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The Treatment of Breast Cancer Tumor Growth and Metastasis With an Anti-MMP9 DNAzyme

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The Treatment of Breast Cancer Tumor Growth and Metastasis With an Anti-MMP9 DNAzyme

Abstract

BACKGROUND: Tumor cell proliferation, invasion and metastasis are known to be mediated, at least in part, through degradation of basement membrane by neutral metalloproteinases (MMP) produced by tumor and stromal cells. MMP-9 is known to play a significant role in breast tumor cell invasion and metastasis via degradation of ECM components and activation of cytokines and chemokines. MMP-9 is known to cleave type IV collagen, one of the most abundant collagens of the extracellular matrix. Our lab has found that the fully processed (68 kDa) form of MMP-9 generated in tumor and stromal cells is also capable of cleaving type I collagen molecule with similar characteristics of MMP-1. This observation raises questions regarding the significance of each MMP in tumor cell invasion and metastases. Thus, in combating tumor growth and invasion, understanding the mechanism of substrate specificity and design of a novel therapeutic compound that is capable of specifically down regulating MMP-9 expression are of interest.

METHOD: DNAzymes are catalytic oligonucleotides that bind to and cleave specific mRNA, resulting in a decreased protein expression. The safety and efficacy of anti-MMP-9 DNAzyme (AM9D) *in vivo* was determined by injecting ^{35}S -labeled AM9D (^{35}S -AM9D) into the tail vein of healthy and MMTV-PyMT transgenic mice and the amount of ^{35}S -AM9D accumulated in different tissues was measured as a function of time. The effect of AM9D treatment on breast tumor progression was tested by four once-weekly intratumoral injections of two concentrations of AM9D into mammary tumors of MMTV-PyMT transgenic mice at early stages of tumor development. The tumor sizes were measured at the end of the experiments. The effect of AM9D treatment on early stage experimental metastasis and survival was evaluated by injecting FVB female mice with 1×10^6 luciferase labeled mammary tumor epithelial cells (luc-MTEC) isolated from MMTV-PyMT mice and treating the animals with AM9D, control DNAzyme, or PBS for 3 weeks or up to 10 weeks, respectively.

The mechanism of substrate specificity of 68 kDa MMP-9 was determined by generating a series of truncated and site directed mutant forms of MMP-9. Specific amino acid residue in sequence $^{444}\text{PRPEPEPRPPTTT}^{456}$ in the hinge region responsible for substrate selectivity of the enzyme was then identified by alanine-scanning site directed mutagenesis. The charged amino acid residues (Arg 445 , Glu 447 , Glu 449 , and Arg 451) were individually replaced by Ala and the proteolytic activity of the mutant enzymes toward Mca-PLGL(Dpa)AR-NH $_2$, gelatin, and type I collagen was determined.

RESULTS: Treatment of MDA-MB-231 breast cancer cell line with AM9D *in vitro* resulted in decreased invasion potential of the cells and intratumoral treatment of MMTV-PyMT mice *in vivo* resulted in delayed rate of tumor growth and retarded final tumor volume by up to 51%. This decrease in tumor growth was correlated with decreased MMP-9 protein production within the treated tumor tissues. Tumors treated with AM9D were also less vascular compared to control and untreated tumors. Furthermore, DNAzyme administered IV is distributed to major organs including lung, without showing any organ toxicity. Intravenous administration of AM9D and control DNAzyme in animals bearing luc-MTECs were able to decrease the number of gross lung macro metastasis. In addition, AM9D treatment increased progression-free survival but did not have an effect on overall survival of animals inoculated with luc-MTECs compared to control DNAzyme and PBS treatment. These data indicate that AM9D can be used individually or as an adjuvant to current chemotherapy for breast cancer.

To further illuminate the role of MMP-9 in tumor growth and metastases the mechanism of substrate

specificity of the enzyme was studied. Site directed and deletion mutagenesis revealed that interaction of Glu⁴¹⁵ in the active site with Glu⁴⁴⁷ and/or Arg⁴⁵¹ in the hinge region makes the active site rigid, preventing full length MMP-9 from cleaving type I collagen. Disruption of this interaction, on the other hand, provides the flexibility necessary for the enzyme's active site to change conformation and be able to bind and cleave type I collagen substrate.

CONCLUSION: Given that MMP-9 is capable of cleaving type I collagen and its down regulation hinders the development of breast cancer in an animal model, AM9D could prove useful as an adjuvant therapy against breast carcinoma cell invasion.

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**THE TREATMENT OF BREAST CANCER
TUMOR GROWTH AND METASTASIS WITH
AN ANTI-MMP9 DNAZYME**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Miranda A. Hallett
May 2011

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DEDICATION

This dissertation is dedicated to my family and friends who provided me with strength, prayer and encouragement in obtaining this degree. A special dedication is extended to my mother who is the epitome of a strong woman. To my father who taught me a conversation without words is the most important conversation. Also, for being there to encourage me through the toughest of times, even when there were dogs in the room.

