER2-amplified breast cancer accounts for 20-25% of all breast cancer cases (54, 90). This type of tumor displays genetic amplification of the ERBB-2 gene. HER2 amplification is positively correlated with lymph node metastases and occurs in up to 60% of in situ carcinomas (91). HER2-amplified breast cancer is associated with poor disease-free survival and correlates strongly with the pathogenesis and pathology of breast cancer (92). Furthermore, HER2 over-expression is linked to increased metastatic incidents (93).
**HER2 Signaling in Breast Cancer**

The ERBB family consists of four genes with similar homology—ERBB (HER1, EGFR), ERBB-2 (HER2, NEU), ERBB-3 (HER3), and ERBB-4 (HER4)—and encode tyrosine kinase receptors. All ERBB receptors, with the exception of HER3, contain cytoplasmic tyrosine kinase activity, and, with the exception of HER2, bind specific ligands at their extracellular domain. The receptors form heterodimers once the ligand binds to the extracellular domain of these receptors. The ligand-bound receptors (HER1, HER3, or HER4) preferentially form heterodimers with HER2 which results in receptor phosphorylation, increased affinity for EGF family ligands, and regulation of the signal transduction pathways of the receptor (94). A key event that occurs upon dimerization is the induction of the tyrosine kinase activity that activates growth stimulatory pathways such as PI3K, resulting in AKT activation and cell survival, and MAP kinases, which results in proliferation and growth (92).

**Detection of HER2-Amplified Breast Cancer**

HER2-amplified breast cancer can be identified in the clinic using two methods: immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). IHC is the most widely used method in which tumor sections are incubated with a specific antibody against HER2. Positive immunoreactivity is then quantified on a scale of 0 (no detectable HER2 staining) to 3+ (high HER2 expression) (95). FISH analysis is more sensitive than IHC and detects HER2 gene amplification. IHC and FISH have about an 80% concordance rate. However, FISH improves the prediction of HER2-targeted therapy responsiveness (96). HER2 immunoreactivity occurs in a high percentage of
tumor cells at both the primary and metastatic tumor sites, indicating that HER2 therapy may target the majority of cancer cells within a patient (97).

**Trastuzumab, Targeted Therapy for HER2 Amplified Breast Cancer**

A monoclonal antibody targeting the HER2 protein, trastuzumab (Herceptin), was approved by the US Federal Drug Administration in 1998 as a targeted therapy for the treatment of HER2-amplified breast cancer. Trastuzumab is a recombinant humanized monoclonal antibody directed against the extracellular domain of the 185 kDa HER2 receptor protein and the cleaved HER2 NH-2 extracellular domain of 95 kDa, known as p95. This humanized antibody physically prevents homodimerization and heterodimerization of the receptor, antibody-dependent cell-mediated cytotoxicity, and endocytosis of the receptor (98), to inhibit PI3K, reduce MAP kinase activity, and decrease angiogenesis (99). The binding of trastuzumab to HER2 results in the activation of natural killer cells, a principle immune cell containing the Fc gamma receptor, which is involved in antibody-dependent cellular cytotoxicity and recognizes the Fc domain of trastuzumab, to initiate natural killer cell-mediated cancer cell lysis (100). Additionally, trastuzumab inhibits the activation of HER2 signaling, which results in decreased MAP kinase and PI3K activities, indicating that trastuzumab treatment results in reduced tumor cell proliferation and stimulation of pro-apoptotic pathways. The full-length HER2 receptor undergoes proteolytic cleavage into a 100 kDa extracellular domain and a 95 kDa truncated membrane-associated fragment. Trastuzumab inhibits the formation of p95, which has kinase properties, thereby inhibiting the growth-promoting properties of the activated kinase (101). Moreover, high serum levels of p95 are correlated with poor
prognosis and increased metastatic potential (102). Preclinical trials also identified trastuzumab as an anti-angiogenic agent; treatment with trastuzumab and paclitaxel reduced tumor microvessel density, normalized tumor vasculature, and improved tumor responsiveness (103).

Even with the multiple anti-tumorigenic properties of trastuzumab, the objective response rates associated with trastuzumab monotherapy range from only 12-34% with a median treatment time of 9 months, indicating that trastuzumab is not an effective monotherapy (104). Trastuzumab is currently administered to patients with HER2-amplified breast cancer in combination with either docetaxel or paclitaxel, which increases the response rates, time to disease progression, and overall survival when compared with trastuzumab therapy alone (105, 106). Even with the improvement in the response rates with combination therapy, the majority of patients that initially respond to trastuzumab-based treatment regimes become resistant within one year of therapy, highlighting the need for new therapeutic options for the treatment of HER2-amplified breast cancer (70, 107, 108).

**Trastuzumab Resistance**

The mechanisms of trastuzumab resistance include mutations in the HER2 receptor, increased activation of downstream HER2 signaling pathways, insensitivity due to enhanced IGF signaling, or reduced expression of cell cycle inhibitors. In a small percentage of lung cancer patients, the HER2 receptor had somatic mutations that altered the extracellular domain, inhibiting the recognition and/or binding of trastuzumab (109).
Cancers with high levels of activated AKT or MAP kinase are resistant to trastuzumab, indicating that elevated downstream HER2 signaling may result in trastuzumab insensitivity (110, 111). Another mechanism linked to trastuzumab resistance is the modulation of the cell cycle inhibitor p27\textsuperscript{kip1}; down-regulation of p27\textsuperscript{kip1} prevents trastuzumab-mediated cell cycle arrest. In studies using small interfering RNA, the reduction in p27\textsuperscript{kip1} expression in SK-BR-3 human HER2-amplified breast cancer cells blocked the G1 arrest normally mediated by trastuzumab (112). Trastuzumab resistance may also occur due to the up-regulation of IGF signaling mechanisms; increased IGF-1 receptor (IGF-1R) activity is positively associated with trastuzumab resistance in HER2-amplified breast cancer cells and over-expression of IGF-1R in SK-BR-3 HER2-amplified breast cancer cells resulted in the loss of the growth inhibitory effect of trastuzumab (113, 114).

Given the multiple mechanism of trastuzumab resistance, there is an increased focus on the development of effective chemotherapeutic agents to treat HER2-amplified breast cancer. One mechanism to overcome resistance is through the development of pharmacological small molecule inhibitors that dually target HER1 and HER2 tyrosine kinase activities. One of these types of drugs is lapatinib, which is the only other FDA approved targeted therapy for HER2-amplified breast cancer. Lapatinib is currently administered to patients that demonstrate disease progression after the combination therapy of trastuzumab and taxane or anthracycline (99). Other agents such as pertuzumab bind to HER2 to prevent dimerization and HER2 signaling. However,
resistance may still occur with these newer drugs, emphasizing the need for additional therapies to treat HER2-amplified breast cancer.

**The Tumor Microenvironment**

The tumor microenvironment plays a critical role in cancer development and progression. Tumor stroma consists of endothelial cells, macrophages, T lymphocytes, fibroblasts, dendritic cells, and effector lymphocytes (115). Cross-talk between these cell types directly impacts tissue architecture and cellular morphology and the extracellular matrix (ECM)-cell interactions directly contribute to cancer progression (116). Interactions of cells and molecules within the tumor microenvironment play an essential role in the development of breast cancer and the progression of an atypical hyperplastic breast lesion to an invasive carcinoma is marked by distinct changes in the tumor stroma (117). Initially, the basement membrane degrades, causing an increase in ECM fibrosis that alters the surrounding epithelium to increase invasiveness. There is a marked increase in infiltrating immune cells, resulting in an increased amount of growth factors and proteases. Newly formed blood vessels carry enriched blood to the tumor site, promoting tumor growth and increased fibroblast activation to promote ECM remodeling (118), (119). These changes in the tumor microenvironment result in a more aggressive cancer, promoting the conversion of hyperplasias to invasive carcinomas (120).

**Role of Reactive Stroma in Breast Cancer**

Over 90% of breast tumor mass is composed of stroma and is characterized by pathological desmoplasia (121). Desmoplasia is a stromal reaction consisting of
increased fibrosis and extracellular matrix (ECM) deposition (122). Infiltrating ductal carcinoma, which represents the most common type of breast cancer, is characterized by a high level of desmoplasia and is often called “scirrhous carcinoma”. The increase in activated stroma results in myofibroblast transformation, secretion of growth factors, increased collagen and fibronectin deposition, and ECM remodeling that in turn stimulate tumor cell growth. Furthermore, reactive stroma generates a supply of blood vessels for the tumor by promoting angiogenesis, to enhance tumor progression. Moreover, α-smooth muscle actin-positive myofibroblast deposition around non-invasive epithelial proliferation in breast ductal carcinoma in situ suggests that the interaction between the epithelium and reactive stroma plays an important role that predates the onset of invasion (116). Therefore, it is not surprising that desmoplastic stroma is associated with poor prognosis (123, 124). Figure 5 illustrates the changes in the tumor microenvironment observed in the progression of breast cancer when compared to healthy breast tissue.

**Cancer as Wounds That Do Not Heal and the Reactive Stroma**

Cancer has been referred to as “wounds that do not heal”. This reference is based upon the similarity between wound healing and cancer with regard to the tumor-stroma interactions (125). In the normal wound healing pathway, when epithelial or endothelial cells undergo damage, the release of cytokines and inflammatory mediators initiates a coagulation cascade that forms a blood clot and provisional ECM deposition. Following exposure to ECM components, platelets trigger coagulation, platelet degranulation promotes vasodilation and increased permeability, and myofibroblasts secrete matrix metalloproteinases to degrade the basement membrane allowing inflammatory cell
Figure 5. Desmoplasia linked with breast cancer progression. Desmoplasia or the increased deposition of reactive stroma has been associated with all stages of breast cancer. In ductal carcinoma in situ (DCIS) as well as in other progressive stages of breast cancer, the deposition of extracellular matrix (ECM) proteins is elevated in association with increased fibroblast density and angiogenesis in the tumor microenvironment.
recruitment (126). Growth factors and cytokines act to recruit and stimulate the proliferation of leukocytes, macrophages and neutrophils that reduce cellular debris, and endothelial cells that create blood vessels. Lymphocytes secrete numerous growth factors such as TGF-β, IL-13, and PDGF that stimulate fibroblasts to cause wound contraction. Finally, epithelial cells divide and migrate to regenerate the damaged tissues, which results in wound healing. However, in the case of chronic inflammation or injury, there is excessive deposition of ECM resulting in the formation of a fibrotic scar (127). This fibrotic or uncontrolled healing process is similar to cancer stroma interactions where there is an imbalance in growth signals, ECM deposition, and angiogenesis. The role that cancer-associated fibroblasts play in these processes will be described in the following sections.

**Characteristics of Cancer-Associated Fibroblasts**

Tumor stroma fibroblasts (often termed “cancer-associated fibroblasts”, “myofibroblasts”, or “activated fibroblasts”) are characterized by their expression profile. Cancer-associated fibroblasts (CAF) differ from normal tissue fibroblasts due to the concurrent expression of α-smooth muscle actin and vimentin. Other physiological indicators of CAF are the presence of stress fibers and a prominent rough endoplasmic reticulum (128). Inflammation and cytokine secretion by the tumor cells recruit fibroblasts to the tumor site. The proposed origin of cancer-associated fibroblasts include host fibroblasts, epithelial-to-mesenchymal transitions, infiltrating mesenchymal precursor/stem cells, and endothelial-to-mesenchymal transitions. In breast cancer, the two major sources of CAF are host fibroblasts and vascular smooth muscle cells. While
pericyte transformation makes up a small percentage of CAF, myoepithelial cells are not involved [15].

**Transforming Growth Factor-β Signaling in CAF Activation**

Transforming growth factor-β (TGF-β) is a potent stimulator of fibroblast to myofibroblast activation and is a prominent activator in the conversion to a reactive stroma phenotype (129). TGF-β is formed by attachment to what is known as the “latent binding protein”, which renders the protein inactive. Cleavage of the latent binding protein by various enzymes (thrombospondin, matrix metalloproteinases-2 and 9, as well as other proteases) allows TGF-β to bind to its receptors, transforming growth factor β type 1 receptor or transforming growth factor β type 2 receptor (TGFβ1R and TGFβ2R). Upon binding, the receptors form a heterodimer causing the activation of two distinct pathways, the Smad-dependent and Smad-independent signaling pathways. The Smad-dependent pathway involves the phosphorylation and activation of Smad2/3, binding to the mediator protein Smad4, and translocation into the nucleus to allow the transcription of ECM proteins. The Smad-independent pathway involves the activation of MAP kinase signaling, which promotes the growth and proliferation of activated myofibroblasts (130, 131). As shown in Figure 6, activated myofibroblasts which are transformed by various cytokines including TGF-β, secrete numerous tumor-promoting growth factors such as hepatocyte growth factor (HGF), platelet-derived growth factors (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor (TGF) α and β. Myofibroblasts also secrete various extracellular matrix components, including collagens, tenacín-C, and fibronectin.
CAF Activation

The TGF-β family of proteins participates in the regulation of cell growth, stimulating mesenchymal cell proliferation, controlling ECM remodeling, influencing immune function, and participating in wound repair (132-134). Depending on the circumstances of activation, TGF-β differentially regulates cell growth. The anti-growth effects of TGF-β are based on early reports where it inhibited the cell cycle and activated apoptosis but only in endothelial cells. During tumor progression, these inhibitory effects of TGF-β can be bypassed by events such as a loss of heterozygosity, mutation of cell cycle inhibitors, or deregulation of intermediary proteins that are involved in cell growth progression. TGF-β can be also be pro-oncogenic based on its ability to increase invasiveness, promote angiogenesis, be immunosuppressive, and increase survival (135).

Studies investigating the duality of TGF-β activity suggest that TGF-β is a tumor suppressor in early breast cancer development while it is pro-oncogenic in late stage and metastatic cancer (136, 137). Moreover increase or complete blockade of TGF-β promotes carcinogenesis and metastasis of breast cancer through different mechanisms. In ER negative breast cancer, an increase in TGF-β is correlated with poor prognosis; in contrast, the loss of TGF-β in ER positive breast cancer is associated with a poor disease outcome (131, 138). When TGF-β signaling is enhanced, there is dysregulation of the production of structural matrix proteins such as collagen that results in growth and dissemination of the primary tumor.
Figure 6: Effects of cancer associated fibroblasts on the tumor microenvironment.

Cancer associated fibroblasts (CAF) secrete numerous growth factors and cytokines that effect multiple cell types. CAF secrete vascular endothelial growth factor (VEGF) which stimulates endothelial cells and promotes angiogenesis. CAF also secrete hepatocyte growth factor (HGF), tenascin-C, and transforming growth factor (TGF)-β which stimulate the proliferation of cancerous epithelial cells. Moreover, CAF secrete matrix metalloproteinases (MMP) that promote tumor invasion and metastases at distant locations.
Main products of TGF-β activated myofibroblasts are collagens. Collagens are the most abundant ECM scaffolding protein, resulting in increased tensile strength of the tissue. ECM stiffness enhances cell growth and promotes cell survival, suggesting that increased collagens in the tumor microenvironment may stimulate tumor growth (139). In particular, collagen I serves as a tissue scaffold, to promote tumor growth. Moreover, collagen I acts upon the tumor cells to stimulate genes that are associated with cellular signaling, cellular metabolism, transcription, and translation, suggesting that collagen I is vital in signaling tumor growth and progression (140). The intratumoral levels of collagen I were linked to increased tumor invasiveness, indicating a potential role for collagen I in metastasis (141). In addition, increased mammographic density was characterized by elevated collagen I deposition, which is associated with an increase in breast cancer risk (142). These data suggest that targeting collagen I deposition in tumors may reduce tumor progression and metastatic potential.

Fibronectin is another component of the ECM that is secreted by TGF-β-stimulated myofibroblasts and is involved in cell-matrix cell-cell adhesions, cell migration, and oncogenic transformation (143). Fibronectin deposition in breast tumor stroma positively correlated with tumor grade, size, and lymph node involvement (144). Furthermore, fibronectin stimulates tumor cells through integrin signaling to promote the activation of focal adhesion kinase (FAK) and MAP kinase. This activation results in cell proliferation, survival, and angiogenesis making fibronectin another attractive target for breast cancer treatment (145).
Myofibroblast activation also results in tenascin-C production. Tenascin-C, a matrix glycoprotein that is often up-regulated in the stroma of solid tumors, promotes cell migration, inhibits cell-cell adhesion, and enhances tumor angiogenesis (146). Tenascin-C is absent from normal adult breast tissue but is often over-expressed in breast carcinomas (147). Tenascin-C expression in breast carcinoma stroma is associated with progressive disease and poor prognosis (144).

Myofibroblasts secrete proteolytic enzymes such as matrix metalloproteinases (MMPs) that alter connective tissue composition and result in tissue remodeling. MMPs are zinc-dependent endopeptidases that degrade ECM proteins and play a vital role in tumor metastases and invasiveness (148). In particular, MMP-3 acts as a natural mammary tumor promoter and elevated levels of sera proMMP-2 and proMMP-9 are found in breast cancer patients (149, 150). Elevated levels of proMMP-2 and proMMP-9 are correlated with poor prognosis, disease stage, lymph node metastases, and tumor size (150). However, clinical trials using MMP inhibitors (MPIs) as cancer therapeutics yielded poor results; Phase II trials designed to measure drug efficacy were complicated by the cytostatic properties of MPIs that prevented the use of conventional measures of efficacy, such as reduction in tumor size (151). Moreover, a Phase III clinical trial using MPIs also proved to be disappointing; Prinomastat, a MPI with preferential activity against MMP-2 and MMP-9, in combination with carboplatin and paclitaxel, provided no significant survival benefit to patients with non-small cell lung carcinoma (152). This negative clinical data may results from the late tumor staging of patients on these clinical
trials, suggesting that MPIs may be more advantageous in early stage of cancer (151). These data indicate that targeting the tumor microenvironment may be beneficial in tumor development and progression.

Tumors must induce angiogenesis, or the formation of new blood vessels from pre-existing blood vessels, to grow beyond 1-2 mm in diameter in size. Angiogenesis is critical in forming metastases (153). Activated myofibroblasts play an important role in angiogenesis by promoting the secretion of vascular endothelial growth factor (VEGF), which stimulates endothelial cell branching and the synthesis of new blood vessels to feed the tumor. Tumor-associated fibroblasts were implicated in mediating resistance to anti-VEGF therapy by stimulating the release of platelet-derived growth factor-C (PDGF-C), which in turns stimulates angiogenesis by overriding the effect of the anti-VEGF treatment (154). Moreover, the secretion of matrix-associated proteins by activated fibroblast provides structural support for blood vessels and over expression of these proteins is involved in the abnormal vascular structure of tumors observed during pathological angiogenesis. These data suggest the important role of tumor-associated fibroblasts in angiogenesis and drug resistance.

Taken together, these studies suggest that a targeted therapy against cancer-associated fibroblasts may be an effective chemotherapeutic strategy to combat cancer. The importance of CAF in the initiation and progression of cancer are under investigation in several ongoing clinical studies and experimental models; however, the effect of
cancer therapies and the development of drugs that target the tumor microenvironment have not been thoroughly explored.