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ABSTRACT

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To further illuminate the role of MMP-9 in tumor growth and metastases the mechanism of substrate specificity of the enzyme was studied. Site directed and deletion mutagenesis revealed that interaction of Glu⁴¹⁵ in the active site with Glu⁴⁴⁷ and/or Arg⁴⁵¹ in the hinge region makes the active site rigid, preventing full length MMP-9 from cleaving type I collagen. Disruption of this interaction, on the other hand, provides the flexibility necessary for the enzyme's active site to change conformation and be able to bind and cleave type I collagen substrate.

CONCLUSION: Given that MMP-9 is capable of cleaving type I collagen and its down regulation hinders the development of breast cancer in an animal model, AM9D could prove useful as an adjuvant therapy against breast carcinoma cell invasion.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
BACKGROUND	1
Breast Cancer	1
Matrix Metalloproteinases (MMP)	1
MMP-9 in Cancer	2
DNAzymes as Novel Treatments for Cancer.....	4
Design and <i>In Vitro</i> Efficacy of Anti-MMP-9 DNAzyme (AM9D).....	5
RESEARCH OBJECTIVES AND SPECIFIC AIMS	7
Objective	7
Specific Aim 1	8
Specific Aim 2	8
Specific Aim 3	8
Specific Aim 4	8
CHAPTER 2. TREATMENT OF TUMOR GROWTH WITH AM9D	10
INTRODUCTION	10
MATERIALS AND METHODS.....	11
DNAzyme	11
MMTV-PyMT Transgenic Mammary Tumor Model.....	11
<i>In Situ</i> Hybridization.....	13
Histology and Immunohistochemistry	13
Statistical Analysis.....	13
RESULTS	14
MMP-9 Is Expressed in Mammary Tumors of MMTV-PyMT Mice.....	14
DNAzyme <i>In Vivo</i> Stability	14
Effect of AM9D Treatment on Final Tumor Load	16
Effect of AM9D on Angiogenesis and Apoptosis	16
DISCUSSION	20
CHAPTER 3. DISTRIBUTION, CLEARANCE, AND CYTOTOXICITY OF DNAZYME	21
INTRODUCTION	21
MATERIALS AND METHODS.....	21
Synthesis of ³⁵ S-radiolabeled Oligonucleotides.....	21
Animal Experiments	22
Systemic Distribution.....	22
Radioactive Sample Analysis	22
Cytotoxicity.....	23
RESULTS	23
Kinetics of DNAzyme in Normal Mice	23
Kinetics of DNAzyme in Mammary Tumor bearing Mice.....	27
Cytotoxicity of Intravenous Injection of AM9D in Healthy Mice	30
DISCUSSION	30

CHAPTER 4. AM9D TREATMENT OF EXPERIMENTAL METASTASIS.....	35
INTRODUCTION	35
MATERIALS AND METHODS.....	36
Establishing eIF1 alpha-Luciferase2 Mammary Tumor Epithelial Cells (luc-MTECs)	36
Animals	36
RESULTS AND DISCUSSION	37
Effect of AM9D Treatment on Early Stage Experimental Metastasis.....	37
Effect of Long Term AM9D Treatment on Lung Tumor Load in Experimental Metastasis and on Survival	40
CHAPTER 5. MMP-9 TRESSPASSES ON MMP-1'S TURF	46
INTRODUCTION	46
MATERIALS AND METHODS.....	48
Mutagenesis	48
Gelatin Zymography	50
Determination of Kcat/Km Values for the Fluorogenic Peptide Substrate Mca-PLGL(Dpa)AR-NH2.	50
Kinetic Analysis of Type I Collagenolytic Activity	50
Analysis of Gelatinolytic Activity of the Enzymes	51
RESULTS	51
Expression, Purification, and Activation of Deletion Mutant Enzymes	51
Characterization of Deletion Mutants MMP-9	51
Glu ⁴⁴⁷ and Arg ⁴⁵¹ Residues in the Hinge Region Play a Role in Type I Collagenolytic Activity of MMP-9	54
Interaction Between Glu ⁴¹⁵ and Glu ⁴⁴⁷ and/or Arg ⁴⁵¹ Dictates Substrate Specificity of MMP-9	57
DISCUSSION	58
CHAPTER 6. SUMMARY.....	62
CHAPTER 7. AM9D: FROM PRECLINIC TO CLINIC	64
AM9D FOR BREAST CANCER PATIENTS	64
AM9D AS AN ADJUVANT TO SURGERY	65
FUTURE AM9D PRECLINICAL STUDIES	65
LIST OF REFERENCES	67
APPENDIX. DEVELOPMENT OF AM9D MATERIALS AND METHODS	77
DNAZYME	77
<i>IN VITRO</i> TRANSCRIPTION AND CLEAVAGE ANALYSIS OF MMP-9 RNA BY DNAZYMES.....	77
CELL TRANSFECTION	77
ANALYSIS OF MMP-9 MRNA AND PROTEIN PRODUCTION IN AM9D TRANSFECTED CELLS	78