**Mitogen-Activated Protein Kinase Signaling**

The molecular mechanisms of the Ang-(1-7)-mediated growth reduction in human lung cancer cells included decreased phosphorylation of the mitogen-activated protein (MAP) kinase extracellular signal regulated kinase (ERK)-1/2 (43). The MAP kinase family consists of at least four distinct members: ERK1/2, c-Jun amino-terminal kinases (JNK-1/2/3), p38 proteins (p38-α/β/γ/δ), and ERK5 (155). As shown in Figure 7, this group of protein kinases is phosphorylated in response to external stimuli and regulates various cellular functions including cell growth, proliferation, survival, and differentiation (156). MAP kinase is regulated by multiple mechanisms including a change in the upstream MAP kinase kinase (MAPKK) or MEKs and the MAP kinase kinase kinases such as raf. A specific MAPKK for each MAP kinase family member activates the particular MAP kinase by phosphorylation. Depending on the external stimuli, various MAPKKs may activate more than one MAP kinase, illustrating the complexity of MAP kinase signaling. MAP kinase activation is also regulated by a change in the MAP kinase phosphatases that dephosphorylate and inactivate MAPK. MAP kinase phosphatases (MKPs) are a group of dual-specificity protein phosphatases (DUSPs) that can dephosphorylate both phospho-threonine and phospho-tyrosine residues, negatively regulating MAP kinase signaling (157). DUSP1/MKP-1 dephosphorylates both threonine and tyrosine residues which deactivate the MAP kinase pathways.
**Figure 7: MAP kinase signaling.** Various extracellular stimuli such as growth factors, cytokines, UV light, and oxidation activate MAP kinase signaling pathways. Activation of the mitogen-activated protein kinase kinase kinases (MAPKKK) leads to the phosphorylation of the mitogen-activated protein kinase kinases (MAPKK). The MAPKK MEK1/2 activates extracellular regulated kinase (ERK) 1/2 that, in turn, phosphorylates cytoplasmic protein and translocates to initiate transcription. MKK7 and MKK4, normally activated through stress induced pathways, activate JNK. MKK3, MKK6, and MKK4 activation leads to the stimulation of p38 signaling.
Cellular Responses: Proliferation, Differentiation, Survival, Apoptosis
ERK Signaling

As previously mentioned, MAP kinase signaling is activated upon an external stimulus, stimulating its receptor tyrosine kinase activity and facilitating guanosine triphosphate binding to Ras-GTPase. Primed Ras-GTPase causes the translocation of Raf kinases (Raf-1, B-Raf, A-Raf) to the plasma membrane. There are three different Raf isoforms which differ in their ability to activate the MAP kinase kinases MEK1/2--B-Raf > Raf-1 > A-Raf with regard to activation of MEK1/2 (158). Activated Raf phosphorylates serine 218 and serine 222 in the activation loop of MEK1/2. The dual specificity kinase activity of MEK1/2 then phosphorylates ERK1/2 at the common –TXY motif, which activates ERK1/2. Once activated, ERK1/2 phosphorylates cytoplasmic substrates such as RSK, which inactivates the pro-apoptotic protein BAD to promote cell survival. Activated ERK1/2 also translocates to the nucleus to initiate transcription and promote cell proliferation and survival. Within the nucleus, ERK1/2 has a number of activities including stimulation of DNA synthesis through the catalyzation of pyrimidine nucleotide biosynthesis, induction of cyclin D1, and reduction of antiproliferative genes, thereby promoting cell cycle progression (159, 160). Another important mechanism of ERK1/2 signaling is the up-regulation of Fra-1, a component of the AP-1 transcriptional factor complex that was showed to dysregulate Rho kinase; the disruption of Rho activity is linked to the promotion of angiogenesis, cell migration, invasion, and metastasis (155, 161-163). These data indicate the vital role that ERK1/2 signaling plays in cancer initiation, progression, and metastasis.
**JNK Signaling**

The JNK pathways are activated by stress including cytokines, UV radiation, growth factor deprivation, DNA-damaging agents, specific G protein-coupled receptors, and serum stimulation (164). There are three different JNK isoforms, JNK1-3; JNK1 and JNK2 are ubiquitously expressed, while JNK3 expression is confined to the brain, heart, and testes. The MAP kinase kinases MEK4 and MEK7 activate JNK isoforms through dual phosphorylation of the threonine and tyrosine of the TPY motif. MEK4 and MEK7 are activated by numerous MAP kinase kinase kinases (MAPKKK), including MEKK1-4, MLL2, MLL3, TAK1, ASK1, and ASK2 (155). The dual phosphorylation and activation of JNK by MEK4 or MEK7 results in the translocation of JNK to the nucleus, phosphorylation of c-Jun as well as other transcription factors, and the enhancement of AP-1 activity. The resulting increase in JNK activity and the duality of JNK signaling in carcinogenesis will be discussed below.

**p38 Signaling**

Various external stimuli including UV and X-ray irradiation, hypoxia, cytokines, and oxidative stress activate the p38-signaling cascade. There are four p38 isoforms--p38-α, p38-β, p38-γ, and p38-δ; p38α is the most abundant isoform. These p38 isoforms are phosphorylated on similar threonine-glycine-tyrosine motifs located in the activation loops, by the MAP kinase kinases MKK3, MKK6, or MKK4. Functional p38 has serine/threonine kinase activity which can phosphorylate cytoplasmic proteins or translocate into the nucleus to initiate transcription. The downstream targets of p38 activation include, but are not limited to, MNK1, MNK2, ASK1, AFT2, ELK1, and p53.
p38 activation is most often correlated with apoptosis and cell cycle arrest; however, a dual modality of p38 signaling was reported and will be further discussed in the following section.

**MAPK Signaling in Cancer**

ERK1/2 signaling is dysregulated in approximately one-third of all human cancers, with the most common mutations in either B-Raf mutations or Ras (155). B-Raf is mutated in over 66% of all malignant melanoma cancers and to a lesser extent in other types of cancers (166). The most common mutation in B-Raf is a V600E amino acid substitution in the activation loop which leads to constitutive activation (167). Over 90% of pancreatic cancer, 45% of colorectal cancer, and 35% of non-small cell lung adenocarcinoma includes a K-Ras mutation (168). The majority of Ras mutations occur in the 12th, 13th, or 61st codon regions and result in the inefficient hydrolysis of GTP, rendering Ras constitutively active (168, 169). Cancer cells may also amplify the transcription factors activated by the ERK1/2 pathway which disrupts normal ERK signaling or alters integrin receptor expression, favoring growth factor activation of Ras and ERK pathway activity. These data highlight the role that ERK signaling plays in cancer initiation, progression, and metastases, indicating that therapeutic targeting of the ERK pathway may be beneficial in the treatment of cancer.

Activation of the JNK signaling pathway can result in both cellular proliferation and apoptosis, depending on the external stimuli. JNK activation and the resulting phosphorylation of c-Jun are also involved in Ras oncogenic transformation and
tumorigenesis (170). In hepatocarcinoma, c-Jun which is activated by JNK, contributes to carcinogen-induced carcinoma by inhibiting p53 function (171). Furthermore, treatment of myeloma and breast cancer cells with either antisense oligonucleotides to JNK or JNK inhibitors inhibits cell growth and increases apoptosis (172, 173). A growing body of literature demonstrating the proliferative and pro-tumorigenic properties of JNK suggests that targeting JNK may be beneficial in the treatment of cancer (174, 175).

However, several reports also suggest that JNK is a tumor suppressor, due to stimulation of apoptosis. JNK signaling was not required for in vivo Ras-induced transformation and tumorigenesis in JNK null mice (176). Moreover, JNK knockout animals developed spontaneous intestinal tumors with significantly lower p21 expression in the intestinal epithelial cells and were highly susceptible to UVA-induced papilloma and TPA-induced skin tumors (177-179). These data demonstrate the growth inhibitory and tumor suppressor properties of JNK signaling and emphasize the duality of JNK signaling in cancer. Due to the dual role of JNK in some types of cancer cells, it is unclear whether targeting JNK would be beneficial in cancer treatment.

Similar to JNK, p38 stress-activated MAP kinase was also implicated in tumorigenesis and may be a tumor suppressor, through negative regulation of the cell cycle. p38α down-regulates cyclins-dependent kinases, up-regulates cyclin-dependent kinase inhibitors, and modulates p53 activity, thereby regulating the cell cycle at both the G0/G1 and G1/G2 checkpoints, reducing proliferation by inhibiting progression through
the cell cycle (180, 181). Furthermore, p38α promotes apoptosis, further supporting a role for p38 as a tumor suppressor (182, 183).

In contrast to these studies, p38 also functions as a tumor-promoting role, playing a role in both the initiation and progression of cancer. For example, p38δ is critical in TPA-induced skin tumorigenesis (184). Moreover, p38α positively regulates proliferation in various prostate, melanoma, and chondrosarcoma cell lines (185-187). These reports demonstrate the variability of p38 signaling, which is dependant upon on cell type and external stimuli, suggesting that p38 may be either pro-carcinogenic or tumor suppressive.

Phosphatase Regulation of MAPK

The physiological response of MAPK signaling is dependent on the balance between upstream kinase activity and the negative regulatory protein phosphatase expression. Studies show that protein tyrosine phosphatases (PTP) such as the striatal enriched phosphatase (STEP), STEP-like phosphatase (PTP-SL), and haemopoietic PTP (HePTP), expressed in neuronal and lymphatic cells, were found to physically associate with activated ERK1/2, resulting in the dephosphorylation and inactivation of ERK1/2 (188-190). While these phosphatases play a role in the regulation of MAPK signaling, dual specificity phosphatases (DUSPs) are predominately implicated in the negative regulation of MAPK signaling in mammalian cells.

Dual Specificity Phosphatase Signaling
MAP kinase phosphatases (MKP), which belong to the family of dual specificity phosphatases (DUSPs), dephosphorylate tyrosine and threonine residues and inactivate various MAP kinases (188). There are 10 different DUSPs, including 4 inducible phosphatases, 3 cytoplasmic phosphatases, and 3 stress-induced phosphatases. All DUSPs contain a C-terminal catalytic domain that is similar in structure as well as a related N-terminal domain that recognizes MAP kinases, known as the kinase interaction domain (KIM) (191).

The gene for the most well-characterized DUSP is human DUSP1, MKP1, that contains four exons and three introns that encode an inducible mRNA of 2.4 kilobases. The promoter region contains several cAMP responsive elements, multiple AP-2 sites, one AP-1 site and a TATA box, demonstrating that the transcription of DUSP1 is regulated by many different factors (192). Various stimuli induce DUSP1, including serum, heat shock, DNA-damaging agents in fibroblasts, dexamethasone in mammary epithelial cells, and arachidonic acid in VSMC (191). DUSP1 is also up-regulated by ERK1/2 in a negative feedback loop used to inhibit kinase activity by acting as an innate “off” switch for MAP kinase signaling.

DUSP1 is an inducible nuclear phosphatase that inactivates JNK, p38, and ERK1/2 MAP kinases. Genetic deletion of DUSP1 suggests a role for the phosphatase in the immune response and knockout animals also display decreased viability and severe hypotension (188, 193). In prostate, bladder and colon cancers, DUSP1 is present at the early stages of carcinogenesis but is diminished with tumor progression (194).
Furthermore, in human breast and lung cancer cells, the induction of DUSP1 and the resulting inactivation of ERK1/2 are required for the stimulation of apoptosis (195, 196). DUSP1 expression in non-small cell lung carcinoma patients was independently correlated with increased survival, suggesting a role for DUSP1 as a tumor suppressor (197). Therefore, up-regulation of DUSP1 may represent a mechanism to arrest cell proliferation and increase survival.

**Cyclooxygenase-2 Signaling and Prostaglandin E<sub>2</sub> Synthase in Cancer**

Previous studies from our laboratory demonstrated that Ang-(1-7) treatment of mice with human lung cancer xenografts was associated with a decrease in cyclooxygenase-2 (COX-2) and that the heptapeptide increased prostacyclin and cyclic AMP in VMSCs, suggesting that Ang-(1-7) may alter the proliferative to anti-proliferative prostaglandin ratio to inhibit cell growth (22, 44). In addition, both inflammation and prostaglandin synthesis are important in the formation and progression of human breast cancers. COX-2 is up-regulated in approximately 40% of human breast cancers and is positively associated with breast cancer angiogenesis, inflammation, invasion, and metastasis (198). Genetic knockdown of COX-2 reduces mammary tumor development, while over-expression of COX-2 induced mammary neoplasia, providing further evidence of the oncogenic activities of COX-2 (199, 200).

The COX-2 signaling cascade is illustrated in Figure 8. COX-2 initially converts arachidonic acid into the precursor prostaglandin PGG<sub>2</sub> and subsequently processes PGG<sub>2</sub> into PGH<sub>2</sub>. PGE<sub>2</sub> is generated by the anabolism of PGH<sub>2</sub> by prostaglandin E<sub>2</sub> synthase (PGES). PGE<sub>2</sub> binds to its prostaglandin E receptors (PTGERs or EP) 1-4 to exert pro-
Figure 8: Arachidonic acid metabolism and cyclooxygenase-2 signaling. Phospholipase A$_2$ metabolizes phospholipids to release arachidonic acid (AA) which is then converted into the precursor prostaglandins PGG$_2$ and then PGH$_2$ by the actions of cyclooxygenase (COX)-1 (constitutively expressed) and COX-2 (inducible). PGH$_2$ is converted by prostaglandin E synthase (PGES) or prostacyclin synthase (PGIS) into PGE$_2$ or PGI$_2$, respectively. The prostanoids subsequently act through selective receptors; PGE$_2$, through activation of EP receptors, stimulates inflammation, proliferation and angiogenesis while the anti-inflammatory, anti-proliferative, and anti-angiogenic biological properties of prostacyclin are mediated by IP receptors.
Inflammation
Proliferation
Angiogenesis

Anti-Inflammatory
Anti-proliferative
Anti-angiogenic
tumorigenic properties, promoting tumorigenesis, angiogenesis, proliferation, and inhibition of apoptosis (201-204). EP receptors are seven-transmembrane G-protein-coupled receptors which activate distinct intracellular signaling pathways. For example, antagonism of the EP4 receptor in various murine mammary cell lines decreased their ability to form lung metastases and increased natural killer cell function, suggesting a role for PGE2 signaling in immunosuppression and metastasis (205). Activation of the EP2 receptor increased malignant cell invasion, angiogenesis, cell cycle progression, and COX-2-mediated mammary hyperplasia (204, 206-208). The antagonism of EP1 may have chemopreventive properties in breast cancer as well as other epithelial cancers, clearly highlighting the importance of PGE2 signaling in cancer (209).

The majority of the actions of COX-2 are mediated by the production of PGE2 by prostaglandin E2 synthase (PGES). There are three different PGESs present in human cells--two inducible microsomal membrane-bound PGESs (mPGES-1 and -2) and one cytosolic PGES (cPGES) (210). PGES-1 is elevated in response to mitogenic and inflammatory stimuli with increased expression in various types of cancer including non-small cell lung cancer, breast cancer, hepatocellular carcinoma, colorectal cancer, and prostate cancer (210-212). mPGES-1 over-expression was reported in 79% of invasive breast cancer cases and was independent of COX-2 expression, ER status, and HER2 amplification (213). Moreover, experimental studies in two different murine models of intestinal cancer showed that genetic deletion of mPGES-1 significantly suppressed the formation of intestinal cancer by 66% (214). These data suggest that COX-2 and mPGES-1 may serve as targets for breast cancer therapies.
Foundation for the Hypothesis that Ang-(1-7) Inhibits Breast Tumor Growth by a Reduction in Growth Signaling and Modulation of the Tumor Microenvironment

Treatment with Ang-(1-7) inhibited the growth of cardiac myocytes, VSMC, and cardiac fibroblasts, in vitro and in vivo (11, 21, 23, 25-27, 215, 216). More importantly, treatment with Ang-(1-7) decreased lung cancer cells in vivo in human lung tumor xenografts in athymic mice. Infusion of the heptapeptide significantly reduced human A549 xenograft tumors with an associated reduction in COX-2. The inhibition of human lung cancer cell growth was associated with a decrease in the activated MAP kinases ERK1/2 (43), suggesting that reduction of the MAP kinase pathway and regulation of prostaglandin signaling may play roles in the molecular mechanism underlying the attenuation in tumor growth associated with Ang-(1-7)-administration (44, 45). Preclinical studies in lung tumor xenografts and the Phase I clinical trial suggest that Ang-(1-7) may reduce tumor growth by inhibiting angiogenesis (45, 46). Additionally, numerous studies showed that infusion of Ang-(1-7) significantly reduced cardiac fibrosis and that mas knockout mice have significantly higher levels of cardiac fibrosis and impaired cardiac function, suggesting an anti-fibrotic role for the heptapeptide (2, 4, 26-28). Based upon these studies, we hypothesize that Ang-(1-7) will inhibit breast tumor growth by down-regulating proliferation, inhibiting angiogenesis, and reducing tumoral fibrosis.
Reference List


(64) Perret S, Dockery P, Harvey BJ. 17beta-oestradiol stimulates capacitative Ca2+ entry in human endometrial cells. Mol Cell Endocrinol 2001;176:77-84.


(94) Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997;16:1647-55.


Chapter II:

Angiotensin-(1-7) Reduces Fibrosis in Orthotopic Breast Tumors

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ABSTRACT

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous seven-amino acid peptide hormone of the renin-angiotensin system with anti-proliferative properties. In this study, human BT-474 epidermal growth factor receptor 2 (HER2) over-expressed, estrogen receptor (ER) positive tumors or ZR-75-1 ER positive tumors in the mammary fat pad of athymic mice were treated with Ang-(1-7), to determine the effect of the heptapeptide on tumor growth. Mice were treated with either saline or 24 µg/kg/h Ang-(1-7) after the tumors grew to 100-200 mm$^3$. Ang-(1-7) administration reduced BT-474 and ZR-75-1 tumor volume by 68% and 78% and tumor weight by 39% and 52%, respectively, indicating that the heptapeptide inhibits breast tumor growth. Treatment with Ang-(1-7) reduced interstitial fibrosis by 75% and 64% in BT-474 and ZR-75-1 tumors, respectively, in association with a significant reduction in collagen I deposition. Ang-(1-7) also decreased perivascular fibrosis by 73% in BT-474 tumors. Ang-(1-7) markedly attenuated the *in vitro* growth of fibroblasts isolated from ZR-75-1 orthotopic breast tumors, reduced fibronectin protein by 38% and decreased transforming growth factor-β (TGF-β) by 45%. The heptapeptide caused a 2.5-fold increase in the mitogen-activated protein (MAP) kinase phosphatase DUSP1 with an associated 47% and 63% decrease in ERK1 and ERK2 MAP kinase activities, respectively, suggesting that Ang-(1-7) increases DUSP1 to reduce MAP kinase signaling. This is the first report that Ang-(1-7) targets the tumor microenvironment, by inhibiting the growth of tumor–associated fibroblasts to reduce tumor fibrosis.
INTRODUCTION

Crosstalk between cancer cells and surrounding tissue is essential for the development and progression of tumors. The interaction between cancerous cells and the adjacent microenvironment transforms the stroma into an abnormal phenotype, altering normal function (1, 2). The altered stromal microenvironment impacts tissue architecture, cellular morphology, and extracellular matrix (ECM)-cell interactions that directly contribute to formation of the neoplasia (1). Solid tumors, in particular breast tumors, are characterized by pathological desmoplasia, resulting in increased fibrosis and ECM deposition (1, 2). About 80% of reactive stroma associated with breast carcinoma is composed of activated myofibroblasts (3) which secrete ECM proteins, resulting in desmoplasia and breast tumor progression (4).

Activated myofibroblasts play a vital role in tumor initiation, growth, and metastases. Tumor stroma myofibroblasts or “cancer-associated fibroblasts” (CAF) are characterized by their expression profile and are distinguished from normal fibroblasts by the expression of α-smooth muscle actin and vimentin (5). Moreover, the presence of α-smooth muscle actin-positive myofibroblasts around non-invasive epithelium in breast ductal carcinoma in situ is strongly correlated with the onset of tumor invasion and poor prognosis (5, 6). Inflammation and cytokine secretion by cancer cells results in the recruitment of CAF to the tumor site. Activated myofibroblasts are transformed by cytokines such as transforming growth factor-β (TGF-β) and secrete tumor promoting growth factors including hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor
(bFGF), and TGF-α and -β (5), which leads to the production of ECM components including collagens, tenacin-C, and fibronectin (7).

CAF play an important role in the initiation and progression of cancer; however, the use of cancer therapies that target the tumor stroma is limited (5, 8). In this study, we assessed whether angiotensin-(1-7) [Ang-(1-7)], a seven-amino acid peptide hormone of the renin-angiotensin system with vasodilatory, anti-proliferative, and anti-fibrotic properties (9), alters the tumor microenvironment to reduce tumor growth and fibrosis. Ang-(1-7) exerts its biological activity through a unique G protein-coupled receptor, mas (9, 10). The heptapeptide decreased mitogen-stimulated growth of vascular smooth muscle cells (VSMCs) in vitro (11) and attenuated neointima formation in a balloon catheter injury model of the rat carotid artery, with no effect on the underlying media layer, indicating that the anti-growth properties of the heptapeptide are limited to proliferating cells (12). More importantly, we showed that Ang-(1-7) significantly decreased the proliferation of human lung cancer cells in vitro by reducing mitogen-activated protein (MAP) kinase activity (13). Ang-(1-7) infusion decreased human A549 lung adenocarcinoma xenograft growth with a corresponding reduction in cyclooxygenase 2 (COX-2) (14). Lung tumors from mice injected with the heptapeptide had reduced vessel density with an associated decrease in VEGF, indicating that Ang-(1-7) inhibited tumor angiogenesis (15). These data suggested that Ang-(1-7) also regulates the tumor microenvironment to inhibit cancer growth.

Fibrosis correlates with the progression and invasion of breast cancer. The increased ECM deposition and secretion of growth factors by myofibroblasts directly contribute to breast tumor growth by stimulating tumor cell proliferation, increasing
angiogenesis, and promoting invasion (5). The purpose of this study was to determine whether Ang-(1-7) could serve as an anti-fibrotic agent that targets the tumor microenvironment to reduce breast cancer tumor growth and fibrosis.

**Materials and Methods**

**Materials:** The following materials were purchased from the companies in the parentheses. Ang-(1-7) and [D-Alanine\(^7\)]-angiotensin-(1-7) or [D-Ala\(^7\)]-Ang-(1-7) (Bachem, King of Prussia, PA); [D-Proline\(^7\)]-angiotensin-(1-7) or [D-Pro\(^7\)]-Ang-(1-7) (GenScript Corporation, Piscataway, NJ); penicillin, RPMI-1640, DMEM/F12, streptomycin, fetal bovine serum (FBS), and Hypoxanthine-Aminopterin-Thymidine (HAT) supplement (Gibco Invitrogen BRL, Carlsbad, CA); TGF-β (Calbiochem, San Diego, CA); Matrigel (BD Biosciences, Bedford, MA); picric acid (Sigma-Aldrich, St. Louis, MO); saturated picric acid (LabChem Inc., Pittsburgh PA); Collagenase, trypsin, and soybean trypsin inhibitor (Worthington Biochemical, Lakewood, NJ). Antibodies were obtained from the following sources: Collagen I (Abcam, Cambridge, MA); phosphorylated ERK 1/2 and TGF-β (Cell Signaling Technology, Danvers, MA); MKP-1 (Upstate Biotech, Lake Placid, NY); fibronectin, vimentin, α-smooth muscle actin, and β-actin (Sigma-Aldrich, St. Louis, MO); Cy2 FITC-coupled donkey anti-rabbit, Cy3 FITC-coupled donkey anti-mouse (Jackson Laboratories, West Grove, PA); and polyclonal and HRP-conjugated secondary antibodies (GE Health care, Buckinghamshire, UK).