CELL INVASION ASSAY	78
VITA	79

LIST OF TABLES

Table 3-1.	Tissue distribution of [^{35}S]-DNAzyme in normal mice presented in average percentage of total DNAzyme.	25
Table 3-2.	Rate of [^{35}S]-DNAzyme initial and final clearance in normal mice.	27
Table 3-3.	Tissue distribution of [^{35}S]-DNAzyme in MMTV-PyMT mice presented in average percentage of total DNAzyme.	29
Table 3-4.	Rate of [^{35}S]-DNAzyme initial and final clearance in mice with mammary tumors.	31
Table 5-1.	Proteolytic activity of deletion mutant enzymes.....	53
Table 5-2.	Proteolytic activity of the Ala mutants	55
Table 5-3.	E415G and DM proteolytic activity.....	58

LIST OF FIGURES

Figure 1-1. Structural domains of matrix metalloproteinases.	3
Figure 1-2. Composition of the 10-23 catalytic motifs.....	5
Figure 1-3. Efficacy of anti-MMP-9 DNAzyme (AM9D) <i>in vitro</i>	6
Figure 2-1. Mouse mammary glands.	12
Figure 2-2. MMP-9 in MMTV-PyMT mice mammary tumors.....	14
Figure 2-3. Stability of DNAzyme in mammary tumors.....	15
Figure 2-4. Effect of AM9D on the rate of tumor growth and final mean tumor volume.....	17
Figure 2-5. Effect of AM9D on MMP-9 protein expression in MMTV-PyMT mammary tumors.	18
Figure 2-6. Fluorescent <i>in situ</i> hybridization.....	18
Figure 2-7. AM9D reduces tumor vasculature.	19
Figure 2-8. AM9D induces apoptosis.....	19
Figure 3-1. Distribution and elimination profile of [³⁵ S]-DNAzyme in normal mice.....	24
Figure 3-2. Distribution profile of [³⁵ S]-DNAzyme in additional organs of normal mice.	26
Figure 3-3. Distribution profile of [³⁵ S]-DNAzyme in MMTV-PyMT mice.	28
Figure 3-4. Distribution of [³⁵ S]-DNAzyme in MMTV-PyMT mammary tumors.	31
Figure 3-5. Pathology of organs from mice injected with AM9D, control DNAzyme, or PBS.	32
Figure 4-1. Representative lungs images extracted from two mice in the PBS treated group at early stage experimental metastasis.....	38
Figure 4-2. Macro and micro metastasis in lungs from early stage experimental metastasis.	39
Figure 4-3. CpG regions in AM9D and control DNAzyme.	41
Figure 4-4. Mouse body weights measured during experimental metastasis	41

Figure 4-5. Analysis of mice organ pathology.	42
Figure 4-6. Effect of various treatments on mice lung tumor burden.	43
Figure 4-7. Survival curve.	44
Figure 5-1. Schematic diagram of MMP-1 and -9.	47
Figure 5-2. MMP-9 recombinant deletion mutants.	52
Figure 5-3. Gelatin- gel zymography of purified mutant enzymes.	53
Figure 5-4. Alanine mutations in $\Delta CT^{(1-456)}$	55
Figure 5-5. Gelatin- gel zymography of purified site directed mutant enzymes.	56
Figure 5-6. Schematic diagram and gelatinolytic activities of E415G and DM.	57
Figure 5-7. Schematic diagram of MMP-9 mutants with type I collagenolytic activity.	60

LIST OF ABBREVIATIONS

α -SMA	Alpha-Smooth Muscle Actin
Δ CT	Carboxyl Terminal Truncated
^{35}S	Radioactive Sulfur
AM9D	Anti-MMP-9 DNAzyme
AS	Active Site
CpG-ODN	Synthetic CpG Containing Oligodeoxynucleotide
DNAzyme	Catalytic Deoxyribonucleotide
ECM	Extracellular Matrix
Luc-MTEC	Luciferase Labeled Mammary Tumor Epithelial Cells
MMP	Matrix Metalloproteinase
MMP-9	Matrix Metalloproteinase-9
MMTV-PyMT	Mouse Mammary Tumor Virus – Polyoma Middle T antigen
MTEC	Mammary Tumor Epithelial Cells
RT	Room Temperature
WT	Wild-Type