**Cell Culture:** ZR-75-1 breast ductal carcinoma cells (ATCC CRL-1500), derived from a 63 year-old Caucasian female, and BT-474 breast carcinoma cells (ATCC HTB-20),
derived from a 60 year-old Caucasian female, were grown in RPMI media containing 10% FBS, 100 µg/mL penicillin, 100 units/mL streptomycin and 10 nM Hepes. Isolated tumoral fibroblasts were grown in DMEM/F12 media containing 10% FBS, 100 µg/mL penicillin, 100 units/mL streptomycin and 10 nM Hepes. Cells were grown at 37°C in a humidified atmosphere of 5% CO2:95% room air.

**Orthotopic Model of Human Breast Cancer**: Female athymic mice (15-20 g, 4-6 weeks of age; Charles River Laboratory, Wilmington, MA) were housed in cages with HEPA-filtered air (12-h light dark cycle) and provided *ad libitum* access to food and autoclaved water. All procedures complied with the policies of the Wake Forest University Animal Care and Use Committee. Female athymic mice were ovariectomized and supplemented with 0.18 mg 17β-estradiol 90-day time release pellets (Innovative America, Saratoga, FL) to prevent cycling of hormones (16, 17). Actively growing ZR-75-1 cells (2 x 10⁶) or BT-474 cells (5 x 10⁶) at 75% confluence were suspended in 50% PBS:50% Matrigel and injected into the inguinal mammary fat pad (18, 19). Tumor size was measured every other day using a caliper and tumor volume was calculated using the formula \( V = \frac{[(\text{Length} \times \text{Width}^2)/2]}{2} \). When tumors reached a volume of 100 mm³ (ZR-75-1) or 200 mm³ (BT-474), the mice were implanted with osmotic minipumps for subcutaneous release of saline or 24 µg/kg/h Ang-(1-7). After 18 days of treatment, the animals were sacrificed and tumors were excised. The study was terminated at 18 days due to the diminished size of Ang-(1-7)-treated ZR-75-1 tumors and the excessive tumor burden of saline-treated BT-474 tumors.
**Immunohistochemistry:** Orthotopic tumors were fixed in 4% paraformaldehyde for 24 h and incubated in 70% ethanol for 48 h prior to embedding in paraffin. The embedded tumors were cut into five micron thick sections and stained with hematoxylin and eosin (H&E) to determine morphology. Interstitial and perivascular tumor fibrosis was measured by picrosirius red histochemical staining (20). Immunostaining was performed with an antibody to collagen I (1:100) using the streptavidin-biotin method (21). Stained sections were visualized with a Leica DM 4000 microscope and photographed with a QImaging Retiga 1300R camera. A computer-assisted counting technique with a pixel counter to select stained fibers was used to quantify picrosirius red and collagen I staining. Four fields or vessels were quantified from tumor sections from each mouse; interstitial fibrosis is expressed as a percentage of reactive fibers/field, while perivascular fibrosis is expressed as a percentage of reactive fibers/blood vessel.

**Tumor Fibroblast Isolation:** Orthotopic ZR-75-1 breast tumors (200 mm³) were excised from mammary fat pads. Minced tumors were digested overnight at 4°C with trypsin (50 µg/mL) and soybean trypsin inhibitor (100 µg/mL) was added to the tumor digest to stop the reaction. The minced tissue was harvested by centrifugation and the supernatant was discarded. The minced tumor pieces were further digested with collagenase (85 U/mL) at 37°C for 30 min. Undigested tumor tissue was removed with a cell strainer and the cells were collected by centrifugation; tumor fibroblasts were isolated by differential plating as previously described (22).
Quantification of Cell Number: Isolated tumor fibroblasts (1 x 10^4 cells/mL) in DMEM/F12 containing 0.5% FBS and 10 ng/mL TGF-\(\beta\) were plated in 24-well tissue culture plates. On day 1 after plating and every 24 h afterwards, cells were treated with 10 ng/mL TGF-\(\beta\) and either PBS or 100 nM Ang-(1-7) in PBS, added daily due to degradation of the heptapeptide. Cells were removed from individual wells on days 4, 7 and 10, using trypsin/EDTA, and counted using a hemocytometer.

Immunofluorescence: Fibroblasts were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated overnight, at 4°C, with antibodies to fibronectin, vimentin, \(\alpha\)-smooth muscle actin (1:100), or collagen I (1:100). Following a 30 min incubation with secondary antibodies, Cy2 or Cy3 (1:100), the cells were counter-stained with 4’’,6-diamidino-2-phenylindole (DAPI) (1:1000). Fluorescent stained cells were visualized with a Leica DM 4000 microscope (\(A_{\text{max}} = 492\) nm Cy2, \(A_{\text{max}} = 550\) nm Cy3) and photographed with a QImaging Retiga 1300R camera.

Western Blot Hybridization: Treated cell monolayers were solubilized in lysis buffer (13) and protein was measured by a modification of the Lowry method (23). Proteins were separated by polyacrylamide gel electrophoresis and transferred to hydrophobic polyvinylidene difluoride membrane. Non-specific binding was blocked by incubation with B\(\text{lotto}\) (Tris-buffered saline with 5% powdered milk and 4% Triton X-100). Membranes were incubated overnight at 4°C with primary antibodies specific to DUSP1 (\(\text{MKP1, 1:1000}\)), fibronectin (1:5000), pERK1/2 or TGF-\(\beta\) (1:1000) followed by a 1 h incubation with polyclonal horseradish peroxidase (HRP)-conjugated secondary
antibodies (1:2000) at room temperature. Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto or Pico West, Pierce Biotechnology, Rockford, IL) and quantified by densitometry using MCID digital densitometry software (Cambridge, UK). Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β-actin (1:2000).

**Statistics:** All data are presented as the mean±standard error of the mean (SEM). Statistical differences were evaluated by Student’s t test or one way analysis of variance (ANOVA) followed by Dunnett’s *post hoc* test. The criterion for statistical significance was set at p < 0.05.

**RESULTS**

**Inhibition of Orthotopic Human Breast Tumors by Ang-(1-7)**

Athymic mice bearing human ZR-75-1 or BT-474 orthotopic breast tumors were administered saline or 24 µg/kg/h Ang-(1-7) via osmotic minipump for 18 days. The ZR-75-1 tumor volume in the two treatment groups was not statistically different at the beginning of treatment [117.3±4.3 mm³ in the saline-treated group when compared to 123.8±7.6 mm³ in the Ang-(1-7)-treated group]. Tumor volume of saline-medicated mice continued to increase, while the growth of tumors in Ang-(1-7)-treated mice was significantly inhibited beginning at day 4 of treatment [Figure 1A, day 18 tumor volumes: 287.4±22.2% change in the saline-treated mice compared to 59.7±9.8% change in the Ang-(1-7)-treated animals when compared to original tumor volume; p<0.001]. By the end of the study, the volume of ZR-75-1 tumors from Ang-(1-7)-medicated mice was
reduced 25% compared to the initial tumor volume, resulting in a %T/C of 22.3%. Due to the diminished size of the tumors from mice administered Ang-(1-7), the study was terminated at 18 days to provide sufficient tumor tissue for mechanistic studies.

The tumor volume of the two treatment groups of athymic mice injected with human BT-474 breast cancer cells was not statistically different at the beginning of treatment [327.0±72.5 mm³ in the saline-treated group compared to 197.7±15.9 mm³ in the Ang-(1-7)-administered group]. The volume of the tumors in saline-treated mice continued to increase, while the growth of tumors from the heptapeptide-medicated animals was inhibited significantly beginning at day 12 [Figure 1B, final tumor volumes: 1818±260.2% change in the saline-treated animals compared to 841.2±24.9% change in the Ang-(1-7)-treated animals when compared to initial tumor volumes; p<0.001]. Ang-(1-7) administration of BT-474 orthotopic human breast tumors resulted in a %T/C of 29.9%. The study was terminated at 18 days for health concerns due to the large tumor burden of the saline-treated mice.

No gross pathological side effects were observed with heptapeptide administration; there was no change in motor function, body weight, and food and water intake. Upon completion of the study, the mice were euthanized and tumors were excised and weighed. The ZR-75-1 tumors from mice treated with Ang-(1-7) weighed approximately 50% less than the tumors from the saline control animals [0.29±0.03 g versus 0.14±0.02 g] (Figure 1C). Similarly, the BT-474 tumors from mice that were medicated with Ang-(1-7) weighed approximately 40% less than the tumors from the mice treated with saline [3.62±0.25 g versus 2.22±0.07 g] (Figure 1D).
**Inhibition of Tumor Fibrosis by Ang-(1-7)**

Interstitial tumoral fibrosis was quantified in orthotopic breast tumor sections stained with picrosirius red, a non-specific collagen stain. ZR-75-1 tumors and BT-474 tumors from saline-treated mice have heavy deposits of collagen which were reduced by Ang-(1-7) administration (Figures 2A and 2B). The relative amount of picrosirius red staining was quantified and the amount of collagen within the tumors is expressed as percent fibrosis per field. Treatment with the heptapeptide reduced interstitial fibrosis by 64% in the ZR-75-1 tumors [23.3±2.4% versus 8.3±0.8% fibrosis per field] (Figure 2A) and by 75% in the BT-474 tumors [4.9±1.0% versus 1.2±0.2% fibrosis per field] (Figure 2B), indicating that Ang-(1-7) reduces tumor fibrosis in orthotopic breast tumors. ZR-75-1 tumors from saline-treated mice had 3-fold more interstitial fibrosis when compared to BT-474 tumors from saline-treated mice, demonstrating a differential deposition of collagen between the two types of breast tumors. The amount of picrosirius red staining surrounding blood vessels in BT-474 tumors was also quantified, to measure perivascular fibrosis. It was not possible to quantify perivascular fibrosis in ZR-75-1 tumors due to pervasive interstitial fibrosis throughout the tumor. Heavy deposits of collagen were visualized around blood vessels in BT-474 tumors from saline-treated animals while tumor sections from Ang-(1-7)-medicated animals had reduced perivascular fibrosis. The heptapeptide decreased collagen deposition around the blood vessels by 73% [49.3±3.2% versus 13.4±2.2% fibrosis per blood vessel] (Figure 2C), suggesting that Ang-(1-7) reduces both interstitial and perivascular fibrosis in breast tumors.

Interstitial fibrosis in orthotopic breast tumors was further characterized by the immunoreactivity of collagen I, one of the main isoforms of collagen found in fibrosis.
breast tissue. Representative pictures of collagen I immunoreactivity in ZR-75-1 and BT-474 tumor sections are shown in Figures 3A and 3B, respectively. Ang-(1-7) reduced collagen I deposition by 80% in ZR-75-1 orthotopic breast tumors [16.2±1.7% per field in the tumors of saline-treated mice versus 3.3±0.9% per field in tumors from Ang-(1-7)-treated mice], while a 78% decrease in collagen I deposition was observed in the BT-474 tumors [3.3±0.7% per field in the tumors of saline-treated animals when compared to 0.7±0.1% per field in tumors from Ang-(1-7)-medicated animals].

Inhibition of Tumor-Associated Fibroblast Growth by Ang-(1-7)

In order to identify the molecular mechanism for the Ang-(1-7)-mediated reduction in tumor fibrosis, fibroblasts were isolated from ZR-75-1 tumors. Tumor-associated fibroblasts were isolated by proteolytic digestion and differential plating and characterized as myofibroblasts by positive immunoreactivity to fibronectin, vimentin, collagen I, and α-smooth muscle actin (Figure 4A). The percent of cells which showed positive immunoreactivity for fibronectin, vimentin, and α-smooth muscle actin was determined in sequentially passaged tumoral fibroblasts, to determine whether the cells maintain their phenotype as activated myofibroblasts with time in culture. As shown in Figure 4B, fibroblasts isolated from orthotopic tumors retained the activated myofibroblast phenotype until passage 5; therefore, only cells from passages 2-4 were used for in vitro tumoral fibroblast experiments.

Isolated tumoral fibroblasts were treated with PBS or 100 nM Ang-(1-7) daily for 10 days and the cells were counted using a hemocytometer, as a measure of cell proliferation. Ang-(1-7) significantly reduced the growth of cultured myofibroblasts
isolated from orthotopic breast tumors at days 4, 7 and 10, with a 33% reduction in cell
growth at day 10 [10,700±400 PBS-treated myofibroblasts versus 7000±200 Ang-(1-7)-
treated myofibroblasts] (Figure 5A).

**Ang-(1-7) Reduces MAP Kinase Activity by Up-regulation of a MAP Kinase Phosphatase**

Phosphorylated-extracellular regulated kinases (pERK1/2) are potent mitogenic
signaling proteins implicated in cell survival, growth, and proliferation. pERK1/2 was
measured by Western blot hybridization in protein homogenates from myofibroblasts
stimulated with 10 ng/mL TGF-β and treated with PBS or 100 nM Ang-(1-7), to
determine if the heptapeptide regulates MAP kinase activities. Ang-(1-7) treatment
decreased pERK1 activity by 47% and pERK2 activity by 63% [1.13±0.23 relative
density units (RDU) in PBS-treated cells versus 0.60±0.06 RDU in Ang-(1-7)-treated
cells for pERK1, and 2.27±0.56 RDU in PBS-treated cells versus 0.84±0.08 RDU in
Ang-(1-7)-treated cells for pERK2] (Figure 5B), indicating that the Ang-(1-7)-mediated
anti-proliferative effect may be due to a reduction in activated ERK1/2.

MAP kinases are phosphorylated and activated by MAP kinases kinases (MEKs)
and dephosphorylated and inactivated by MAP kinase phosphatases. Dual specificity
phosphatase 1 (DUSP1), a MAP kinase phosphatase that dephosphorylates and
inactivates ERK1/2, was up-regulated 2.52±0.29-fold by Ang-(1-7) in tumor-associated
fibroblasts, suggesting that the heptapeptide may reduce pERK1/2 activities by up-
regulation of the MAP kinase phosphatase DUSP1 (Figure 5C). Pretreatment with the
Ang-(1-7) receptor (AT_{1,7}R) antagonist D-[Ala^{7}]-Ang-(1-7) or D-[Pro^{7}]-Ang-(1-7) (100
nM) completely blocked the Ang-(1-7)-mediated increase in DUSP1 (Figure 5D), whereas the antagonists alone had no effect, indicating that Ang-(1-7) activated an AT\(_{(1-7)}\) receptor to increase DUSP1 in tumor-associated fibroblasts.

**Ang-(1-7) Reduces Active TGF-β1 and Fibronectin in Isolated Tumoral Fibroblasts**

TGF-β1 is a potent stimulator of fibroblast activation that transforms fibroblasts to secreting myofibroblasts. Active TGF-β1 was quantified by Western blot hybridization in protein homogenates from myofibroblasts isolated from orthotopic breast tumors and treated with PBS or 100 nm Ang-(1-7) for 24 h. Ang-(1-7) reduced TGF-β1 by 45.4±11.7% in myofibroblasts compared to controls (Figure 6A). Since TGF-β1 stimulates myofibroblasts to synthesize and deposit ECM proteins, myofibroblasts were stimulated with 10 ng/mL TGF-β1 and treated with either PBS or 100 nM Ang-(1-7) for 24 h, to determine if the heptapeptide regulated fibronectin synthesis. Ang-(1-7) administration decreased fibronectin by 37.5±12.0% compared to the control (Figure 6B), suggesting that Ang-(1-7) reduces fibronectin synthesis to attenuate ECM protein deposition, fibrosis, and fibronectin signaling.

**DISCUSSION**

The link between fibrosis and breast cancer is well established. Collagen I deposition in the breast leads to increased mammographic density, which correlates with breast cancer risk (24). Increased ECM remodeling and stiffening by collagen and fibronectin enhances tumor cell survival and proliferation (25). Moreover, the secretion of growth factors, cytokines, and pro-angiogenic peptides by myofibroblasts promotes breast tumor growth (5). The present study is the first to show that Ang-(1-7) reduces the
growth of human breast orthotopic tumors with a corresponding decrease in tumoral fibrosis. The reduction in interstitial and perivascular fibrosis was associated with a decrease in collagen I deposition. The heptapeptide reduced the serum-stimulated proliferation of isolated tumoral fibroblasts, suggesting that Ang-(1-7) inhibits myofibroblast growth to reduce tumor fibrosis. The reduction in fibroblast proliferation by Ang-(1-7) was associated with increased DUSP1 and a corresponding decrease in phosphorylated ERK1/2 activities. The up-regulation of DUSP1 was blocked by the AT(1-7) receptor antagonist, indicating that DUSP1 induction by Ang-(1-7) was a receptor-mediated process. Ang-(1-7) also reduced production of active TGF-β and synthesis of fibronectin in the isolated tumoral fibroblasts. This ability of Ang-(1-7) to prevent production of TGF-β1 as well as decrease TGF-β1-stimulated ERK1/2 activation and fibronectin synthesis indicate that the heptapeptide is an antagonist of TGF-β1-mediated fibrosis in breast cancer. Of note, Ang-(1-7) also reduced the proliferation of breast tumor cell in vitro; the molecular mechanisms involved in the inhibition of breast cancer cell proliferation are detailed in a separate manuscript (in preparation).

Collagen I deposition was significantly decreased by Ang-(1-7), to reduce tumor fibrosis. However, the inhibition of collagen synthesis by the heptapeptide may also participate in the reduction in breast tumor growth. Collagen I forms a tissue scaffold to promote tumor growth and stimulates expression of genes associated with cellular metabolism, transcription and translation, including eukaryotic translation initiation factor 4B, glutamyl-prolyl-tRNA polymerase, and Poly (a) polymerase, playing a role in both tumor structure as well as growth and progression (26). Intratumoral collagen I modulates e-cadherin-mediated cell-to-cell contact, to increase tumor invasiveness and
metastases (27, 28). This suggests that the Ang-(1-7)-mediated decrease in collagen I production may also participate in the reduction in tumor proliferation as well as the inhibition of tumor metastases.

Fibronectin, another component of the ECM secreted by activated myofibroblasts, is involved in cell-matrix cell-cell adhesions, cell migration, and oncogenic transformation (29). The deposition of fibronectin in breast tumor stroma is positively correlated with tumor grade, size, and lymph node invasion (30). Fibronectin stimulates tumor cells through activation of integrin signaling to activate both focal adhesion kinases (FAKs) and MAP kinases, increasing cell proliferation, survival, and angiogenesis (31). We observed a 40% decrease in fibronectin in Ang-(1-7)-treated isolated tumoral fibroblasts, suggesting that the heptapeptide may attenuate ECM deposition, tumor invasion and proliferation by reducing fibronectin.

TGF-β binds to its receptors on fibroblasts to activate both MAP kinase and Smad signaling pathways and stimulate cell proliferation and fibrosis. Activation of the Smad signaling pathway by TGF-β results in ECM synthesis and deposition, tenascin-C production as well as its own production, creating an autocrine cycle of fibroblast activation and ECM deposition (5). TGF-β1 expression in breast cancer biopsies positively correlated with the rate of disease progression, independent of node status, tumor stage, age, and ER status, suggesting its role in tumor progression (32). Since Ang-(1-7) reduced TGF-β1 in isolated tumoral fibroblasts, the heptapeptide may attenuate myofibroblast activation by reducing TGF-β1 synthesis and signaling.

The exact molecular mechanism for the Ang-(1-7) mediated down-regulation of TGF-β1 is not known. However, we previously showed that Ang-(1-7) increased cAMP
in vascular smooth muscle cells; (33) since an increase in cAMP inhibited TGF-β-stimulated collagen synthesis by ERK1/2 signaling in cardiac fibroblasts, (34) Ang-(1-7) may increase cAMP in tumoral fibroblasts to reduce TGF-β. In addition, the DUSP1 promoter contains a cAMP-responsive element (35), suggesting that Ang-(1-7) may increase cAMP in tumoral fibroblasts to up-regulate DUSP1, reduce MAP kinase activities, and inhibit fibrosis.

The amount of interstitial fibrosis was 3-fold higher in ZR-75-1 tumors than in BT-474 tumors, as assessed by comparing the basal level of picrosirius red staining in saline-treated mice. Several factors could account for this difference. BT-474 cells express the c-Met receptor, while ZR-75-1 cells do not. Since activation of the c-Met receptor by HGF reduces fibrosis, (36, 37) HGF signaling through the c-Met receptor may be responsible for the decreased total interstitial fibrosis observed in the BT-474 tumors (38, 39). On the other hand, HER2 signaling increases the expression of the Wilms’ tumor suppressor gene (WT1) product through activation of protein kinase B (Akt); (40) WT1 is implicated in tumor suppression and progression as well as inhibition of TGF-β signaling, which may reduce interstitial fibrosis (41, 42). The over-expression of HER2 in BT-474 cells and increased WT1 signaling could also contribute to reduced interstitial fibrosis. Further investigation is warranted to determine the molecular mechanism for the differences in interstitial fibrosis in the ZR-75-1 and BT-474 tumors.