CHAPTER 1. INTRODUCTION

BACKGROUND

Breast Cancer

Breast cancer is one of the leading causes of cancer deaths in women, second only to lung cancer [1]. Breast cancer is divided into three subtypes based on the tumor cells expression of estrogen receptor (ER), progesterone receptor (PR), and/or HER2. The aforementioned subtypes of breast cancer are known as luminal A (ER or PR⁺/HER2⁻), luminal B (ER or PR⁺/HER2⁺), and triple negative (ER⁻/PR⁻/HER2⁻) [2]. The American Cancer Society estimated that in 2010 there would be 207,090 new cases of invasive and 57,010 new cases of non-invasive breast cancer diagnosed in women. In 2010, about 39,840 women were expected to die as a result of breast cancer. The spread of cancer cells to distant organs, metastasis, is responsible for the majority of morbidity and mortality [3-5]. In the case of breast cancer, metastasis affects the lungs, regional lymph nodes and bone, while less frequently affecting brain and liver [6]. This metastasis and eventual tumor growth at distant sites depends on interaction between tumor cells, stromal cells, and the host microenvironment [7]. Since the main event separating those that survive breast cancer and those who fall victim to breast cancer is metastasis, it is as important to develop therapeutic strategies against the metastatic process as for any other process in the breast cancer spectrum.

It has been widely hypothesized that metastasis occurs when cells from the primary tumor undergo sequential steps to make them more adaptable to seed tumors in distant organs. These sequential steps include the transition of epithelial cells to mesenchymal like cells (epithelial to mesenchymal transition; EMT), degradation of the extracellular membrane (ECM), intravasation, extravasation, and finally transition back to epithelial like cells (mesenchymal to epithelial transition; MET) [8]. This transition has been shown to involve matrix metalloproteinases (MMP) produced by tumor and stromal cells [9]. These enzymes enable the invasion of carcinoma cells into adjacent normal tissue by degrading the extracellular (ECM) and basement membrane components.

Matrix Metalloproteinases (MMP)

MMPs are a family of over 24 proteolytic enzymes and essential regulators of cells microenvironment [10]. These enzymes are secreted as catalytically latent species, which are processed to their activated forms *in vivo* by other proteinases [11]. Most human MMPs are categorized into five main subclasses based on their substrate specificity, collagenases (MMP1, MMP8, and MMP13), stromelysin (MMP3, MMP10, MMP11, MMP7 and MMP26), gelatinases (MMP2 and MMP9), membrane type MMPs (MMP14, MMP15, MMP16, MMP17, MMP24, and MMP25) [10, 11] and metalloelastase (MMP-12) [12]. MMPs cleave virtually all components of the ECM and

overlap in their substrate specificity. Collagenases can cleave native fibrillar collagen types I, II, and III [13]. Although less well studied, the collagenases can also cleave collagen types VII and X as well as gelatin, aggrecan, entactin, and tenascin [14]. Stromelysins digest native collagen types III, IV, V, IX, X, and XI, as well as, tenascin, gelatin, aggrecan, and many other substrates [14]. Gelatinases digest elastin, aggrecan, and collagen types IV, V, and XI [14]. They also act synergistically with interstitial collagenase in the degradation of fibrillar collagens. Recent studies have shown that MMP-2 can cleave native type I collagen in the same position as MMP-1 [15], and MMP-9 cleaves $\alpha 2$ chains of type I collagen [16]. Finally, a few of the MT-MMP substrates are collagens type I, II, and III, gelatin, casein, fibronectin, laminin, vitronectin, and entactin [14].

As evidenced by their substrate specificity, MMPs play a significant role in normal and pathological conditions that involve matrix degradation and remodeling [17]. This includes, but is not limited to, wound healing, post partum involution, congestive heart failure, rheumatoid arthritis, osteoarthritis, and cancer (reviewed in [18-22]). In cancer, degradation of the ECM by MMPs not only enhances tumor invasion, but also affects tumor cell behavior and leads to cancer progression [10].

Metastasis, a multi step process, involves dissemination of cells from the primary tumor to distant organs or tissues and the formation of secondary tumors. Expression of MMPs apparently leads to dissociation of cell-cell and cell-ECM adhesion through the processing of cadherins, thereby leading to EMT initiation [8, 23, 24]. This transition can lead to increased extracellular proteolytic activity further promoting migration. MMPs are also capable of affecting cell regulatory signaling, directly or indirectly, within the extracellular microenvironment, which ultimately affects the cells intracellular signaling [25]. This is achieved by their role in the bioavailability of multiple growth factors and cytokines [10, 25]. MMPs also cleave insulin-like growth factors and release transmembrane precursors of growth factors, including TGF- β , which aids in proliferation [10]. Thus, MMPs are important through their ability to affect tumor initiation and growth via cell adhesion independency, evasion of apoptosis, disruption of proper cell division, and as mediators of metastasis during late stage malignancy [25].

MMP-9 in Cancer

It has been suggested that the production of MMPs in general and MMP-9 in particular is one of the most important factors for migration of tumor cells into the bloodstream or lymphatic vessels, or into adjacent normal tissues [26, 27]. MMP-9 (gelatinase B) has been shown to be closely associated with invasion and metastasis [28, 29]. MMP-9 degrades type IV collagen, one of the most abundant collagens in the ECM [22], and is involved in angiogenesis and proliferation. With the use of multistage pancreatic carcinogenesis in a transgenic RIP1-Tag2 mouse model, MMP-9 was shown to be up-regulated in angiogenic islets, leading to an increase in the bioavailability of VEGF [30]. Xu, et al. evidenced that MMP-9's cleavage of type IV collagen exposes an otherwise hidden HUIV26 cryptic site which is important in angiogenesis and tumor

growth *in vivo* [31]. Rao, et al. have demonstrated the down regulation of MMP-9 increased β 1-integrin which leads to ERK activation and apoptosis through one of two mechanisms: (1) release of cytochrome C into the cytosol and/or (2) increase in NF- κ B activation, which both lead to subsequent activation of caspase 3 [32]. Although few cell types express MMP-9 constitutively, the majority of human metastatic tumor cells (i.e. melanoma, fibrosarcoma, breast adenocarcinoma, glioma, etc.) that have been tested consistently show elevated MMP-9 activity compared with benign control cells [33-36]. In addition, tumor cells stably expressing the MMP-9 cDNA have been shown to metastasize in nude mice [37]. Furthermore, inhibition of MMP-9 activity has been demonstrated to block the invasive behavior of breast carcinoma cells, MDA-MB-231 [38]. Thus, inhibition of MMP-9 expression can be used as a therapeutic modality to decrease growth and invasion of tumor cells.

Analyses of the primary structures of MMPs revealed that these proteins could be divided into several distinct domains with apparent specific functions (Figure 1-1). A typical secreted MMP has three major domains: NH₂-terminal prodomain, central catalytic domain, and COOH-terminal hemopexin-like domain [18, 27]. The characterization of MMP-9 revealed two additional domains, one with homology to the gelatin-binding region of fibronectin and the other with a collagen-like sequence [39]. The fibronectin-like gelatin binding domain has been shown to be important for the gelatinolytic and type V collagenolytic activity of the enzyme [40]. Structural similarities among MMP members have made it very difficult to develop specific synthetic inhibitor for MMP-9 as a potential cancer therapeutic agent. The lack of specificity of most active site directed MMP inhibitors that entered clinical trials made

Figure 1-1. Structural domains of matrix metalloproteinases.

This is a schematic diagram showing the main structural domains of Collagenases (MMP-1) and Gelatinases (Gelatinase B, MMP-9; Gelatinase A, MMP-2). Gelatinases contain two additional domains compared to collagenases, the gelatin-binding domain and the collagen-like domain.

them more harmful than helpful [41]. For example, Batimastat, the first synthetic MMP inhibitor studied in humans, showed reduced growth of primary tumor, onset of distant metastases, and even prolonged survival of animals with pancreatic, orthotopic colon, or orthotopic liver tumor [42-44]. In addition, Marimastat, a derivative of Batimastat significantly increased progression-free survival in the patients with inoperable gastric cancer, without improving overall survival [45]. Despite favorable results of these synthetic MMP inhibitors in animal systems and human clinical trials, they lead to significant side effects, which include but are not limited to fatigue, anorexia, nausea, vomiting, asthenia, and inflammatory polyarthritis [46, 47]. These multiple side effects associated with usage of the non-specific MMP inhibitors in clinical trials are comprehensible since as stated above, MMPs are required for normal physiological processes such as cell growth and remodeling, ovulation, and wound healing. In addition, two MMP members, MMP-3 and -8, have been identified as cancer anti-targets because their down regulation results in initiation of the disease or deleterious alteration in disease progression [27]. In order to overcome this problem, the mechanism of substrate specificity of MMP-9 must be identified and considered in the design of the specific inhibitor to be used for cancer therapy.

The role of the hemopexin-like carboxyl terminal domain of MMP-9 is of interest, since we have recently shown that it dictates substrate specificity of the enzyme (please see below). The role and significance of collagen-like and hemopexin-like carboxyl terminal domains in substrate specificity of MMP-9 has been investigated in Chapter 5.

DNAzymes as Novel Treatments for Cancer

Over the past few years, DNAzymes have been used *in vitro* to selectively inhibit or modify specific mRNA levels in mammalian cell cultures [48]. DNAzymes are catalytic DNA molecules that in the absence of proteins bind to and cleave specific mRNA sequences, which results in the decreased expression of the encoded protein [49] (Figure 1-2).