We observed a significant reduction in perivascular fibrosis in BT-474 tumors from Ang-(1-7)-medicated mice when compared to tumors from saline-treated mice. It was not possible to quantify perivascular fibrosis in the ZR-75-1 tumors due to pervasive interstitial fibrosis. The heptapeptide-mediated reduction in perivascular fibrosis in BT-
474 tumors may lead to an overall decrease in the tumoral interstitial fluid pressure. High interstitial fluid pressure, found in breast tumors as well as other types of tumors, is associated with vessel leakiness, lymph vessel abnormalities, and perivascular fibrosis, leading to matrix rigidity and fibroblast contractility and resulting in increased fiber tension (43, 44). This results in decreased transcapillary transport, limiting chemotherapeutic drug delivery to the tumor. These results suggest that Ang-(1-7) may enhance the delivery of chemotherapeutic agents when administered in combination with other therapeutic drugs (43).

The inhibition of tumor fibrosis by Ang-(1-7) is supported by previous studies demonstrating the anti-fibrotic effect of Ang-(1-7) in cardiac and renal cells and tissues. Ang-(1-7) infusion prevented cardiac fibrosis in deoxycorticosterone acetate salt-induced hypertension with a significant decrease in left ventricular wall fibrosis and reduced perivascular fibrosis, (45) in agreement with studies showing that the heptapeptide reduced collagen formation and TGF-β in rat cardiac fibroblasts (46). Studies by our group showed that Ang-(1-7) infusion reduced cardiac fibrosis in Ang II-treated rats, further illustrating the role of Ang-(1-7) as an anti-fibrotic agent (47). Mice with ablated mas, the Ang-(1-7) receptor, have impaired cardiac function with increased cardiac collagen I, collagen III, and fibronectin deposition, (48) while mas deletion increased collagen III, collagen IV, and fibronectin in the renal cortex and medulla (49). Our results demonstrate that Ang-(1-7), through activation of mas, also inhibits tumoral fibrosis.

In a recently reported Phase I clinical trial assessing Ang-(1-7) as a chemotherapeutic agent, we showed that the heptapeptide reduced plasma placental
growth factor (PIGF) in patients with clinical benefit, (50) in agreement with our preclinical studies demonstrating that Ang-(1-7) inhibits angiogenesis (15). Since activated myofibroblasts secrete cytokines which stimulate blood vessel formation, (5) Ang-(1-7) may reduce angiogenesis by inhibiting the growth of tumor-associated fibroblasts. Taken together, these results suggest that Ang-(1-7) has pleiotropic effects on the tumor microenvironment, to decrease tumor fibrosis, inhibit angiogenesis, and reduce tumor growth.

Disclosure of Potential Conflict of Interest
Drs. Gallagher and Tallant hold a patent for the treatment of cancer with Ang-(1-7).

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REFERENCES


McCollum LT, Gallagher E, Tallant EA. Angiotensin-(1-7) Attenuates Cardiac Fibrosis and Vascular Hypertrophy in a Model of Angiotensin II-Dependent Hypertension. Hypertension 2008;52:e34.


Figure 1. Effect of Ang-(1-7) on orthotopic human breast cancer growth. The volumes of human ZR-75-1 (Panel A) or BT-474 (Panel B) orthotopic breast tumors from mice treated with saline or Ang-(1-7) were measured using a caliper, calculated as \( V = \frac{1}{2} (L \times W^2) \), and presented as the percent change compared to initial tumor volume. ZR-75-1 (Panel C) or BT-474 (Panel D) tumor weight from mice infused with saline or Ang-(1-7) was determined at time of sacrifice. * denotes \( p<0.05 \); \( n=5-6 \).
**Figure 2.** Inhibition of breast tumor fibrosis by Ang-(1-7). Representative pictures of picrosirius red-stained sections of ZR-75-1 (*Panel A*) and BT-474 tumors (*Panel B*) from mice treated with saline or Ang-(1-7) are shown at x200 magnification. Interstitial fibrosis was calculated as the percent fibrosis per field in sections of ZR-75-1 or BT-474 tumors. Representative pictures of picrosirius red-stained blood vessels from BT-474 tumors (*Panel C*) at x200 magnification. Perivascular fibrosis was calculated as percent fibrosis per vessel. * denotes p<0.05; n=5-6.
**Figure 3.** Ang-(1-7) reduction in collagen I deposition in breast tumors. ZR-75-1 (Panel A) or BT-474 tumors (Panel B) from mice treated with saline of Ang-(1-7) were incubated with an antibody to collagen I and representative pictures are shown at x200 magnification. Collagen deposition was quantified as the percent collagen I per field in ZR-75-1 (Panel C) and BT-474 tumors (Panel D). * denotes p<0.05; n=5-6.
Figure 4. Characterization of isolated tumoral fibroblasts. **A:** Isolated tumoral fibroblasts were incubated with antibodies to vimentin, fibronectin, collagen I, and α-smooth muscle actin (α-SMA). **B:** The percent of cells which showed positive immunoreactivity for fibronectin, vimentin, and α-SMA was determined in sequentially passaged tumoral fibroblasts. n=2-3.
Figure 5. Ang-(1-7) inhibition of isolated tumor myofibroblast growth. **A:** Tumoral fibroblasts were treated with PBS or 100 nM Ang-(1-7) and 2% FBS and cell number was determined on days 4, 7 and 10, using a hemocytometer. **B:** Tumoral fibroblasts were serum starved overnight, pre-treated with PBS (control) or 100 nM Ang-(1-7) for four h and stimulated with 10 ng/mL TGF-β1 for 2 h. pERK1/2 was assessed by Western blot hybridization and is presented as a function of β-actin. **C:** Tumoral fibroblasts were serum starved overnight and treated with PBS (control) or 100 nM Ang-(1-7) for four h. DUSP1 was assessed by Western blot hybridization and presented as a function of β-actin. **D:** Tumoral fibroblasts were incubated for 4 h with PBS (Control), 100 nM Ang-(1-7) (A7), 1 µM D-[Ala7]-Ang-(1-7) (D-Ala), 100 nM Ang-(1-7) and 1 µM D-[Ala7]-Ang-(1-7) (D-Ala + A7), 1 µmol/L D-[Pro7]-Ang-(1-7) (D-Pro), and 100 nM Ang-(1-7) and 1 µmol/L D-[Pro7]-Ang-(1-7) (D-Pro + A7). DUSP1 was assessed by Western blot hybridization. * denotes p<0.05; n=3-4 of cells from different passage numbers.
**Figure 6.** Effect of Ang-(1-7) on TGF-β and fibronectin in isolated tumor myofibroblasts.  

**A:** Isolated tumoral fibroblasts were serum starved overnight and incubated with 100 nM Ang-(1-7) [A7] for 24 h. TGF-β was assessed by Western blot hybridization and quantified as a function of β-actin.  

**B:** Isolated tumoral fibroblasts were serum starved overnight and treated with 100 nM Ang-(1-7) [A7] and 10 ng/mL TGF-β1 for 24 h. Fibronectin was assessed by Western blot hybridization and quantified as a function of β-actin. * denotes p<0.05; n=3-4.
Chapter III:

Angiotensin-(1-7) Inhibits Estrogen Receptor Positive Breast Tumor Growth with an Associated Reduction in Prostaglandin E Synthase

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ABSTRACT

Over 40,000 American women died from breast cancer last year, despite advances in new therapeutics which target the estrogen receptor or estrogen production. We previously showed that the heptapeptide angiotensin-(1-7) [Ang-(1-7)] inhibits lung tumor growth in association with a reduction in cyclooxygenase 2 (COX-2). The purpose of this study was to determine whether Ang-(1-7) inhibits the growth of estrogen receptor (ER) positive breast cancer. Orthotopic ZR-75-1 human ER positive breast tumors in the mammary fat pad of athymic mice were treated with saline or Ang-(1-7). After 18 days, Ang-(1-7) reduced tumor volume by 40% compared to the volume at treatment initiation, while tumors from control mice continued to grow. Tumor weight decreased 54%, in association with a 61% reduction in Ki67 immunoreactivity. Ang-(1-7) markedly reduced MAP kinase activities [saline, 0.51±0.13 compared with Ang-(1-7), 0.16±0.06 for pERK1 and saline, 1.17±0.33 compared with Ang-(1-7), 0.30±0.07 for pERK2, p<0.05; n= 5-6] with a corresponding 2-fold increase in the MAP kinase phosphatase DUSP1. Ang-(1-7) decreased COX-2 and prostaglandin E synthase (PGES) with no apparent effect on prostacyclin synthase, suggesting that Ang-(1-7) alters the ratio of proliferative to anti-proliferative prostaglandins. Pre-treatment of ZR-75-1 with phosphatase inhibitors or siRNA to DUSP1 prevented the Ang-(1-7)-mediated reduction in phospho-ERK1/2 and PGES, demonstrating that Ang-(1-7) up-regulates DUSP1 to prevent MAP kinase activity and prostaglandin E production. Based on the anti-proliferative and anti-inflammatory properties of the heptapeptide, these results suggest that Ang-(1-7) may be an effective, first-in-class compound for the treatment of breast cancer.
INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and is the second leading cause of cancer deaths in American women, with over 40,000 women in the United States dying from breast cancer last year (1). Estrogen receptor (ER) positive breast cancer is the most prevalent type of breast cancer, accounting for 60-70% of all breast cancer cases (2). Estrogen regulates numerous target genes as well as stimulates acute non-genomic effects, many of which are implicated in cell survival and enhanced proliferation (3, 4). Breast cancers that express ERs can be sensitive to hormonal therapies, such as selective ER modulators (SERMs), or aromatase inhibitors. However, these treatments have limitations and adverse side effects which hinder their use; aromatase inhibitors are only effective in postmenopausal women and SERMs may be ineffective due to drug resistance and cardiotoxicities (5, 6). This suggests that additional targeted therapies for the treatment of ER positive breast cancer are needed.

Angiotensin-(1-7) [Ang-(1-7)], an endogenous, seven amino acid peptide hormone of the renin-angiotensin system, inhibits both cell proliferation and tissue fibrosis through activation of a unique (AT_{1-7}) G protein-coupled receptor mas (7-10). Ang-(1-7) significantly inhibited the growth of human lung cancer cells in vitro and decreased the size of human A549 lung cancer tumor xenografts with a reduction in cell proliferation and tumor angiogenesis (11-13). We recently reported that Ang-(1-7) administration to patients with solid tumors had clinical benefit in 4 out of 15 evaluable patients, indicating that the heptapeptide may serve as a novel chemotherapeutic drug for the treatment of cancer (14).
The Ang-(1-7)-mediated reduction in human lung tumor xenografts was associated with a significant decrease in cyclooxygenase 2 (COX-2) (12). Ang-(1-7) stimulated the production of cAMP and increased prostacyclin in VSMCs, suggesting that the heptapeptide may alter the proliferative to anti-proliferative prostaglandin ratio, to reduce inflammation and inhibit cell growth (15). COX-2 is up-regulated in approximately 40% of invasive breast cancers and positively associates with breast cancer angiogenesis, inflammation, invasion, and metastasis (16). Administration of non-steroidal, anti-inflammatory drugs (NSAIDs) to 5,882 Canadian women for 2-5 years correlated with a 24% reduced risk of developing invasive breast cancer (17). These data suggest that COX-2 has an important role in tumorigenesis.

COX-2 catalyzes both the conversion of arachidonic acid into the precursor prostaglandin PGG\textsubscript{2} as well as the processing of PGG\textsubscript{2} to PGH\textsubscript{2}, while PGES converts PGH\textsubscript{2} into PGE\textsubscript{2} (18). There are four known isoforms of PGES - two cytosolic isoforms, glutathione transferases (GSTM2-2 and GSTM3-3), primarily found in the brain, and two microsomal PGES isoforms, mPGES-1 and mPGES-2. mPGES-1 is an inducible enzyme that is up-regulated in response to numerous pro-inflammatory and mitogenic stimuli, including interleukin-1\textbeta, tumor necrosis factor \alpha, or lipopolysaccharide (19). mPGES-1 is also regulated by activation of MAP kinase signaling pathways in cardiac fibroblasts and myocytes (20). The inducible mPGES-1 has a higher catalytic activity when compared to the other PGES isoforms, suggesting that mPGES-1 has a key role in the synthesis of PGE\textsubscript{2} from PGH\textsubscript{2} (18, 21). mPGES-1 is over-expressed in 79% of breast cancers and amplification of mPGES-1 is independent of COX2, suggesting that dual
inhibition of COX2 and mPGES-1 may be more beneficial in the treatment of breast cancer than COX-2 or mPGES-1 inhibition alone (22).

We investigated the effect of Ang-(1-7) on the growth of ZR-75-1 ER positive breast cancer cells and tumors, using an orthotopic mouse model and examined the effect of the heptapeptide on MAP kinase ERK1/2 and the prostaglandin synthesizing enzymes.

Materials and Methods

Materials: The following materials were purchased from the companies in parentheses. Ang-(1-7) and [D-Alanine]-Ang-(1-7) (Bachem, King of Prussia, PA); [D-Pro]-Ang-(1-7) (GenScript Corporation, Piscataway, NJ); penicillin, RPMI-1640, OptiMem, streptomycin, fetal bovine serum (FBS), dialyzed FBS, and hypoxanthine-aminopterin-thymidine (HAT) supplement (Gibco Invitrogen BRL, Carlsbad, CA); Matrigel (BD Biosciences, Bedford, MA); TRIzol reagent (Life Technologies/Invitrogen, Carlsbad, CA); RQ1 DNase (Promega, Madison, WI); Taqman Universal PCR Master Mix and mas primer probe sets (Applied Biosystems, Foster City, CA); Tween-20 (Fisher Scientific, New Jersey); sodium vanadate and okadaic acid (Merck KGaA, Darmstadt, Germany). Antibodies were purchased from the following sources: mas (Alamone Labs, Israel), Ki67 (Abcam, Cambridge, MA), phosphorylated ERK 1/2 (Cell Signaling Technology, Danvers, MA), DUSP1 (Millipore, Eschborn, Germany), COX2, PGES, PGIS (Cayman Chemical, Ann Harbor, MI), β-actin (Sigma-Aldrich, St. Louis, MO), and polyclonal and HRP-conjugated secondary antibodies (GE Health Care, Buckinghamshire, UK).

Cell Culture: ZR-75-1 breast ductal carcinoma cells (ATCC CRL-1500), derived from a 63 year old Caucasian female, were grown in RPMI media containing 10% FBS, 100
µg/mL penicillin, 100 units/mL streptomycin and 10 nM Hepes, at 37°C in a humidified atmosphere of 5% CO₂ and 95% room air. Cells were incubated for 24 h with serum-deficient, phenol red-free media, prior to each treatment and were stimulated with 1% dialyzed FBS phenol-free media containing 10 nM estradiol.

**Cell Growth:** ZR-75-1 cells (1 x 10⁴) in phenol-free RPMI containing 0.5% FBS and 10 nM estradiol were plated in 24-well tissue culture plates. On the first day after plating and every 24 h afterwards, the cells were treated with 10 nM estradiol and either PBS [phosphate-buffered saline, 50 mmol/L NaPO₄ (pH 7.2), 100 mmol/L NaCl]) or 100 nM Ang-(1-7) in PBS, added daily due to degradation of the heptapeptide. Every two to three days, cells treated with either PBS or Ang-(1-7) were removed from individual wells using trypsin/EDTA and counted using a hemocytometer, to determine cell number.

**Orthotopic Model of Human Breast Cancer:** Female athymic mice (15-20 g, 4-6 weeks of age, Charles River Laboratory, Wilmington, MA) were housed in cages with HEPA-filtered air (12-h light dark cycle) and provided *ad libitum* access to food and autoclaved water. All procedures complied with the policies of the Wake Forest University Animal Care and Use Committee. Mice were ovariectomized to prevent cycling of hormones and supplemented with 0.18 mg 17β-estradiol 90-day time release pellets (Innovative America, Saratoga, FL). Actively growing ZR-75-1 cells (2 x10⁶) were suspended in 50% PBS and 50% Matrigel and injected into the inguinal mammary fat pad of 5 to 7 week old athymic female mice. Tumor size was measured every other day using a caliper and tumor volume was calculated using the formula \( V = \frac{1}{2} \times \text{Length} \times \text{Width}^2 \).
When tumors reached an approximate volume of 100 mm³, the mice were implanted with an osmotic minipump for subcutaneous release of either saline or Ang-(1-7) at a concentration of 24 μg/kg/h. After 18 days of treatment, the animals were sacrificed and tumors were excised for subsequent analysis.

Reverse transcriptase real time polymerase chain reaction (RT real-time PCR): RNA was isolated from orthotopic breast tumors using TRIzol as directed by the manufacturer. The RNA was incubated with RQ1 DNase to eliminate any residual DNA that would amplify during the PCR. The RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). RT real-time PCR assay was performed as described (13). The results were quantified as C_t values, where C_t is defined as the threshold cycle of PCR at which the amplified product is first detected, and defined as relative gene expression (the ratio of target/control).

Immunohistochemistry: Tumors were fixed in 4% paraformaldehyde for 24 h and incubated in 70% ethanol for 48 h prior to embedding in paraffin. The embedded tumors were cut into five micron thick sections and stained with hematoxylin and eosin to determine morphology. Tumor cell proliferation was measured by immunostaining with an antibody to Ki67 (1:100) using the streptavidin-biotin method, as described (23, 24). Stained sections were visualized with a Leica DM 4000 microscope and photographed with QImaging Retiga 1300R camera. A computer-assisted counting technique with a grid filter to select cells was used to quantify the immunohistochemical staining of Ki67.
The number of immunostained cells is expressed as a percentage of the total cell number examined (100 cells counted from each tissue site within a tumor section).

**Western Blot Hybridization:** Tumor tissue (~ 1 to 2 mm² in size) was homogenized in PBS using a Qiagen TissueLyser (Retsc, Hann, Germany) and Western blot hybridization was performed as described (13). Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto West or Pico West,) and quantified by densitometry. Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β-actin (1:2000).

**Transfection of siRNAs:** siRNAs duplexes that targeted mRNA sequences encoded by DUSP1 were synthesized as follows - (5'-CCAAUUGUCCCAACAUUU-3' 5'-GCAUAACUGCCUUGAUCAA’ 5'-GCACAAUCUUCUUCCUCA-3’ 5'-GAAGGGUGUUGUCCACUG-3’). ZR-75-1 breast cancer cells in OptiMEM media (at 50 to 60% confluence) were transfected with siRNA using Oligofectamine at a final concentration of 0.24 µM. An siRNA to cyclophilin, which has no known homology to any gene sequences in mice, rats or humans; was used to determine non-specific effects of transfection. Following a 48 h incubation with siRNAs, the cells were made quiescent by treatment with serum-deficient OptiMem for 24 h. Quiescent cell monolayers were treated with 100 nM Ang-(1-7) for 8 h and harvested in lysis buffer. DUSP1, pERK1/2, and PGES were quantified by Western blot hybridization, as described (13).
Statistics: All data are presented as the mean ± standard error of the mean (SEM). Statistical differences were evaluated by Student’s t test or one way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. The criterion for statistical significance was p < 0.05.

Results
Inhibition of human ER positive breast cancer cell growth by Ang-(1-7). Actively growing ZR-75-1 cells were treated daily with 100 nM Ang-(1-7) or saline and cell number was determined at days 4, 7 and 10 using a hemocytometer, to determine the effect of Ang-(1-7) on cell growth. Ang-(1-7) caused a significant reduction in cell growth at day 4, 7, and 10, [Figure 1A; day 4 control 6.92±0.16 x 10^4 compared to Ang-(1-7), 4.08±0.45 x 10^4 cells; day 7 control, 9.62±0.63 x 10^4 compared with Ang-(1-7), 5.62±0.44 x 10^4 cells; day 10 control, 11.62±1.02 x 10^4 compared with Ang-(1-7), 6.75±0.59 x 10^4 cells; p<0.01; n = 4] and a 42% decrease in cell count at day 10.