DNAzymes have emerged as novel, highly selective inhibitors or modulators of gene expression [50]. Khachigian and colleagues have reported that the DNAzymes targeting early growth response factor-1 (Egr-1) mRNA inhibits neointimal formation after balloon injury to the rat carotid artery wall and reduce intimal thickening after stenting pig coronary arteries. DNAzyme targeting c-Jun caused repair of injured rat carotid arteries [51]. Upregulation of Aurora kinase A has been shown to be important in carcinogenesis of prostate cancer. DZ2, an Aurora kinase A specific DNAzyme was shown to strongly inhibit Aurora kinase A in a prostate cancer cell line *in vitro* and inhibit tumor growth of human prostate cancer xenografts in nude mice [52]. Finally, a DNAzyme targeting vascular endothelial growth factor receptor 2 (VEGFR2) significantly inhibited the growth of breast tumors derived from xenografting MDA-MB-435 cells into nude mice by inducing apoptosis [53]. These data demonstrate that these small nucleotide mRNA cleaving sequences can potentially be designed and administered to target specific genes with relative lack of toxicity. DNAzymes have

Figure 1-2. Composition of the 10-23 catalytic motifs.

The DNA enzyme (bottom strand) binds the RNA substrate (top strand) through Watson-Crick pairing. Cleavage occurs at the position indicated by the arrow. R=Purine (A or G); Y=Pyrimidine (U or C). Reprinted with permission from Santoro SW, Joyce GF (1997). A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci U S A* **94**: 4262-4266.

excellent potential as a biotherapy against matrix metalloproteinases.

As stated above, since DNAzyme molecules can be designed to target a specific gene with relative lack of toxicity, it is expected that specific down regulation of MMP-9 by DNAzyme will prevent tumor cell invasion and metastasis while generating a minimum side effect.

Design and *In Vitro* Efficacy of Anti-MMP-9 DNAzyme (AM9D)

To test whether down regulation of MMP-9 by an MMP-9 mRNA targeted DNAzyme could be used as a treatment strategy for cancer, Dr. Hasegawa, a postdoctoral fellow in our laboratory, designed several anti-MMP-9 DNAzymes (AM9D) according to putative DNAzyme target sites in the predicted secondary structure of MMP-9 mRNA and selected the most efficient AM9D with respect to its activity toward RNA substrate. As shown in Figure 1-3a, only AM9D-2 (lane 3) and AM9D-5 (lane 6) were able to efficiently cleave over 85% of the RNA substrate in 2 h at 37 °C as determined by the density of the RNA fragment products. These reactions were time dependent as a longer incubation of the RNA substrate with the AM9D-2 and AM9D-5 led to complete cleavage of the RNA molecule (data not shown). The AM9D-1, scrambled control, and sense control DNAzymes (lanes 2, 7 and 8, respectively) at the same concentration did not have any activity toward the RNA substrate. AM9D-3 and -4 (lanes 4 and 5) were able to cleave only ~ 25% of the RNA substrate. AM9D-5 (referred to in this body of work as AM9D) and scrambled control DNAzyme was used for further studies.

Figure 1-3. Efficacy of anti-MMP-9 DNzyme (AM9D) *in vitro*.

(a) Urea-Polyacrylamide gel electrophoresis of cleaved MMP-9-RNA by DNzyme. Anti MMP-9 DNzymes, scrambled, or sense control DNzymes (12.5 μ M) were incubated with MMP-9 RNA substrate (764 nt, 0.48 μ M) for 2 h at 37 $^{\circ}$ C and the products were analyzed by polyacrylamide-urea gel. Lane 1, RNA substrate alone; lane 2, AM9D-1; lane 3, AM9D-2; lane 4, AM9D-3; lane 5, AM9D-4; lane 6, AM9D-5; lane 7, Scrambled control; and lane 8, Sense control DNzymes. The RNA bands were visualized by Ethidium Bromide staining. The lowest molecular weight bands are DNzyme. (b) AM9D reduced MMP-9 mRNA level. MMP-9 stably transfected COS-7 cells (COS-7 NG) were transfected with Oregon Green 488 labeled DNzymes. Positively transfected cells were identified by flow cytometry. Total RNA was isolated and MMP-9, MMP-2, and β -actin mRNA were amplified by RTPCR. The PCR products were subjected to agarose gel and visualized by Ethidium Bromide staining. Lane 1, AM9D-5; lane 2, control DNzyme; lane 3, Cells treated with DOTAP transfection reagent only and lane 4, the PCR product from parental COS-7 cells (non-transfected with MMP-9). (c) AM9D decreased MMP-9 production. Culture media from MMP-9 stably transfected COS-7 cells (COS-7 NG) treated with DOTAP alone (lane 1), control DNzyme (lane 2), and AM9D-5 (lane 3) were separated on gelatin zymogram gel. As shown, AM9D-5 down regulates production of MMP-9 in COS-7 NG cells. (d) AM9D decreased the invasive behavior of human MDA-MB-231 breast cancer cells. The percentage ratio of carcinoma cells invading the Matrigel Matrix after treatment with AM9D-5 against that of cells treated with control DNzyme after 24 h. The AM9D-5 transfected cells showed a 43% reduction in cell invasion compared to control DNzyme transfected cells.

To assess the effect of AM9D-5 on MMP-9 production *in vitro*, monkey kidney cells lacking MMP-9 (COS-7 cells, Figure 1-3b, lane 4) were stably transfected with MMP-9 cDNA to constitutively express the protein. The effect of AM9D-5 on the production of MMP-9 in the stably transfected cells (COS-7 NG) was then examined by transiently transfecting the cells with different amounts of either control DNAzyme or AM9D-5 using Gene PORTER (Genlantis, San Diego, CA). Twenty-four h after transfection, the cells were harvested, total RNA was extracted, and condition media were examined for the presence of MMP-9 mRNA and protein by RT-PCR (Figure 1-3b) and by gelatin gel zymography (Figure 1-3c), respectively. As demonstrated in Figure 1-3b and c, AM9D-5 was able to significantly reduce the level of the MMP-9 mRNA (Figure 1-3b, lane 1) and protein as evidenced by the low enzyme activity in AM9D-5 treated COS-7 NG cells (Figure 1-3c, lane 3) compared to non-treated and control DNAzyme treated cells (Figure 1-3c, lanes 1 and 2, respectively).

To demonstrate the specificity of AM9D toward MMP-9 mRNA its activity toward MMP-2 mRNA, a closely related enzyme to MMP-9, was determined. COS-7 cells constitutively express MMP-2 (Figure 1-3b and c). Stably transfecting COS-7 cells with MMP-9 cDNA (COS-7 NG) and treating the cells with AM9D-5 did not have any effect on MMP-2 mRNA (Figure 1-3b) or protein (Figure 1-3c) expression levels. This data clearly demonstrates that the AM9D-5 is very specific and it only affects the MMP-9 production in cells.

To determine the effect of decreased MMP-9 mRNA expression on the invasive behavior of cancer cells, MDA-MB-231 highly invasive breast cancer cells were transiently transfected with fluorescently labeled AM9D-5 or control DNAzyme, FACS sorted and the invasive behavior of the sorted cells was determined using ECMatrix™ (Millipore, Billerica, MA) invasion chamber. The mean invasion potential of MDA-MB-231 cells decreased by 43% when transfected with AM9D-5 compared to control DNAzyme treated cells (Figure 1-3d). These data indicate that MMP-9 significantly contributes to the invasive behavior of MDA-MB-231 tumor cells and provides evidence that AM9D treatment significantly and specifically reduces MMP-9 production and suppresses the invasive behavior of breast tumor cells *in vitro*. Thus, AM9D may be a useful anti-tumorigenic and anti-metastatic agent *in vivo*.

RESEARCH OBJECTIVES AND SPECIFIC AIMS

Objective

The objective of this proposal was to evaluate the efficacy of an MMP-9 targeted DNAzyme as a therapy in treating breast carcinoma and preventing lung metastasis. If anti-MMP-9 DNAzyme (AM9D) therapy is proven useful in reducing tumor growth and preventing metastasis in an animal model of breast cancer, this will be an important step in the preclinical development of a new biotherapy with excellent potential for expanding the standard of care for breast cancer patients.

Specific Aim 1

The first aim of this body of work was to test the efficacy of anti-MMP-9 DNAzyme oligonucleotides on tumor growth in MMTV-PyMT transgenic mice. This was accomplished by intratumorally injecting control DNAzyme or AM9D into mammary tumors once a week, for 4 weeks, at an early palpable tumor size. Tumor sizes were measured weekly and the animals were sacrificed at a selected time point (one week after the 4th injection). The efficacy of MMP-9 downregulation by AM9D on the rate of tumor growth, final tumor load, development of new vasculature, and tumor cell survival was evaluated.

Specific Aim 2

The second aim of this body of work was to determine the distribution, clearance, and cytotoxicity of AM9D. This was accomplished by intravenously injecting ³⁵S-labeled DNAzyme ([³⁵S]-DNAzyme) molecule into normal and tumor-bearing MMTV-PyMT mice, sacrificing mice at various time points (2 h -7 days), and determining the amount of DNAzyme in organs and mammary tumors. To determine organ toxicity by DNAzyme in general and the anti-MMP-9 DNAzyme (AM9D) in particular, mice were injected with PBS or escalating concentrations (200 µg, 650 µg, or 1500 µg) of AM9D or control DNAzyme. Mice were sacrificed on day 7 post treatment and a Pathologist evaluated major organs for any signs of toxicity.