Reduction in ER positive breast cancer tumor growth by Ang-(1-7). The effect of Ang-(1-7) on tumor growth was examined in female athymic mice with orthotopic ZR-75-1 ER positive human breast tumors. Athymic mice were ovariectomized to prevent the normal cycling and supplemented with estrogen pellets for the continuous release of the hormone. Seven days after ovariectomy, actively proliferating ZR-75-1 cells (2 x10^6 in Matrigel) were injected into the right 3rd inguinal fat pad. When the tumors were approximately 100 mm^3, osmotic minipumps were implanted between the shoulder blades for the subcutaneous administration of either saline or Ang-(1-7), at a dose of 24 µg/kg/h. Tumor volumes at the initiation of treatment were similar in both groups
Tumors from mice treated with saline increased in volume over time, whereas the tumors from mice administered Ang-(1-7) were markedly reduced in size, reaching statistical significance by day 6 [211.3±17.5 mm$^3$ in saline-treated animals compared with 95.3±9.0 mm$^3$ in the Ang-(1-7)-treated group; p<0.001; n=5-6]. By day 18, tumors from mice treated with Ang-(1-7) were reduced approximately 40% as compared to the initial tumor volume (to 74.8 ±13.6 mm$^3$), while the tumors from saline-treated mice grew to a final volume of 337.4±28.6 mm$^3$. Due to the diminished size of the Ang-(1-7)-treated tumors, the mice were euthanized at day 18 of treatment to have adequate tissue for further analysis. Mice administered Ang-(1-7) had a 54% decrease in tumor wet weight compared to tumors from saline-treated animals [saline, 0.28±0.03 g compared with Ang-(1-7), 0.13±0.02 g; p<0.005, n=5-6]. A representative photograph is shown in Figure 1C. Mice maintained their body weight, food and water intake was normal, and there was no evidence of impaired motor function during treatment. At time of sacrifice, the mice showed no signs of gross pathological abnormalities, indicating a lack of adverse toxicities due to administration of Ang-(1-7).

Mas in tumors from mice treated with saline or Ang-(1-7) was measured by Western blot hybridization and RT real-time PCR, to determine whether the heptapeptide regulated the AT$_{1a}$ receptor. Ang-(1-7) administration had no effect on either transcriptional regulation of mas or the amount of mas in the tumors (Figure 1D).
Decrease in proliferation in Ang-(1-7) treated-breast tumors. A section from the tumors of mice treated with either saline or Ang-(1-7) was fixed in formalin for immunohistochemical analysis and 5-µm sections from each tumor were stained with an antibody to Ki67. Ki67 is present in all actively growing phases of the cell cycle and is absent from G0 phase, making Ki67 a molecular marker for actively proliferating cells. Representative pictures of Ki67 stained sections of tumors from mice treated with saline or Ang-(1-7) are shown in Figure 2A. Mice infused with the heptapeptide had reduced Ki67 immunoreactivity when compared to the animals treated with saline (Figure 2B; saline, 68.2±3.8% compared with Ang-(1-7), 26.8±2.0; p<0.05; n=5-6).

Ang-(1-7) decreases phosphorylated ERK1/2 and increase DUSP1. The MAP kinases ERK1 and ERK2 play significant roles in the regulation of cell proliferation and their activities are increased in association with aberrant cell growth. Sections of ZR-75-1 tumors from mice treated with saline or Ang-(1-7) were analyzed by Western blot hybridization using an antibody to phosphorylated ERK1/ERK2, to determine whether Ang-(1-7) administration reduces MAP kinase activities. Ang-(1-7) reduced phospho-ERK1 by 67% and phospho-ERK2 by 74% when compared to tumors from mice treated with saline [Figure 3A; saline, 0.51±0.13 relative density units (RDU) compared with Ang-(1-7), 0.17±0.06 RDU for phospho-ERK1; saline, 1.17±0.33 RDU compared with Ang-(1-7), 0.30±0.07 RDU for phospho-ERK2; p<0.05, n=5-6]. In contrast, no significant difference in unphosphorylated ERK1/2 was observed [Figure 3B; saline, 1.01±0.06 RDU versus Ang-(1-7), 1.17±0.10 RDU for ERK1; saline, 0.68±0.08 RDU compared with Ang-(1-7), 0.59±0.15 RDU for ERK2; n=5-6]. MEK1/2, the kinase
which phosphorylates and activates ERK1/2, was also quantified by Western blot hybridization, to determine whether the reduction in phospho-ERK1/2 is a result of down-regulation of MEK1/2. There was no difference in MEK1/2 between tumor homogenates from mice treated with saline or Ang-(1-7) [Figure 3C; saline, 1.09±0.04 RDU versus Ang-(1-7), 1.11 ± 0.05 RDU; n=5-6], suggesting that the heptapeptide does not down-regulate MEK1/2 to reduce ERK1/2 activity.

The reduction in ERK1/2 phosphorylation and activation could result from an increase in a phosphatase that dephosphorylates and inactivates the kinases. Ang-(1-7) increased DUSP1 (dual-specificity phosphatase or MAP kinase phosphatase MKP-1) by more than 2-fold [Figure 3D; saline, 0.23 ± 0.03 RDU compared to Ang-(1-7), 0.51 ± 0.06 RDU; p<0.003, n=5-6]. In parallel studies in the parental ZR-75-1 cell line, Ang-(1-7) treatment caused a 3.6-fold increase in DUSP1 after 4 h (Figure 4A) which correlated with a significant decrease in ERK1/2 phosphorylation. ZR-75-1 cells were pretreated with 100 nM Ang-(1-7) for 4 h, followed by stimulation with 1% FBS for 2, 5, and 10 min (Figure 4B and Figure 4C). Ang-(1-7) pretreatment significantly decreased both phosphorylated ERK1 and ERK2 following the 4 h pretreatment, with or without serum stimulation. The maximal decrease in MAP kinases phosphorylation was observed following treatment with serum for 10 min [phosphorylated ERK1 - control, 3.02±0.55 RDU compared to Ang-(1-7)-treated, 0.86±0.21 RDU; phosphorylated ERK2 - control, 2.40 ± 0.43 RDU compared to Ang-(1-7)-treated, 0.91±0.25 RDU; p<0.05; n=4].

**Reduction of COX-2 and PGES by Ang-(1-7).** Inhibition of lung tumor growth by Ang-(1-7) was accompanied by a significant decrease in COX-2, suggesting that the
heptapeptide regulates prostaglandin synthesis to reduce tumor size (12). COX-2, PGES, and PGIS were quantified by Western blot hybridization of tumor homogenates from mice treated with saline or Ang-(1-7), to determine the effect Ang-(1-7) on these prostaglandin-synthesizing enzymes. Ang-(1-7) significantly reduced COX-2 [Figure 5A; saline, 1.12±0.22 RDU compared with Ang-(1-7), 0.32±0.05 RDU; p<0.02; n=4-6] and PGES [Figure 5B; saline, 4.60±1.35 RDU compared with Ang-(1-7), 1.03±0.48 RDU; p<0.05; n=5-6] in ZR-75-1 breast tumors. In contrast, Ang-(1-7) had no significant effect on PGIS [Figure 5C; saline, 2.15±0.44 compared with Ang-(1-7), 3.10±0.90 RDU units; n=5-6]. PGES was also significantly reduced in ZR-75-1 cells treated with Ang-(1-7). Quiescent ZR-75-1 cells were treated with 100 nM Ang-(1-7), for 1, 2, 4, 8, and 24 h. Western blot hybridization demonstrated that Ang-(1-7) significantly reduced PGES protein by 54% after 8 h (Figure 5D).

**Ang-(1-7) inactivates MAP kinase and reduces PGES through an up-regulation of DUSP1.** Protein phosphatase inhibitors and siRNAs to the MAP kinase phosphatase DUSP1 were used to determine whether the up-regulation of DUSP1 by Ang-(1-7) reduces ERK1/2 activities to regulates PGES. ZR-75-1 cells were treated with a serine/threonine phosphatase inhibitor, okadaic acid (100 nM), and the tyrosine phosphatase inhibitor, sodium vanadate (10 µM), for 30 min, followed by incubation with 100 nM Ang-(1-7) and 1% serum for 8 h. Pretreatment with phosphatase inhibitors significantly blocked the Ang-(1-7)-mediated down-regulation of PGES (Figure 6A), suggesting that Ang-(1-7) up-regulates a phosphatase to down-regulate PGES. Since Ang-(1-7) up-regulates the MAP kinase phosphatase DUSP1 in ZR-75-1 cells and
tumors, a siRNA to DUSP1 was used to determine the role of DUSP1 in the Ang-(1-7)-mediated regulation of PGES. Transfection of ZR-75-1 cells with DUSP1 siRNA reduced DUSP1 protein by 60% in ZR-75-1 cells when compared to cells transfected with cyclophilin, used as a control (Figure 6B). ZR-75-1 cells transfected with siRNA to DUSP1 were stimulated with serum and treated with 100 nM Ang-(1-7); Western blot hybridization using a phospho-ERK1/ERK2 antibody showed that the decrease in DUSP1 prevented the reduction in ERK1 by Ang-(1-7) in control cells or cells transected with cyclophilin siRNA; similar results were obtained with ERK2 (Figure 6C). Western blot hybridization using an antibody to PGES was used to determine whether the increase in DUSP1 is associated with the reduction in PGES. Ang-(1-7) reduced PGES in ZR-75-1 cells treated with saline or a siRNA to cyclophilin. In contrast, the inhibition of DUSP1 by siRNA prevented the Ang-(1-7)-mediated reduction in PGES, as observed under saline-treated cells or cells transfected with the siRNA to cyclophilin. These results suggest that Ang-(1-7) up-regulates DUSP1 to inhibit both MAP kinase activation as well as prostaglandin E2 synthase.

DISCUSSION

The present study is the first to demonstrate that Ang-(1-7) reduces ER positive human breast tumor growth with a corresponding decrease in phosphorylated ERK1/2, COX-2, and PGES, suggesting Ang-(1-7) has pleiotropic effects, inhibiting growth and inflammation. A significant decrease in immunoreactive Ki67 was also observed in tumors from mice treated with Ang-(1-7), suggesting that Ang-(1-7) inhibits tumor cell proliferation to reduce tumor size. Activated ERK1/2 are key regulators of many cellular
functions including growth, proliferation, and differentiation and are implicated in cancer progression and tumor growth (25). Ang-(1-7) reduced phospho-ERK1/2 in ZR-75-1 human ER positive breast cancer tumors, in agreement with previous reports from our group and others demonstrating that the heptapeptide reduced serum-stimulated phosphorylation of ERK1/2 in human lung cancer cells, rodent VSMCs, cardiac myocytes, and renal proximal tubule cells (10, 11, 15, 26). MAP kinase activities are regulated by multiple mechanisms including phosphorylation and activation by MEKs as well as dephosphorylation to reduce activity (25). We recently reported that the Ang-(1-7)-mediated decrease in phosphorylated ERK1/2 involved the up-regulation of a phosphatase in VSMCs (27). In ER positive human breast cancer cells, the reduction in ERK1 and ERK2 MAP kinase activities was associated with a 2-fold increase in the MAP kinase phosphatase DUSP1. No changes in total ERK1/2 or MEK1/2 were detected following treatment with Ang-(1-7). Down-regulation of DUSP1 by treatment of ZR-75-1 cells with a siRNA to the phosphatase prevented Ang-(1-7) reduction in ERK1/ERK2 phosphorylation, demonstrating that DUSP1 dephosphorylates and inactivates the kinases in ER positive breast cancer cells.

DUSP1 or MKP-1 is an inducible nuclear phosphatase that inactivates JNK, p38, and ERK1/2 (28). DUSP1 had a 10-25-fold higher expression in ovarian epithelial cells than in ovarian cancer cell lines, in agreement with an increase of the phosphatase in non-cancerous ovarian tissue compared to malignant tissue (29). In addition, over-expression of DUSP1 in ovarian cancer cells attenuated cell growth in vitro and reduced intra-peritoneal tumor growth in athymic mice (29). In lung cancer, increased DUSP1
expression correlated with improved survival (30). Taken together, these studies suggest a role for DUSP1 as a tumor suppressor.

We previously showed that the Ang-(1-7)-mediated reduction in human A549 in human lung cancer xenografts was associated with a significant decrease in COX-2, suggesting that Ang-(1-7) inhibits a pro-inflammatory mechanism to reduce tumor growth (12). We observed a similar reduction in COX-2 in human ER positive breast tumors. Increased COX-2 and the production of prostaglandins is associated with numerous human neoplasias, including 40% of invasive breast carcinomas (16, 31). COX-2 over-expression in human breast tumors correlates with aggressive disease, increased tumor grade, decreased overall survival, and an elevation in the proliferation index (32, 33). Genetic knockdown of COX-2 in mice decreased mammary tumor development, while over-expression of COX-2 induced mammary neoplasias (34, 35). Inhibition of COX activity with NSAIDs lowered the risk of colorectal adenocarcinomas (36, 37) and treatment with selective COX-2 inhibitors dramatically reduced the growth of both colorectal and lung adenocarcinoma xenograft tumors (18, 38, 39). Moreover, COX-2 inhibitors reduced chemically-induced skin and mammary gland tumorigenesis in rodents (18, 38). Collectively, these studies suggest that induction of COX-2 and the association production of prostaglandins participate in tumorigenesis and the dysregulation of cell growth in numerous cancers, including breast cancer.

Based upon evidence demonstrating a role for the COX-2-mediated production of prostaglandins in tumor development and growth, selective or non-selective COX-2 inhibitors may be useful chemotherapeutic agents. However, many selective COX-2 inhibitors were removed from the market due to an increased risk of cardiovascular
events associated with long term treatment (40) and non-steroidal anti-inflammatory drugs (NSAID) must carry warnings describing the cardiovascular toxicities associated with their use. The cardiovascular side effects observed with the use of selective COX-2 inhibitors may be due to the inhibition of prostacyclin production. COX-2 catalyzes arachidonic acid to a precursor prostaglandin for prostacyclin synthesis. Inhibition of COX-2 leads to the subsequent reduction prostacyclin-mediated vasodilation and the inhibition of platelet aggregation that may participate in the cardiovascular toxicities associated with the use of selective COX-2 inhibitors (40). In contrast, Ang-(1-7) is cardioprotective and reduces thrombosis (41, 42). Since we observed a significant reduction in PGES with no change in PGIS in breast tumors from mice treated with Ang-(1-7), the heptapeptide provide the beneficial effects of a COX-2 inhibitor without the associated adverse cardiovascular events (41, 43-45). No significant cardiovascular toxicities were associated with our recently completed Phase I clinical trial using Ang-(1-7) as a single agent therapy for the treatment of cancer or in a previous Phase I clinical trial examining Ang-(1-7) as a myeloprotective agent(14, 46).

The majority of the oncogenic activities resulting from COX-2 induction are associated with the synthesis of PGE$_2$ and PGE$_2$-mediated signaling events (16, 37). Treatment with PGE$_2$ reversed the anti-proliferative activity NSAIDs in colon cancer cells and reversed the NSAID-mediated inhibition of intestinal adenoma tumor growth in Apc$^{Min}$ mice (47). Since COX-2 tumorigenic properties result from an increase in PGE$_2$ synthesis, research has focused on the PGE$_2$ synthases as potential drug targets for chemotherapy. mPGES-1 is increased in many different types of cancer, including breast cancer. Approximately 79% of invasive breast cancers have mPGES-1 expression, while
mPGES-1 is undetectable in normal breast epithelial cells (22). Genetic deletion of mPGES-1 suppressed intestinal tumorigenesis and genetic knockdown of PGES in lung and prostate cancer cells increased xenograft tumor latency in athymic mice (48, 49). These data suggest that targeting mPGES-1 may be an effective strategy to combat cancer initiation and progression. Since mPGES-1 expression is independent of COX-2, dual targeting of COX-2 and mPGES-1 may be beneficial in the treatment of breast cancer (22).

Ang-(1-7) significantly up-regulated DUSP1 to decrease ERK1/2 activities and reduced mPGES-1 in ZR-75-1 cells and tumors. Supporting data from cardiovascular studies showed that incubation of cardiac fibroblasts with inhibitors of ERK1/2 or JNK MAP kinases prevented the IL-1β-stimulated induction of mPGES-1, indicating a role for MAP kinases in mPGES-1 regulation (20). Collectively, these results suggest that the Ang-(1-7)-mediated increase in DUSP1 regulates both the anti-proliferative and anti-inflammatory effects of the heptapeptide in ER positive breast cancer. Since PGE_2 also promotes estrogen biosynthesis through up-regulating aromatase, the Ang-(1-7) reduction in PGE_2 may also decrease paracrine and autocrine estrogen production to inhibit ER positive breast cancer growth (16, 50).

Based upon our preclinical studies in human lung cancer cells and tumors, a Phase I clinical trial for the use of Ang-(1-7) to treat patients with solid tumors was conducted at the Comprehensive Cancer Center of Wake Forest University (12-14, 27). Four patients displayed clinical benefit of the 15 evaluable patients treated with Ang-(1-7). A Phase II clinical trial for patients with sarcoma is currently in the recruitment phase. The findings reported in this paper demonstrate that Ang-(1-7) inhibits the growth of human
ER positive breast tumors, through a reduction in proliferation and inflammation. These results suggest that Ang-(1-7) should also be considered as a novel therapeutic drug for breast cancer.
Disclosure of Potential Conflict of Interest

Drs. Gallagher and Tallant hold a patent for the treatment of cancer with Ang-(1-7).

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Reference List


(45) Tallant EA, Ferrario CM, Gallagher PE. Cardioprotective role for angiotensin-(1-7) and angiotensin converting enzyme 2 in the heart. Future Cardiol 2006;2:335-42.


Figure 1. Ang-(1-7) reduces ZR-75-1 cell proliferation and orthotopic tumor growth. A. ZR-75-1 cells were treated with 10 nM 17-β-estradiol (E2) and 100 nM Ang-(1-7) or saline daily for 10 days; ZR-75-1 cell number was determined in triplicate wells from 4 different passages with a hemocytometer. * denotes p < 0.05  
B. Orthotopic human ZR-75-1 breast tumors from mice treated with saline or 24 µg/kg/h Ang-(1-7) were measured using a caliper and volume was calculated as $V=\frac{1}{2} L \times W^2$.  
C. Photographs of representative ZR-75-1 tumors from mice infused with either saline or Ang-(1-7) at the time of sacrifice.  
D. mas protein and mRNA were quantified by Western blot hybridization and RT real-time PCR, respectively, in tumors from mice treated with saline or Ang-(1-7). * denotes p<0.05; n=5-6.
Figure 2. Ang-(1-7) decreases proliferation of ZR-75-1 orthotopic tumors. A. Representative photographs of tumor sections from mice treated with saline or Ang-(1-7) and stained with an antibody to Ki67. B. Ki67 immunoreactivity was quantified using a computer-based grid counting system and quantified as the percentage of stained cells per 100 total cells in each tissue section. * denotes p<0.05; n=5-6.
A.

Saline

Ang-(1-7)

B.

Ki67 Immunoreactivity (%)

Saline

Ang-(1-7)

*
**Figure 3.** Ang-(1-7) increases DUSP1 with a corresponding reduction MAP kinase activity. A. Phosphorylated ERK1/2 were measured in homogenates of tumors from mice treated with either saline or Ang-(1-7) by Western blot hybridization.  B. Unphosphorylated ERK1/2 in tumor homogenates from Ang-(1-7)- and saline-treated mice were measured by Western blot hybridization. C. MEK1/2 were measured in homogenates of tumors from Ang-(1-7)-and saline-treated mice by Western blot hybridization. D. DUSP1 was measured in homogenates of tumors from mice treated with saline or Ang-(1-7) by Western blot hybridization. The data are presented as the relative density, as a function of β–actin as the loading control. * denotes p<0.05; n=5-6.
Figure 4. Ang-(1-7) up-regulates DUSP1 with an associated decrease in pERK1/2 activity in ZR-75-1 cells.  

A. Quiescent ZR-75-1 cells were treated with 100 nM Ang-(1-7) for 1, 2, 4, and 8 h. DUSP1 was measured by Western blot hybridization of protein homogenates from treated ZR-75-1 cells.  

B. Quiescent ZR-75-1 cells pretreated with 100 nM Ang-(1-7) for 4 h were stimulated with 1% fetal bovine serum for 2, 5, and 10 min. Phosphorylated ERK1 (pERK1) and ERK2 (pERK2) were measured by Western blot hybridization in protein homogenates from treated ZR-75-1 cells. The data are presented as the relative density, as a function of β–actin as the loading control. * denotes p<0.05; n=cells from 3 to 4 different passage numbers.
Figure 5. Ang-(1-7) reduces COX-2 and PGES with no effect on PGIS. A. COX-2 was measured in homogenates of tumors from mice treated with saline or Ang-(1-7) by Western blot hybridization. B. PGES in tumor homogenates from Ang-(1-7)- and saline-treated mice was measured by Western blot hybridization. C. PGIS were measured in homogenates of tumors from mice treated with saline or Ang-(1-7) and saline treated mice by Western blot hybridization. * denotes p < 0.05, n = 5-6. D. ZR-75-1 cells were treated with 100 nM Ang-(1-7) for 1, 2, 4, 8, and 24 h. PGES was measured by Western blot hybridization in lysates of treated ZR-75-1 cells. The data are presented as the relative density, as a function of β–actin as the loading control. * denotes p<0.05; n = cells from 3 different passages.
Figure 6. Inhibition of Ang-(1-7)-mediated down-regulation of PGES by inhibition or reduction of DUSP1. A. ZR-75-1 cells pretreated for 30 min with the phosphatase inhibitors (PI) okadaic acid (100 nM) and sodium orthovanadate (10 µM) were incubated with 100 nm Ang-(1-7) and 1% serum for 8 h; PGES was measured by Western blot hybridization. B. ZR-75-1 cells were transfected with siRNA to DUSP1 (Du siRNA) or cyclophilin siRNA (CP siRNA) for 2 days; DUSP1 was measured by Western blot hybridization. * p<0.05; n = 7-11. C. ZR-75-1 cells were transfected with either an empty vector (Control), an siRNA to cyclophilin (CP siRNA), or an siRNA to DUSP1 (DU siRNA) and treated with 100 nM Ang-(1-7) for 8 h. Phospho-ERK1 was measured by Western blot hybridization. D. ZR-75-1 cells were transfected with either an empty vector (Control), an siRNA to cyclophilin (CP siRNA), or an siRNA to DUSP1 (DU siRNA) and treated with 100 nM Ang-(1-7) for 8 h. PGES was measured by Western blot hybridization. The data are presented as the relative density, as a function of β–actin as the loading control. * denotes p<0.05; n = cells from 3-6 different passages.
Chapter IV: General Discussion

Our group was the first to show that Ang-(1-7) inhibits cells growth, in the vasculature as well as in cancer cells and tumors (1-4). Ang-(1-7) inhibited the growth of human lung cancer cells \textit{in vitro}, by activation of the unique \textit{AT}_{(1-7)} receptor, \textit{mas}, and a reduction in MAP kinase activity. Moreover, infusion with Ang-(1-7) decreased the growth of human A549 non-small cell lung adenocarcinoma xenograft tumors with an associated decrease in COX-2 and VEGF expression, indicating that the heptapeptide has anti-inflammatory and anti-angiogenic properties (4, 5). The purpose of the studies reported in this thesis was to determine whether Ang-(1-7) inhibits the growth of human ER-positive and HER2-amplified breast cancer and to identify the molecular mechanisms for the Ang-(1-7)-mediated growth reduction in breast tumors.

Ang-(1-7) Modulates the Tumor Microenvironment in Breast Cancer

The tumor microenvironment plays an important role in all stages of cancer. The stromal microenvironment, in particular the CAF, is implicated in tumor initiation, progression, and metastasis (6). CAF secrete numerous growth factors such as HGF, tenascin-C, PDGF, and TGF-β that influence tumor cell growth. Furthermore, these tumoral fibroblasts are responsible for the deposition of ECM proteins including fibronectin and collagen I, which stimulate tumor growth. Activated myofibroblasts promote metastases by the synthesis of MMPs and stimulate angiogenesis by the secretion of growth factors such as VEGF and PDGF (7, 8). This suggests that activated
fibroblasts present within the microenvironment of the tumor are important in not only the regulation of the growth of the tumor itself but also metastases to distant sites.

Our group first showed that Ang-(1-7) modulated the tumor microenvironment by reducing angiogenesis in human lung tumor xenografts (5). In the current study, we found that infusion of Ang-(1-7) into mice with orthotopic breast tumors significantly reduced tumor fibrosis in association with a decrease in intratumoral collagen I deposition. In isolated tumor-associated fibroblasts, Ang-(1-7) treatment markedly reduced cell growth as well as activation of the MAP kinases ERK1/2 and the production of TGF-β and fibronectin. In contrast, the heptapeptide increased DUSP1 2-fold, which was inhibited by pre-incubation with an AT(1-7)R antagonist, indicating that the increase in DUSP1 is mediated by the Ang-(1-7) receptor. Taken together, these data suggest that Ang-(1-7) also modulates the tumor microenvironment by reducing tumor fibrosis.

We showed that Ang-(1-7) administration inhibited the proliferation of fibroblasts isolated from orthotopic breast tumors. This decrease in CAF proliferation may result in reduced desmoplasia and ECM deposition (9), to attenuate the collagen scaffolding which provides support for the tumor and ECM-stimulated integrin signaling (10, 11). The diminished reactive stroma may also attenuate tumor growth by reducing the production of cytokines which stimulate angiogenesis; CAF secrete numerous cytokines and pro-angiogenic proteins including VEGF, PDGF, HGF, and TGF-β (8). In addition, CAF participate in the regulation of invasive cancer growth (12). Genes that encode invasion-associated proteins are up-regulated in myofibroblasts in breast and basal cell carcinoma.
and an increase in disease reoccurrence was associated with α-smooth muscle actin expression in colorectal cancer (7). More importantly, co-injection of activated myofibroblasts with breast tumor cells in a xenograft mouse model, increased invasive tumor growth when compared to the tumor invasiveness of the breast cancer cells alone, demonstrating the promoting role CAF play in tumor invasion (7). Taken together, these data suggest that the inhibition of CAF proliferation by Ang-(1-7) may reduce tumor growth, angiogenesis, and invasiveness (7).

Ang-(1-7) reduced TGF-β production by fibroblasts isolated from ZR-75-1 tumors. TGF-β has both growth inhibitory and growth stimulatory properties, which are dependent upon its concentration. The Ang-(1-7)-mediated reduction in TGF-β expression may inhibit the pro-oncogenic activities of TGF-β such as growth stimulation, increased angiogenesis, suppression of immune surveillance, and increased cancer cell motility and invasiveness (13). A decrease in TGF-β expression in breast tumors was associated with increased patient survival (14). On the other hand, TGF-β signaling has tumor suppressor activities which include growth inhibition, activation of apoptosis, induction of replicative senescence, and maintenance of tissue architecture (13, 15). However, these tumor suppressor activities of TGF-β are lost with tumor progression and the loss of suppressor activity is associated with increased expression of TGF-β in the tumor microenvironment (16).

Unlike most tumor suppressors, TGF-β is not a simple on/off mechanism, where homozygous loss of the TGF-β allele promotes carcinogenesis. The loss of TGF-β
suppressor response may be due to a dosage effect; for example in endothelial cells, increased levels of TGF-β differentially effects distinct type I receptors, leading to activation of diverse downstream signaling (13). In mice, heterozygous loss of one TGF-β allele is sufficient to inhibit the growth suppressor activity of TGF-β. In addition, TGF-β stimulatory activities become the predominate signaling output promoting tumor growth when MAP kinases are hyper-activated, Smad activity is modulated, or cell cycle inhibitors are suppressed (13). Given the dose-dependent complexity of TGF-β signaling, the Ang-(1-7)-mediated reduction in TGF-β synthesis by CAF suggests that the heptapeptide may inhibit the pro-oncogenic properties of TGF-β to possibly restore TGF-β tumor suppressor activities.

TGF-β is synthesized inside the cell as a large precursor protein; it undergoes various processing steps, one of which is the endopeptidase-catalyzed conversion of the large latent TGF-β to two protein dimers (17). The 65-75 kDa dimer protein on the N-terminal region is the latency-associated peptide while the 25 kDa dimer is the mature TGF-β. The presence of the latency-associate peptide facilitates the transportation of TGF-β from the cell. Found in an inactive form in the extracellular matrix, TGF-β can be activated by cleavage of the latency-associated peptide by numerous factors including MMP-2, MMP-9, thrombospondin (TSP)-1, β6-integrin, plasmin, and reactive oxygen species (ROS) (17, 18). Once activated, TGF-β binds to one of its receptor, TGFβRI or TGFβRII, heterodimerizes and activates one of two distinct pathways. Activated TGF-β receptors phosphorylate Smad 2/3 proteins, which then bind to a mediator protein Smad 4, translocate into the nucleus and stimulate the transcription of matrix associated genes.
TGF-β signaling also activates the MAP kinase pathway, through phosphorylation of ras and rho (17, 18); activated MAP kinases stimulate the proliferation of activated fibroblasts and facilitate transcription of extracellular matrix proteins.

The MAP kinases ERK1/2 are implicated as pro-oncogenic signaling moleculares which are responsible for cancer cell proliferation, growth, and survival (19). Up-regulation of ERK1/2 results in the loss of TGF-β tumor suppressor capabilities. Moreover, increased ERK1/2 activities alter TGF-β downstream signaling to favor proliferation and ECM deposition. Studies investigating the role of ERK1/2 in TGF-β-stimulated transcription in fibroblasts showed that increasing DUSP1 or decreasing MEK by transfection with a dominant negative MEK, both of which would decrease phospho-ERK1/2, ablates TGF-β-stimulated transcription of collagen I, demonstrating the vital role of ERK1/2 in the pro-tumorigenic properties of TGF-β (20). Moreover, pre-treatment with PD-98059, a MEK1/2 inhibitor, significantly blocked TGF-β-stimulated angiogenesis and endothelial cell survival, implicating ERK1/2 as mediators of the pro-angiogenic activity of TGF-β (21). In addition, inhibition of ERK1/2 reduced TGF-β-stimulated TSP-1 expression in mouse fibroblasts, suggesting that the Ang-(1-7)-mediated reduction of ERK1/2 activities may inhibit TSP-1 synthesis to alter the self-promoting TGF-β autocrine feed-back loop (22). Taken together, these data demonstrate the importance of ERK1/2 signaling in the pro-carcinogenic activity of TGF-β, suggesting that the Ang-(1-7)-mediated reduction in TGF-β and phospho-ERK1/2 may restore TGF-β tumor suppressor activities.
Ang-(1-7) administration may also affect immune function since TGF-β suppresses immune function (23). TGF-β inhibits T-cell function and neutrophil activation, prevents natural killer cell stimulation, and promotes the differentiation of monocytes to macrophages (16, 24). Since Ang-(1-7) reduces TGF-β and TGF-β suppresses immune function, the heptapeptide may increase immune surveillance and function to inhibit tumor initiation and growth, which could be explored in syngenic models.

In contrast to the Ang-(1-7)-mediated reduction in the tumor microenvironment and its effects to reduce tumor growth, Ang II and the Ang II-mediated stimulation of the AT₁ receptor also affect the tumor microenvironment. Comparing wild type and AT₁R knock-out mice injected with syngeneic tumors, host stroma AT₁R contributed directly to host angiogenesis by induction of VEGF (25). The AT₁R is highly expressed on tumor-associated macrophages (TAMs). Macrophage infiltration and TAM-secreted VEGF was significantly reduced in AT₁R null mice when compared to wild-type mice. Since Ang-(1-7) often opposes the effects of Ang II, the heptapeptide may also play a role in inflammation-related tumor processes to contribute to the reduction in tumor growth (26).

**Regulation of Fibrosis by Angiotensin Peptides**

The role of Ang II and Ang-(1-7) in modulating the TGF-β pathway in the cardiovascular system has been investigated in a number of different models. Ang II stimulates both cardiac and renal fibrosis (27), by increasing TSP-1 in human mesangial cells and MMP-2 and MMP-9 in cardiac myocytes, to induce TGF-β activation and
promote fibrosis (28-30). In addition, Ang II directly increases TGF-β production in cardiac myocytes, fibroblasts, and proximal tubule cells, suggesting that the pro-fibrotic activity of Ang II may be due, in part, to TGF-β activation (31-33).

In contrast, Ang-(1-7) reduces cardiac fibrosis. Studies by our group and others showed that infusion of Ang-(1-7) inhibited cardiac fibrosis in an Ang II-stimulated model of hypertension, with both reduced perivascular and interstitial fibrosis. Infusion of the heptapeptide prevented cardiac fibrosis in a deoxycorticosterone acetate (DOCA)-salt induced model of hypertension, with a significant decrease in left ventricular wall fibrosis and reduced perivascular fibrosis (34). Furthermore, Ang-(1-7) reduced collagen formation as well as TGF-β, endothelin-1, and leukemia inhibitory factor expression in isolated adult rat cardiac fibroblasts (35). Infusion of the heptapeptide into New Zealand white rabbits following angioplasty in the abdominal aorta significantly decreased neointimal thickness with an associated reduction in TGF-β and phospho-Smad2 as well as a decrease in collagen I and collagen III mRNA, suggesting that the Ang-(1-7)-mediated activation of TGF-β signaling inhibited vascular fibrosis. The anti-fibrotic responses to Ang-(1-7) following angioplasty were attenuated by infusion of an AT₁ receptor antagonist, suggesting that the Ang-(1-7)-mediated reduction in fibrosis was mediated by the AT₁ receptor (36). Development of an AT₁ receptor knock-out mouse provided further evidence of the heptapeptide’s anti-fibrotic effects. Mas receptor knockout mice have impaired cardiac function with increased cardiac collagen I, collagen III, and fibronectin deposition (37), suggesting that Ang-(1-7) regulates the production of these fibrotic proteins in the heart. Ang-(1-7) prevents fibrosis in a number of other
tissues, including the kidney and liver. In the mouse kidney, mas receptor deletion resulted in a marked increase in collagen III, collagen IV, and fibronectin in the cortex and medulla of the kidney (38), demonstrating the anti-fibrotic effects of the heptapeptide in the kidney. The activity of the ACE2/Ang-(1-7)/Mas axis is critical in regulating renal protection since Ang-(1-7) infusion prevented glomerular and tubular fibrosis following ovariectomy (39). Ablation of the ACE2 gene led to glomerulosclerosis, demonstrating the necessity of the enzyme for maintenance of normal kidney structure (40). An elevation in the RAS, particularly an increase in Ang II, is also implicated in the pathogenesis of liver disease, characterized by inflammation and fibrosis. Chronic infusion of the heptapeptide attenuated fibrosis in a rat model of liver cirrhosis induced by bile duct ligation (41). Furthermore, liver injury, induced by bile duct ligation or exposure to toxic compounds, resulted in increased liver fibrosis in ACE2 knockout mice compared to wild-type mice and treatment with recombinant ACE2 markedly attenuated hepatic fibrosis in both models of liver injury (42), suggesting that increased ACE2 limits liver fibrosis. Elevation in circulating Ang-(1-7) by exogenous infusion or by ACE2 over-expression may protect against tissue remodeling and scarring in multiple organs.

The Tumor Microenvironment as a Target for Chemotherapeutic Drugs

Since the tumor microenvironment plays an important role in both tumor growth and progression, drugs were developed to target the stromal microenvironment and inhibit tumor growth. One of the first drugs targeting the tumor microenvironment was bevacizumab (Avastin ®), a monoclonal antibody against the pro-angiogenic growth factor VEGF; bevacizumab binds to VEGF, preventing binding and activation of its
receptor (43), and is the first FDA-approved drug to target the tumor microenvironment. VEGF is the most potent regulator of tumor angiogenesis, activating a receptor tyrosine kinase on the surface of endothelial cells to stimulate vessel growth. Activation of the VEGF receptor leads to stimulation of many cellular signaling processes and results in the formation of blood vessels; in addition, VEGF regulates the proliferation, migration and survival of endothelial cells and cancer cells as well as other types of cells. The pleiotropic effects of VEGF in inhibiting tumor growth make it an attractive target for chemotherapy.

In animal models of colorectal cancer, bevacizumab was effective at inhibiting the pro-angiogenic effects of VEGF and reducing tumor growth (43, 44). Although the same effectiveness for bevacizumab as a single agent was not observed in the clinical setting (45), bevacizumab is effective when used in combination with other chemotherapeutic agents. A Phase III clinical trial showed a 4.7-month increase in overall survival when bevacizumab was used in combination with chemotherapy (irinotecan/5-fluorouracil/leucovorin) in previously untreated, metastatic colorectal cancer patients (46). Combinations of bevacizumab with different chemotherapeutic agents is approved for the treatment of specific types of cancer— in combination with 5-FU for the treatment of colorectal cancer, with paclitaxel in metastatic breast cancer, with carboplatin and paclitaxel in non-squamous non-small cell lung cancer, and with interferon alpha in metastatic renal cell carcinoma.
Targeting angiogenesis with bevacizumab was originally focused on the endothelium; however angiogenesis requires the crosstalk between the extracellular matrix, the endothelium, and cells derived from the tumor stroma, suggesting that bevacizumab therapy also modulates these molecules to regulate their interactions (47). VEGF receptors are present on VSMCs, macrophages, dendritic cells, as well as hematopoietic stem cells/bone marrow-derived cells. Perhaps the most obvious effect of bevacizumab therapy on the tumor microenvironment is the normalization of the tumor vasculature by restoring oxygen levels and easing tumor hypoxia. In a Phase I clinical trial, a single dose of 5 mg/kg of bevacizumab decreased microvascular density which was associated with a reduction in tumor interstitial fluid pressure and increased pericyte coverage (48). This stabilization of the tumor microenvironment resulted in normalization of the tumor vasculature and may explain why bevacizumab is more effective in combination rather than as a monotherapy.

The responses to bevacizumab when used in other non-cancer pathologies can be extrapolated to predict other effects of the drug on the tumor microenvironment. In a model of glaucoma in rabbits, animals treated with bevacizumab that underwent surgery had reduced scar formation and decreased fibroblast proliferation which was related to a reduction in collagen deposition and fibrosis when compared to untreated animals (49). A reduction in tumor fibrosis by bevacizumab therapy may lead to normalization of the tumor stroma which might improve the delivery of chemotherapy to the tumor. The pleiotropic effects of VEGF and the presence of VEGF receptors on many cell types suggest that bevacizumab may affect numerous processes associated with the tumor.
microenvironment. Inhibition of VEGF or blockage of its receptor is not sufficient to reduce tumor growth in patients. However, inhibition of VEGF by bevacizumab has a significant increase in the overall survival of cancer patients; this improvement is associated with a profound effect on the cells and growth factors that are associated with the tumor stroma. This suggests that further studies are needed to identify novel drugs which target the tumor microenvironment to inhibit cancer growth.

Preclinical studies also investigated the effect of known therapeutics on the growth of tumor stroma and CAF and showed promising results. Halofuginone, a collagen-I inhibitor that is FDA approved for the treatment of scleroderma, is currently undergoing clinical trials in Europe as a potential chemotherapeutic agent for the treatment of solid tumors (50). Administration of halofuginone to mice with either prostate cancer or Wilms’ tumor xenografts significantly reduced tumor size with an associated decrease in collagen I and α-smooth muscle actin immunoreactivity (51). Commonly used as adjuvant therapy in breast cancer, tamoxifen was given to female rats to determine the effect of the anti-estrogen on the mammary stroma (52); tamoxifen suppressed mammary epithelial cell proliferation. In addition, primary cultures of fibroblasts treated with tamoxifen had decreased motility, reduced fibronectin production, and were quiescent, suggesting that tamoxifen suppressed the reactive stroma phenotype (52). Moreover, ECM excised from mammary tissue of tamoxifen-treated rats had reduced breast cancer cell motility, invasion, and organoid size in a 3-dimensional culture model when compared to ECM from untreated rats, demonstrating the growth-promoting role of the tumor stroma during tumor development and the importance of treating both
the tumor cells as well as the tumor microenvironment (52). A study investigating the humanized HER2-targeted antibody, trastuzumab or Herceptin®, to treat the tumor stroma showed that trastuzumab inhibited the proliferation of L87/4 and L88/5 stroma cells (53). These data suggest that trastuzumab not only affects the growth of HER2-amplified cancer cells, but also modulates the reactive stroma. Treatment of L88/5 stromal cells with trastuzumab significantly decreased the production of VEGF, suggesting that the anti-angiogenic activity of trastuzumab may be due to a reduction in the secretion of angiogenic factors from tumor-associated stromal cells (53). Imatinib mesylate, also known as Gleevec®, has anti-fibrotic and anti-proliferative effects on human breast stromal fibroblasts (54). In fibroblasts isolated from surgically removed breast tumors, Imatinib mesylate significantly reduced DNA synthesis, measured by ³H-thymidine incorporation, and progression through the cell cycle, measured by flow-cytometry (54). Taken together, these studies demonstrate that chemotherapeutic agents which are currently in use for their ability to inhibit the growth of malignant cells or reduce angiogenesis also modulate the tumor microenvironment; this suggests that the anti-growth activity of these drugs may be due, in part, to suppression of the growth of fibroblasts in the reactive stroma.

Clinical studies directly targeting the reactive stroma also showed some success in reducing tumor growth. A Phase I dose escalation study of sibrotuzumab, a humanized monoclonal antibody to the fibroblast activation prote in (FAP), evaluated 26 patients with non-small cell lung cancer or colorectal cancer (55, 56). The antibody was localized to the tumor, demonstrated using a gamma camera, and the objective response was not
reached after three months of treatment resulting in the cessation of the study; however, two patients showed stable disease, one of them for over 2 years. The results of this study suggest that CAFs promote tumor growth and that drugs which target activated fibroblasts may be effective in the treatment of cancer.

**Ang-(1-7) as an Anti-inflammatory Agent in the Treatment of ER Positive Breast Cancer**

Chronic inflammation is a risk factor for cancer. For example, the human papilloma virus causes cervical cancer, helicobacter pylori bacterial infection is associated with gastric adenocarcinoma, hepatitis B viral infection is associated with cirrhosis, and hepatocellular carcinoma, and asbestos induces inflammation and mesothelioma (57). Chronic inflammation induces a remodeling of surrounding tissue and suppression of the immune system, supporting tumor development. The metabolism of arachidonic acid to pro-inflammatory prostaglandins, catalyzed by COX-2 and prostaglandin E\(_2\) synthase, is a key initiating step in inflammation. COX-2 was up-regulated in 40% of invasive breast carcinomas and mPGES-1 was significantly increased in 79% of breast cancers, suggesting a key role of inflammation in breast cancer (58, 59).

We previously demonstrated that Ang-(1-7) infusion inhibited the growth of human A549 non-small cell lung adenocarcinoma xenografts with an associated reduction in COX-2 (4). In ZR-75-1 human ER positive orthotopic breast tumors, treatment with the heptapeptide significantly reduced COX-2 as well as mPGES-1 with
no apparent change in PGIS, suggesting that the heptapeptide alters the PGE$_2$ to PGI$_2$ ratio to favor the anti-proliferative, anti-inflammatory prostacyclin. Ang-(1-7) increased the MAP kinase phosphatase, to reduce activation of the MAP kinase activities, and inhibition of phosphatase activity with either phosphatase inhibitors or siRNA to DUSP1 prevented the Ang-(1-7)-mediated reduction in mPGES-1 expression, demonstrating that the heptapeptide stimulated DUSP1 to reduce mPGES-1.

The oncogenic properties of prostanoids and leukotrienes, which are produced from the cyclooxygenase-catalyzed increase in arachidonic acid, were investigated to determine their role in the inflammation associated with cancer. In particular, pro-inflammatory PGE$_2$ was identified as playing a predominant role in promoting tumor growth, proliferation, and angiogenesis (60, 61). In colon cancers, PGE$_2$ supported tumor growth in several mouse models. Exogenous PGE$_2$ administration dramatically increased both small and large intestinal adenomas in Apc$^{min/+}$ mice, and deletion of 15-PGDH, the enzyme that catalyzes PGE$_2$ to an inactive metabolite, in Apc$^{min/+}$ mice significantly promoted colon tumor growth (62, 63). Conversely, genetic deletion of endogenous PGES in Apc$^{min/+}$ mice suppressed intestinal tumorigenesis (64). Genetic deletion of the EP2 receptor inhibited chemically-induced murine lung carcinogenesis and suppressed COX-2-stimulated murine mammary hyperplasia (65, 66). Furthermore, in a chemically-induced of breast cancer rat model of breast cancer, EP1 antagonism markedly inhibited mammary tumorigenesis (67). Taken together, these studies demonstrate the oncogenic role of PGE$_2$ in colon, lung, and breast cancers, suggesting that drugs which targeting PGE$_2$ synthesis or activity may be beneficial in the treatment of cancers.
In contrast to the oncogenic properties of PGE\(_2\), an increase in prostacyclin (PGI\(_2\)) or enzymes that generate prostacyclin reduces tumor growth, by increasing apoptosis or inhibiting proliferation, angiogenesis, and invasion (68-70). Over-expression of PGIS successfully protected against lung tumorigenesis in a smoke-induced model of lung cancer (71). PGI\(_2\) analogs induced PPAR\(\delta\) receptor resulting in inhibition of the growth of human A549 non-small cell lung carcinoma cells (72-74). It is important to note that there are conflicting data on the anti-carcinogenic properties of PGI\(_2\), one report showed that PGI\(_2\) activation of PPAR\(\delta\) led to the acceleration of tumorigenesis in the Apc\(^{min/+}\) mice, suggesting a tumor subtype specificity to anti-growth PGI\(_2/\) PPAR\(\delta\) signaling (75).

Ang-(1-7) administration to mice with orthotopic breast tumors reduced mPGES-1 with no apparent change in PGIS, suggesting that Ang-(1-7) may decreases the pro-inflammatory PGE\(_2\) signaling. This reduction in PGE\(_2\) would be expected to reduce the synthesis of PGE\(_2\), altering the PGE\(_2\) to PGI\(_2\) prostaglandin ratio in favor of the anti-proliferative properties of PGI\(_2\). Modulation of the ratio of proliferative to anti-proliferative prostaglandin may be a molecular mechanism for Ang-(1-7) growth inhibition in orthotopic breast tumors.

We showed that Ang-(1-7) resulted in a two-fold increase in DUSP1 with a corresponding decrease in phospho-ERK1/2. MAP kinases are implicated in the regulation of numerous pathways including inflammation. Since MAP kinases regulate COX-2 at both the transcriptional and posttranslational levels, the reduction in COX-2
expression by Ang-(1-7) could be due to the observed reduction in MAP kinase activity (76-78). Up-regulation of DUSP1, which inactivates MAP kinases, may reduce COX-2, as we observed with Ang-(1-7) treatment. DUSP1 knock-out mice exposed to the gram negative bacteria E.coli expressed higher levels of COX-2 when compared to wild-type mice (79). Moreover, silencing of DUSP1 in human chondrocytes ablated aurothiomalate (a drug used to treat rheumatoid arthritis)-stimulated inhibition of COX-2, further suggesting a role for DUSP1 in the regulation of COX-2 (80). The COX-2 promoter contains binding sites for various transcription factors including AP-1, which may be regulated by MAP kinases, and also NF-κB, which may be regulated by COX-2 expression. Ang II up-regulated COX-2 by inducing NF-κB; since many of the actions of Ang-(1-7) oppose the actions of Ang II, the heptapeptide may down-regulate NF-κB to inhibit COX-2 signaling (81, 82). These data suggest that Ang-(1-7) may down-regulate COX-2 by reducing MAP kinase activities or by inhibiting NF-κB.

**COX-2 Signaling and the Tumor Microenvironment**

Inhibition of COX-2 activity improves cardiac function after myocardial infarction (83); both COX-2 and PGE₂ were up-regulated in the infarct region of the left ventricle while administration of NS-398, a selective COX-2 inhibitor, significantly reduced TGF-β expression. Moreover, treatment with COX-2 inhibitors significantly ameliorated uretic damage in rats with obstructed uropathy by reducing fibrosis and TGF-β expression in the smooth muscle layer of ligated ureters (84). COX-2 inhibitors also inhibited MMP-2 and MMP-9, suggesting that COX-2 participates in both ECM remodeling and TGF-β-stimulated fibrosis (85) (86). Stimulation of COX-2 signaling
through the prostaglandin E$_2$ receptor, EP2, suppresses the anti-proliferative properties of TGF-β and facilitates its oncogenic activities (87, 88). These studies suggest that targeting COX-2 signaling may restore the tumor suppressor properties of TGF-β in cancer (89). Collectively, these data suggest that the reduction in COX-2 may be another potential pathway by which Ang-(1-7) inhibits fibrosis and restores TGF-β-mediated growth inhibitory signaling.

COX-2 inhibitors also suppress aromatase expression in breast cancer cells, suggesting that the heptapeptide-mediated reduction in COX-2 and modification of PGE$_2$ signaling may influence aromatase expression and estradiol synthesis (90). In fact, localized production of PGE$_2$ by tumor cells, CAF, and TAM increases aromatase expression in both the stroma and in tumor epithelial cells (91, 92). The activation of aromatase by PGE$_2$ would increase intra-tumoral estrogen biosynthesis, promoting the growth of ER positive breast cancer cells. Ang-(1-7) down-regulation of COX-2 and mPGES-1, in turn, may result in a decreased aromatase expression and inhibition of estradiol signaling.

**Ang-(1-7) as a Single Agent Chemotherapeutic Phase I Clinical Trial and Potential Pitfalls in the Drug Development of Ang-(1-7)**

A recent clinical trial investigating Ang-(1-7) as a single chemotherapeutic agent for the treatment of cancer was completed at Wake Forest University Comprehensive Cancer Center (93). In this study, 18 patients were enrolled and treated with four different doses of Ang-(1-7). At the 700 µg/kg dose, one instance of stroke, a grade 4
toxicity, and 1 grade 3 toxicity consisting of a reversible cranial neuropathy were reported; a deep vein thrombosis was also reported in a patient at the 400 µg/kg dose. Other reported toxicities were mild and treatment with the heptapeptide was generally well-tolerated. The 400 µg/kg dose was determined to be an effective dose for continuation to a Phase II clinical trial. Of the 15 evaluable patients, 4 showed clinical benefits; one patient had a minor response to Ang-(1-7) treatment with a 19% reduction in tumor mass, and 3 patients showed stable disease lasting over 3 months on treatment. One patient was on the heptapeptide for over 14 cycles, just under 300 days, before disease progression. In patients showing clinical response, Ang-(1-7) administration led to a significant decrease in plasma levels of the pro-angiogenic peptide, PlGF, in agreement with pre-clinical studies that first demonstrated the anti-angiogenic activity of Ang-(1-7) in breast cancer.

Recent work by our group indicated that Ang-(1-7) has potent anti-angiogenic properties in the treatment of non-small cell lung adenocarcinoma (5). In this study, athymic mice with human A549 xenograft tumors were treated with subcutaneous injections of Ang-(1-7). Administration of Ang-(1-7) over a period of 30 days resulted in a reduction in tumor growth; histological assessment indicated that tumors of animals treated with Ang-(1-7) had reduced vessel density, demonstrating that Ang-(1-7) inhibited tumor angiogenesis to reduce tumor growth. This decrease in vessel density was associated with a reduction in VEGF expression. Ang-(1-7) also reduced VEGF in A549 cells, which was blocked by pre-treatment with an Ang-(1-7) receptor antagonist, showing that the reduction in VEGF by Ang-(1-7) was mediated by the AT_{(1-7)} receptor.
Ang-(1-7) reduced tubule formation of human endothelial cells in Matrigel as well as decreased vessel branching in the *in vivo* chick chorionic allantoic membrane assay; both the reduction in tubule formation and branch point formation by Ang-(1-7) were reversed by co-treatment with the Ang-(1-7) receptor antagonist, indicating that the anti-angiogenic effect of the heptapeptide is mediated by the activation of an AT$_{1R}$ receptor. Subcutaneous infusion of Ang-(1-7) significantly reduced vessel density in ZR-75-1 ER positive and BT-474 HER2 amplified orthotopic breast tumors (as shown in Figure 1 and Figure 2 in the Appendix). Moreover, administration of Ang-(1-7) markedly reduced PlGF and VEGF expression in breast tumor-bearing mice (as shown in Figure 3 and Figure 4 in the Appendix). Taken together, these data clearly supports an anti-angiogenic role of Ang-(1-7) in cancer.

Ang-(1-7), as a chemotherapeutic drug, is delivered to patients by subcutaneous injection, with an average half-life in human plasma of about 30 minutes (93, 94). Future drug development of Ang-(1-7) would include analogs to improve drug delivery and prolong drug half-life. Second-generation Ang-(1-7) homologs are being developed and studied. An orally available compound, AVE 0991, was developed by Aventis pharmaceuticals as an AT$_{1R}$ agonist (95). Sequential studies comparing Ang-(1-7) to AVE 0991 activities showed similar bioactivities. AVE 0991 was cardio-protective and attenuated heart failure induced by myocardial infarction (96), similar to Ang-(1-7). Moreover, AVE 0991 vasodilator function was abolished in the aorta of *mas*-knockout mice, suggesting that AVE 0991 binds to the Ang-(1-7) receptor *mas* to exert its biological properties (97). Even though AVE 0991 binds to the *mas* receptor and has
cardiovascular protective properties similar to Ang-(1-7), studies demonstrate that
treatment with AVE 0991 resulted in a 5-fold increase in total nitric oxide released, when
compared to Ang-(1-7) administration, and that AVE 0991-stimulated nitric oxide release
was significantly inhibited by both pre-treatment with an AT(1-7) receptor antagonist as
well as an AT1R or AT2R antagonist (98). Collectively, these data indicate that AVE
0991 can act through both the mas receptor and the Ang II receptors, AT1R or AT2R,
suggesting a lack of receptor specificity. Moreover, elevated nitric oxide increases the
growth of tumor xenografts by stimulating angiogenesis with an associated increase in
VEGF, suggesting that AVE 0991 may also increase angiogenesis (99). This suggests
that AVE 0991 would not be a more efficacious alternative to Ang-(1-7) for the treatment
of cancer since AVE 0991 may stimulate growth through the activation of the AT1R as
well as increase angiogenesis. These data also indicate the complexity of designing 2nd
generation Ang-(1-7) homologs due to potential receptor cross-reactivity and the
opposing responses to Ang-(1-7) and Ang II.

A thioether bridge linking position 4 to 7 on the N-terminal of Ang-(1-7) was
recently developed [cAng-(1-7)] (100). cAng-(1-7) was more resistance to proteolytic
cleavage by ACE when compared to endogenous Ang-(1-7), with more than a 2-fold
decrease in plasma clearance. Furthermore, the group showed that the thioether linked
Ang-(1-7) had enhanced vasodilator properties as measured by % relaxation of
phenylephrine-contracted aortic rings when compared to endogenous Ang-(1-7). The
vasodilatory effect of cAng-(1-7) was blocked by an AT(1-7) receptor antagonist,
indicating that the cAng-(1-7) vasodilator activities are mediated by an AT(1-7) receptor.
These data suggest that adding a thioether bridge linkage to the heptapeptide increases its vasodilator potency and half-life. However, future studies investigating the bridged heptapeptide’s ability to bind to the \textit{mas} receptor and conserve the endogenous peptides anti-proliferative properties as well as potential cross reactivity with Ang II receptors need to be explored.

In the phase I clinical trial, one patient who was given the lowest Ang-(1-7) dose (100 µg/kg) had the highest plasma Ang-(1-7) level, one hour after peptide administration, and the slowest observed peptide half-life, indicating the variability of Ang-(1-7) metabolism (93). Since ACE converts Ang-(1-7) to Ang-(1-5), variabilities in the breakdown of the heptapeptide could be due to differences in plasma ACE in people with different ACE polymorphisms. Reduced ACE activity would increase circulating Ang-(1-7) and extend the half-life of the heptapeptide. In the National Center for Biotechnology Information database, there are more than 160 reported ACE gene polymorphisms, most of which are single nucleotide changes (101). About 21% of the polymorphisms are contained within the coding region of the ACE gene; only 34 polymorphisms are within the coding region and 18 of these are missense mutations (101). One of the most common ACE polymorphisms involves the presence (insertion, I) or absence (deletion, D) of a 287-base pair sequence of DNA in intron 16 of the gene (SNP ID: rs1799752). Carriers of the D/D allele have a two-fold increase in ACE activity, while people with I/D allele polymorphism have intermediate ACE levels indicating allele co-dominance; individuals with I/I allele expression have the lowest ACE activity (101). Moreover, over 47% of the variance in ACE activity was due to the
presence of the I/D polymorphism. A similar distribution of ACE activity was observed with the A-240T polymorphisms with A/A having the lowest ACE activity, A/T having intermediate activity, and T/T having the highest mean plasma ACE levels (102). Furthermore, population studies investigating the role of ACE polymorphisms in cancer showed a significant correlation between ACE activity and breast cancer risk. In the Rotterdam Study in which 4,117 women were genotyped, D/D carriers had a significantly increased risk of developing breast cancer when compared to I/I carriers (103). In addition, in the Singapore Chinese Health Study Cohort in which 189 breast cancer cases and 671 control female subjects were compared, one or two copies of the A allele showed significantly reduced risk when compared to T/T genotype (104). When the two polymorphisms (I/D and A-240T) were considered simultaneously, individuals with both the A and I alleles had a reduced breast cancer risk of over 50%, clearly indicating the important role of ACE activity in breast cancer and suggesting that RAS modulation may be a therapeutic target in the treatment of cancer (104).

Another concern during the development of Ang-(1-7) as a chemotherapeutic agent was the potential for the elevated circulating Ang-(1-7) to bind to and activate other Ang II receptors. Micromolar levels of the heptapeptide activate the AT$_1$ receptor in cultured VSMCs, demonstrating that Ang-(1-7) binds to the AT$_1$ receptor with low affinity (105). In the clinical trial at WFUCCC, using the 400 µg/kg dose of Ang-(1-7), the highest plasma level of the heptapeptide was around 1 µM, which peaked one hour after injection; since the plasma heptapeptide half-life ranged from 0.42 to 0.61 hours, Ang-(1-7) was substantially elevated over baseline Ang-(1-7) plasma levels (about 35
pg/mL), six hours after administration (93). Due to variations in the half-life of Ang-(1-7) and plasma heptapeptide levels, which may be the result of ACE polymorphisms or other Ang-(1-7) degrading enzyme variability, it is critical to monitor blood pressure during Ang-(1-7) administration, to ensure that Ang-(1-7) does not elevate blood pressure by activation of the AT1R. There were no reported changes in blood pressure during the Phase I clinical trial (93).

**Future Clinical Applications of Ang-(1-7)**

There is increased interest in designing rational chemotherapeutic drug combinations for the treatment of cancer. It has becoming increasingly clear that single agent will likely not be effective due to drug resistance. Even if the tumor is initially responsive to treatment, tumor molecular heterogeneity leads to resistant cell population growth (106, 107). Examples of successful adjuvant chemotherapeutic drugs used in combination therapy for the treatment of breast cancer include: doxorubicin and cyclophosphamide (AC), AC and docetaxel (AC + T), and cyclophosphamide, epirubicin, and fluorouracil (CEF). Drugs are often first approved as single agent therapies, then combined based upon lack of overlapping toxicity profiles and difference in tumor targeting mechanism. Many studies focus on sequence and scheduling of drugs rather than determining molecular interactions.

The studies discussed in this thesis highlight the molecular mechanisms that participate in the Ang-(1-7) inhibition of breast cancer. Some of the signaling pathways that are down-regulated by administration of Ang-(1-7) are often up-regulated in breast
cancer cells that are resistant to tamoxifen, aromatase inhibitors, or Herceptin. Pathways implicated in drug resistance including activation of MAP kinases, Akt, or IGF, are modulated by the renin-angiotensin system (108-110). Ang-(1-7) reduces phospho-ERK1/2 in various types of cells including human lung cancer cells, human breast cells, and cancer associated fibroblasts. The heptapeptide decreases phosphorylated Akt in MDA-MB-231 (triple negative breast cancer), SK-Br-3 (HER2-amplified breast cancer), and ZR-75-1 (ER positive breast cancer) cell lines (Figure 5 in the Appendix). However, Ang-(1-7) increases phospho-Akt in the BT-474 (HER2-amplified, ER-positive breast cancer) cell line indicating a cell line specific response of Ang-(1-7) on Akt signaling. The down-regulation of phospho-Akt and ERK1/2 activity by Ang-(1-7) administration may result in a re-sensitizing of the cancer cells to the primary therapeutic agent. Patients on ACE inhibitors had significantly increased IGF-binding protein 3 (IGFBP-3) which binds circulating IGF and prevents receptor activation; however, there is no direct evidence of the role for Ang-(1-7) in IGF signaling (111). Taken together, these data suggests that Ang-(1-7) may be used in combination with aromatase inhibitors, tamoxifen, or trastuzumab to prevent drug resistance, but also may be used to restore drug sensitivity to the cancers.

Combination therapy of commonly used chemotherapeutic agents with Ang-(1-7) may reduce adverse side effects associated with single agent administration. For example, tamoxifen as administration can cause uterine cancer and blood clots that may limit their use as adjuvant therapy in the clinic (112). Infusion of Ang-(1-7) into rats that were stimulated to develop deep venous thrombosis significantly reducing thrombosis
weight, by 50-70%, demonstrating that Ang-(1-7) is anti-thrombotic; the reduction in thrombosis formation was inhibited by co-infusion with an Ang-(1-7) receptor antagonist, demonstrating that the anti-thrombotic effect was mediated by an \( \text{AT}_{(1-7)} \) receptor (113). In addition, incubation of Ang-(1-7) with platelets markedly decreased adhesion of the platelets to fibrillar collagen, providing further evidence of the anti-thrombotic activities of the heptapeptide (114). Since Ang-(1-7) inhibits the growth of various types of cancer (lung and breast), the combination of Ang-(1-7) with tamoxifen (in breast cancer patients) may also prevent the development of uterine cancer that sometimes occurs with tamoxifen mono-therapy.

Another example of a chemotherapeutic drug-induced toxicity that may be prevented by Ang-(1-7) co-administration is trastuzumab-associated cardiac toxicity. Trastuzumab, especially when used in combination with anthracycline, causes cardiomyopathy in approximately 27% of treated patients (115). The importance of HER2 signaling in normal cardiac development was demonstrated using HER2 cardiac-specific knock-out mice, where knockout of HER2 resulted in the development of cardiac myopathy in adult mice (116-118). Moreover, treatment with the HER2 antibody resulted in a loss of mitochondrial membrane potential in cardiomyocytes, promoting cell death and decreasing survival. Administration of the HER2 antibody increases the formation of reactive oxygen species (ROS) in cardiomyocytes and increases cardiac cell death, suggesting that trastuzumab cardiac toxicity may be regulated by increased ROS signaling resulting in mitochondrial apoptosis and cardiomyocyte death (117, 119). Ang-(1-7) is cardio-protective (120); treatment of rats with Ang-(1-7) after myocardial
infarction attenuated the development of heart failure and reduced the incidence and duration of reperfusion arrhythmias (121). Ang-(1-7) decreased Ang II-stimulated ROS production in thoracic aortic rings (122), suggesting that co-administration of Ang-(1-7) and trastuzumab may counter-regulate the cardiotoxicity produced by the HER2 antibody and reduce the production of ROS to prevent mitochondrial depolarization.

Although research has resulted in a decrease in cancer mortality rates, cancer is still a leading cause of death in the United States, second only to cardiovascular disease. Approximately 192,000 new cases of breast cancer were diagnosed last year and over 40,000 American women died from breast cancer, demonstrating the need to develop more effective chemotherapeutic agents to treat the disease (123). We showed that Ang-(1-7) significantly inhibits ER positive and HER2 amplified orthotopic breast tumor growth. This inhibition of growth was associated with a significant reduction in cell proliferation, inflammation, and reactive stroma. Since the heptapeptide mediates its biological activity by the activation of a unique G protein-coupled receptor, mas, Ang-(1-7) may serve as an effective targeted chemotherapeutic agent in the treatment of breast cancer.
Reference List


(50) de Jonge MJ, Dumez H, Verweij J, Yarkoni S, Snyder D, Lacombe D, et al. Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone...


(57) Smith GR, Missailidis S. Cancer, inflammation and the AT1 and AT2 receptors. J Inflamm (Lond) 2004;1:3.


(94) Rodgers KE, Oliver J, Dizerega GS. Phase I/II dose escalation study of angiotensin 1-7 [A(1-7)] administered before and after chemotherapy in patients with newly diagnosed breast cancer. Cancer Chemother Pharmacol 2006;57:559-68.


(97) Lemos VS, Silva DM, Walther T, Alenina N, Bader M, Santos RA. The endothelium-dependent vasodilator effect of the nonpeptide Ang(1-7) mimic


APPENDIX

Ang-(1-7) and Tumor Angiogenesis

Studies by our group demonstrated that Ang-(1-7) is a potent anti-angiogenic agent in the treatment of non-small cell lung adenocarcinoma (1). Ang-(1-7) treatment of athymic mice with human A549 xenograft tumors significantly reduced tumor growth and vessel density, suggesting that Ang-(1-7) inhibited tumor angiogenesis to reduce tumor growth. The reduction in vessel density was associated with a decrease in vascular endothelial growth factor (VEGF) in extracts of tumors treated with Ang-(1-7). VEGF was also reduced in A549 tumor cells, which was blocked by an AT(1-7) receptor antagonists, demonstrating that the Ang-(1-7) reduction in VEGF was mediated by the AT(1-7) receptor. Ang-(1-7) attenuated human endothelial cell tubule formation in Matrigel and reduced vessel branching in the in vivo chick chorionic allantoic membrane assay. The reduction in tubule formation and branch point formation by Ang-(1-7) was reversed by co-treatment with the Ang-(1-7) receptor antagonist demonstrating that the anti-angiogenic effects of the heptapeptide are mediated by activation of an AT(1-7) receptor (1). A recently completed phase I clinical trial, conducted in the Wake Forest University Comprehensive Cancer Center, investigated the use of Ang-(1-7) as a single chemotherapeutic agent for the treatment of patients with metastatic or unresectable solid tumors. In this study, Petty et. al. reported that Ang-(1-7) significantly reduced plasma placental growth factor (PlGF), a potent angiogenic protein, in patients that showed clinical benefit, supporting the preclinical evidence that the heptapeptide is an anti-angiogenic agent (2).
The effect of Ang-(1-7) on angiogenesis was studied in BT-474 HER2-amplified and ZR-75-1 ER-positive orthotopic breast tumors, treated with subcutaneous infusion of Ang-(1-7) or saline for 18 days. After treatment, the excised tumors were fixed in 4% paraformaldehyde for 24 h and incubated in 70% ethanol for 48 h prior to embedding in paraffin. The embedded tumors were cut into five micron thick sections and stained with hematoxylin and eosin (H&E) to determine morphology. Sections of tumors were incubated with an antibody to CD34, an endothelial cell marker, and tumor vessels were identified based upon vessel morphology. Clusters of vessels or “hot spots” were located around the periphery of the tumor. Even though the total number of hot spots was similar in tumors from both the saline-treated mice and the Ang-(1-7)-treated mice, the number of vessels within each cluster varied between treatment groups. ZR-75-1 (Supplemental Figure 1) and BT-474 (Supplemental Figure 2) tumor sections from saline-treated mice showed numerous blood vessels which were significantly reduced by Ang-(1-7) administration. As illustrated in Supplemental Figure 1 and Figure 2, treatment with the heptapeptide reduced tumor vessel density in ZR-75-1 and BT-474 orthotopic breast tumors by approximately 50% when compared to the saline treated mice [ZR-75-1 saline tumors, 16.6 ± 1.9 vessels per field when compared to ZR-75-1 Ang-(1-7) tumors, 7.2 ± 1.1 vessels per field; BT-474 saline tumors, 12.5 ± 1.1 vessels per field compared with BT-474 Ang-(1-7) tumors, 5.0 ± 0.9 vessels per field; p < 0.002; n = 4-5], suggesting that Ang-(1-7) reduces breast cancer angiogenesis.
Vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) are potent angiogenic signaling proteins implicated in tumor growth, vascularization and proliferation. VEGF and PIGF were measured by Western blot hybridization of protein homogenates isolated from BT-474 orthotopic breast tumors. Tumor tissue (~1 to 2 mm$^2$ size pieces) was homogenized in PBS using a Qiagen TissueLyser (Retsc, Hann, Germany). Protein was solubilized by adding hot sodium dodecyl sulfate (SDS)/β-mercaptoethanol to tissue homogenates to a final concentration of 3% SDS-10% β-mercaptoethanol. Protein was measured by a modification of the Lowry method (3). Proteins were separated by electrophoresis on a 10% or 15% SDS polyacrylamide gels followed by transfer to hydrophobic polyvinylidene difluoride membrane. Non-specific binding was blocked by incubation with Blotto (Tris-buffered saline with 5% powdered milk and 4% Triton X-100). Membranes were incubated overnight at 4°C with primary antibodies specific to VEGF (1:10,000 Santa Cruz Biotechnology, Santa Cruz, CA) or PIGF (1:1000 Novus Biologicals, Littleton, CO) followed by a 1 h incubation with polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 GE Health care, UK) at room temperature. Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto West or Pico West, Pierce Biotechnology, Rockford, IL) and quantified by densitometry using MCID digital densitometry software (Cambridge, UK). Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β-actin (1:2000 Sigma-Aldrich, MO) overnight followed by monoclonal horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h and analyzed by digital densitometry. As shown in Figure 3, Ang-(1-7) administration resulted in 83% decrease in immunoreactive VEGF and as shown in Figure 4, a 57%
reduction in immunoreactive PlGF protein [0.92 ± 0.01 in tumors from saline-treated mice versus 0.16 ± 0.06 in tumors from Ang-(1-7)-treated mice for VEGF, and 1.40 ± 0.13 in tumors from saline-treated mice versus 0.61 ± 0.27 in tumors from Ang-(1-7)-treated mice for PlGF; n = 5; p < 0.05], indicating that the Ang-(1-7)-mediated reduction in tumor vessel density may be due to a inhibition of the pro-angiogenic peptides VEGF and PlGF.

Ang-(1-7) in Protein Kinase B (Akt) Signaling

Akt pro-oncogenic signaling is implicated in numerous stages of tumor development and progression. Akt regulates the cell cycle by sequestering the cell cycle inhibitors p21 and p27 in the cytoplasm and stabilizing cyclin D1, thereby promoting cell cycle progression and proliferation (4). In addition, Akt inhibits apoptosis by reducing Bcl-2 and Bad pro-apoptotic proteins and forkhead transcription factor which activates apoptotic genes. Akt, by phosphorylating IκB kinase, results in NFκB-mediated pro-survival gene expression. Moreover, Akt signaling is linked to increased invasion by promoting the expression of MMPs and upregulation of β1-integrins (4). Taken together, these data indicate that Akt signaling promotes cancer cell growth.

Ang-(1-7) stimulates Akt signaling in cardiovascular cells and tissues. The heptapeptide increased phosphorylated Akt in Chinese hamster ovary cells transfected with the mas receptor (5). Furthermore, injections of Ang II and Ang-(1-7) into the rat heart increased phosphorylated Akt, in a time-dependent manner. The increase in phosphorylated Akt upon injection with Ang-(1-7) was inhibited by co-injection with an AT(1-7) receptor antagonist, suggesting that Ang-(1-7) stimulation of Akt signaling in the
heart is mediated by an AT(1-7) receptor (6). These data suggest that Ang-(1-7) may stimulate Akt activity and support tumor proliferation, survival, and invasion.

MDA-MB-231, BT-474, ZR-75-1, and SK-Br3 human breast cancer cells were serum starved overnight, incubated with 100 nM Ang-(1-7) and 1% serum for 1, 2, 4, and 8. Cell monolayers were solubilized in lysis buffer as described previously (7). BT-474, MDA-MB-231, and ZR-75-1 tumor tissue (~ 1 to 2 mm² size pieces) was homogenized in PBS using a Qiagen TissueLyser (Retsc, Hann, Germany). Protein was solubilized by adding hot sodium dodecyl sulfate (SDS)/β-mercaptoethanol to tissue homogenates to a final concentration of 3% SDS-10% β-mercaptoethanol. Protein was measured by a modification of the Lowry method (3). Proteins were separated by electrophoresis on a 10% SDS polyacrylamide gels followed by transfer to hydrophobic polyvinylidene difluoride membrane. Non-specific binding was blocked by incubation with Blotto (Tris-buffered saline with 5% powdered milk and 4% Triton X-100). Membranes were incubated overnight at 4ºC with primary antibodies specific to Akt or phosph-Akt (Ser473) (1:1,000 Cell Signaling Technology, Danvers, MA) followed by a one h incubation with polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 GE Health care, UK) at room temperature. Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto West or Pico West, Pierce Biotechnology, Rockford, IL) and quantified by densitometry using MCID digital densitometry software (Cambridge, UK). Protein loading was visualized by incubation of stripped membranes with a monoclonal antibody to β-actin (1:2000 Sigma-Aldrich, MO) overnight followed by monoclonal horseradish peroxidase (HRP)-conjugated
secondary antibody for 1 h and analyzed by digital densitometry. Phospho-Akt protein expression was measured by Western blot hybridization; as shown in Figure 5. Ang-(1-7) administration resulted in time-dependent decrease in p-Akt protein in the MDA-MB-231, ZR-75-1, and SK-Br3 cells; conversely, Ang-(1-7) treatment resulted in a time-dependent increase in phospho-Akt in the BT-474 breast cancer cells, indicating that the effect of Ang-(1-7) on Akt activation is tumor type-specific. However, the BT-474 cell line has a PIK3CA mutation that results in a constitutively active PI3K, which may explain the differential effect of Ang-(1-7) on Akt signaling in this cell line. This differential activation of Akt by Ang-(1-7) was also observed in heptapeptide-treated mice bearing BT-474 HER2 amplified ER positive orthotopic breast tumors when compared to Ang-(1-7)-treated mice bearing MDA-MB-231 triple negative or ZR-75-1 ER positive orthotopic breast tumors, as shown in Figure 6 [0.16 ± 0.03 RDU in BT-474 tumors from saline-treated mice versus 0.51 ± 0.06 RDU in BT-474 tumors from Ang-(1-7)-treated mice for phospho-Akt, 0.96 ± 0.05 RDU in MDA-MB-231 tumors from saline-treated mice versus 0.47 ± 0.13 RDU in MDA-MB-231 tumors from Ang-(1-7)-treated mice, and 0.28 ± 0.03 RDU in ZR-75-1 tumors from saline-treated mice versus 0.15 ± 0.04 RDU in ZR-75-1 tumors from Ang-(1-7)-treated mice for phospho-Akt, n= 5-6, *p<0.05]. These findings suggest that Ang-(1-7) administration results in a differential regulation on Akt signaling and may result in breast cancer inhibition or survival.

**Ang-(1-7) Effect on MAP Kinases in BT-474 HER2 Amplified Breast Tumors**

MAP kinases are activated by numerous external stimuli, including growth factors, UV light, oxidation, and cytokines, and are key regulators of cell growth and
proliferation. The MAP kinase family consists of three members, ERK1/2, JNK, and p38, whose activation leads to various cellular responses including growth, proliferation, differentiation, survival, and apoptosis. A review of the MAP kinase signaling pathways in cancer was discussed in greater detail in Chapter I.

Ang-(1-7) administration reduced ERK1/2 activity in lung cancer cells (8), cancer associated fibroblast (Chapter II, Figure 5B), and ZR-75-1 cells and tumors (Chapter III, Figure 3A, 4B, and 4C), suggesting a role for the heptapeptide in MAP kinase regulation. Moreover, treatment with Ang-(1-7) up-regulated DUSP1 in CAF (Chapter II, Figure 5C) and ZR-75-1 cells and tumors (Chapter III, Figure 3B and 4A), suggesting that up-regulation of the MAP kinase phosphatase DUSP1 may account for the heptapeptide-mediated down-regulation of ERK1/2 activity. In addition, transfection of ZR-75-1 with DUSP1 siRNA ablated the Ang-(1-7) mediated down-regulation of pERK1/2 (Chapter III, Figure 6D), further demonstrating the role of DUSP1 in the Ang-(1-7)-mediated regulation of ERK activity in ZR-75-1 cells.

HER2 amplified BT-474 cells in Matrigel were injected into the mammary fat pad of ovariectomized athymic mice supplemented with a subcutaneous time release estradiol pellet. Tumors grew to a uniform sized before being treated with either saline or Ang-(1-7) at a dose of 24 µg/kg/h for 18 days. Tumors were excised, homogenized, and solubilized using the methods described in the above. Western blot hybridization was performed, using the following antibodies, to determine whether Ang-(1-7) regulates the activities of specific MAP kinases in BR-474 orthotopic breast tumors: pERK1/2, pJNK, p-p38, DUSP1, pMEK1/2, MEK1/2, MKK4, MKK7, and MKK3/6 (1:1000, Cell Signaling Technologies, Danvers, MA). Administration of the heptapeptide significantly
reduced pERK1/2 activity in BT-474 tumors [saline, 0.75 ± 0.36 RDU compared with Ang-(1-7), 0.15 ± 0.06 RDU for pERK1 and saline, 1.35 ± 0.18 RDU compared with Ang-(1-7), 0.53 ± 0.07 RDU for pERK2 in BT-474 tumors, *p<0.05, n = 4] (Figure 7A). Moreover, treatment with Ang-(1-7) had no effect of the MAP kinase phosphatase DUSP1 (Figure 7B) but reduced MEK1/2 in BT-474 tumors (Figure 7C) [saline, 1.01 ± 0.22 RDU compared with Ang-(1-7), 0.32 ± 0.15 RDU, *p < 0.05, n = 4-5], indicating that the Ang-(1-7)-mediated reduction in pERK1/2 is due to a decrease in the MAP kinase kinase (MEK1/2) that phosphorylates the enzyme. This is in contrast to results in ZR-75-1 tumor cells or CAFs, where the heptapeptide up-regulated the MAP kinase phosphatase to reduce MAK kinase activities. In addition, pretreatment of BT-474 cells with the phosphatase inhibitors okadaic acid and sodium orthovanadate did not alter the Ang-(1-7)-mediated reduction in pERK1/2 (Figure 7D) [control, 1.87 ± 0.15 RDU compared to Ang-(1-7)-treated, 0.75 ± 0.02 RDU, phosphatase inhibitor-treated, 1.97 ± 0.07 RDU compared with phosphatase inhibitor plus Ang-(1-7)-treated, 0.58 ± 0.17 RDU, *p<0.05, n = 3], suggesting that the Ang-(1-7)-mediated down-regulation of pERK1/2 is not dependent on phosphatase activity in BT-474 tumors. Ang-(1-7) regulation of the stress-activated MAP kinases JNK and p38 were also investigated in BT-474 tumors. Heptapeptide administration to BT-474 tumor-bearing mice reduced pJNK expression [saline-treated, 1.10 ± 0.15 RDU compared with Ang-(1-7) treatment, 0.37 ± 0.14, * demonstrates p<0.05; n = 5] (Figure 8A) and increased p-p38 [saline, 0.52 ± 0.12 RDU compared with Ang-(1-7) treated, 0.91 ± 0.05 RDU, p < 0.05; n = 4-5] (Figure 8B) in the treated tumors, suggesting that Ang-(1-7) modulates multiple MAP kinase signaling pathways.
Figure 1. Vessel Density is Reduced by Ang-(1-7) in ZR-75-1 Orthotopic Breast Tumors. A: representative pictures of ZR-75-1 stained sections of tumors from mice treated with saline or Ang-(1-7) following incubation with an antibody to the endothelial cell marker CD34 at x200 magnification. Blood vessels were identified based upon morphology in vessels highlighted by CD34-immunoreactive endothelial cells. B: average vessel density defined as the number of intra-tumoral vessels from four 0.3 mm² fields in sections of ZR-75-1 tumors from mice treated with saline or Ang-(1-7). * denotes p < 0.05; n = 4-6.
Figure 2. Infusion with Ang-(1-7) Reduces Vessel Density in BT-474 Orthotopic Breast Tumors. A: representative pictures of stained sections of tumors from mice treated with saline or Ang-(1-7) following incubation with an antibody to the endothelial cell marker CD34 in BT-474 tumors at x200 magnification. Blood vessels were identified based upon morphology in vessels highlighted by CD34-immunoreactive endothelial cells. B: average vessel density defined as the number of intra-tumoral vessels from four 0.3 mm² fields in sections of BT-474 tumors from mice treated with saline or Ang-(1-7). * denotes p < 0.05; n = 5.
Figure 3. Ang-(1-7) Treatment Reduces VEGF in BT-474 Orthotopic tumors. VEGF was measured in protein homogenates of tumors from Ang-(1-7)- and saline-treated mice by Western blot hybridization. * denotes p < 0.05; n = 5.
Figure 4. Ang-(1-7) Reduces PlGF in BT-474 Orthotopic Breast Tumors. PlGF was measured in protein homogenates of tumors from Ang-(1-7)- and saline-treated mice by Western blot hybridization. * denotes p < 0.05; n = 5.
Figure 5. Differential Regulation of Phospho-Akt by Ang-(1-7). Quiescent ZR-75-1, MDA-MB-231, BT-474, and SK-BR3 cells were treated with 100 nM Ang-(1-7) and stimulated with 1% serum for 1, 2, 4, and 8 h. Phosphorylated Akt was measured by Western blot hybridization. * denotes p < 0.05; n = 3-4.
Figure 6. Differential Regulation of Akt by Ang-(1-7) in Orthotopic Human Breast Tumors. Akt and phospho-Akt (Ser473) were measured in protein homogenates of BT-474, MDA-MB-231, and ZR-75-1 tumors from Ang-(1-7) and saline-treated mice by Western blot hybridization. * denotes p < 0.05; n = 5-6.
Figure 7. Ang-(1-7) Reduces ERK1/2 Activity in BT474 Orthotopic Breast Tumors.

A: Phosphorylated ERK1/2 were measured in protein homogenates of BT-474 tumors from mice treated with Ang-(1-7) or saline, by Western blot hybridization. * denotes p < 0.05; n = 4. B: DUSP1 was measured by Western blot hybridization, in protein homogenates of BT-474 tumors from mice treated with saline or Ang-(1-7). n = 4. C: MEK1/2 was measured in BT-474 tumor homogenates from mice treated with saline or Ang-(1-7). * denotes p < 0.05; n = 4-5. D: BT-474 cells were pre-treated with 10 µM sodium vanadate (NaV) and 100 nM okadaic acid (OK) for 30 min prior to the addition of 100 nM Ang-(1-7) (A7) and serum for 2 h. Pretreatment with phosphatase inhibitors had no significant effect on the down-regulation of ERK1/2 activity by Ang-(1-7). * denotes p < 0.05; n = 3.
Figure 8. Ang-(1-7) differentially regulates JNK and p38 in BT-474 orthotopic breast tumors. **A:** Phosphorylated JNK was measured in protein homogenates of BT-474 tumors from mice treated with Ang-(1-7) or saline, by Western blot hybridization. * denotes p < 0.05; n = 5. **B:** Phosphorylated p38 was measured by Western blot hybridization, in protein homogenates of BT-474 tumors from mice treated with saline or Ang-(1-7). * denotes p < 0.05; n = 4-5.
Reference List


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Cook Katherine L, Tallant EA, Gallagher PE. Angiotensin-(1-7) Inhibits Breast Tumor Growth in an Orthotopic Murine Model by Reducing Angiogenesis and Fibrosis. 5th International Tumor Microenvironment Meeting, accepted for oral presentation, October 2009.


Publications:


Cook KL, Gallagher PE and Tallant EA. Angiotensin-(1-7) inhibits estrogen receptor positive breast tumors with an associated reduction in prostaglandin E2 synthase. (Submitted to Clinical Cancer Research April 2010)


Soto-Pantoja DR, Menon J, Cook KL, Chacko S, Gallagher PE and Tallant EA. Angiotensin-(1-7) inhibits growth of triple negative breast cancer in a mouse orthotopic model. (To be submitted to Cancer Research May 2010)

Soto-Pantoja DR, Cook KL, Gallagher PE and Tallant EA. Angiotensin-(1-7) inhibits the growth of triple negative breast cancer through the reduction in angiogenesis. (In preparation)

Cook KL, Gallagher PE and Tallant EA. Inhibition of HER2 over-expressing orthotopic breast tumor growth by regulation of MAP kinase signaling pathways. (In preparation)

Gallagher PE, Maglic D, Cook KL and Tallant EA. Inhibition of leukemia cell proliferation by angiotensin-(1-7). (In preparation)