Specific Aim 3

The third aim of this body of work was to determine the effect of AM9D on experimental metastasis. To accomplish this goal normal FVB mice were injected with luciferase labeled mammary tumor epithelial cells (luc-MTEC) derived from the MMTV-PyMT transgenic mice used in the experiments described in specific aim 1. Mice received weekly intravenous injections of AM9D, control DNAzyme, or PBS. These mice were sacrificed on day 21 or when they displayed signs of lethargy due to lung tumor burden to assess the role of AM9D on early stage experimental metastasis and overall survival, respectively.

Specific Aim 4

The fourth aim of this body of work was to determine the mechanism of substrate specificity of MMP-9. As stated above, degradation of basement member components by MMP is one of the first steps in tumor cell invasion and metastasis. Our data indicate that contrary to a general belief that MMP-9 only cleaves types IV and V and XI collagen, MMP-9 generated by tumor and stroma cells is also capable of cleaving type I collagen. Thus, understanding the mechanism behind collagen substrate specificity of MMP-9 sheds light on its role in metastasis, the major cancer-surviving determinant. Specific

domain(s) and amino acid residues responsible for substrate specificity of the enzyme were identified by deletion and Ala scanning site directed mutagenesis of the MMP-9 molecule.

CHAPTER 2. TREATMENT OF TUMOR GROWTH WITH AM9D

INTRODUCTION

Accumulating evidence suggests that production of matrix metalloproteinase-9 (MMP-9) by tumor and stroma cells is one of the most important factors for metastatic behavior of tumor cells [8, 23, 54]. MMP-9 is a member of the matrix metalloproteinase family of enzymes, which play an important role in normal physiological responses, including wound healing and bone formation [55]. Some MMPs, including MMP-9, become deregulated during tumorigenesis and are associated with pro-oncogenic events such as neo-angiogenesis, tumor cell proliferation and metastasis [9]. For example, MMP-9 was shown to be upregulated in angiogenic islets of the RIP-Tag2 transgenic mouse model of pancreatic cancer, leading to an increase in the distribution of VEGF, a potent pro-angiogenic factor [30]. Moreover, high levels of MMP-9 expression in breast cancers have been shown to be positively correlated with enhanced tumor cell invasion and metastasis [28, 29] and with enhanced progression and poor prognosis [9].

MMP-9 is conserved across several species (human, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and *A.thaliana*). MMP-9 degrades type IV collagen, one of the most abundant collagens in the extracellular matrix (ECM) [56], which may stimulate local invasion, the first step in metastasis. In addition, MMP-9 also cleaves pro-cytokines, chemokines, and growth factors, thereby modifying their biological activity [10, 25, 57]. In addition, tumor cells that stably express MMP-9 cDNA have been shown to have enhanced metastatic ability [37]. Thus, inhibition of MMP-9 expression by our Anti-MMP-9 DNazymes could be a useful therapeutic modality to decrease the growth and invasive properties of tumor cells.

As described previously, DNazymes have been used by several investigators to selectively inhibit or modulate different gene expressions *in vivo* [50]. Khachigian and colleagues have reported that the DNazymes targeting early growth response factor-1 (Egr-1) mRNA inhibits neointimal formation after balloon injury to the rat carotid artery wall and reduce intimal thickening after stenting pig coronary arteries. DNzyme targeting c-Jun caused repair of injured rat carotid arteries [51]. Finally, a DNzyme targeting vascular endothelial growth factor receptor 2 (VEGFR2) significantly inhibited the growth of breast tumors derived from xenografting MDA-MB-435 cells into nude mice [53].

The purpose of the present study was to evaluate the effect of AM9D on breast tumor growth in the MMTV-PyMT mouse model of breast cancer. It is demonstrated for the first time that once weekly intratumoral injection of AM9D, in the absence of any carrier molecule, for four weeks was sufficient to significantly reduce the rate of tumor growth and final tumor load in a dose dependent and statistically significant manner ($p \leq 0.05$). Together, the data presented here justify the further development of AM9D for its potential as an anti-tumor agent and as an ideal candidate for breast cancer therapy.

MATERIALS AND METHODS

DNAzyme

All DNA oligonucleotides used in these experiments were synthesised by Integrated DNA Technology (Coralville, IA). The sequence of the DNAzyme targeting mRNA of human and mouse MMP-9 is, 5'-GTGGTGCCAGGCTAGCTACAACGA TTGAGGCG-3'. In the control DNAzyme, 5'-CTAGTCAGCGGCTAGCTACAACG ATAAGCTGCT-3', the catalytic sequence of DNAzyme is flanked by 9 bases randomly chosen and not specific for any MMP coding sequence. DNAzyme used in some of the experiments was labeled with Oregon Green™ 488 C5-maleimide (Invitrogen, Carlsbad, CA). Oregon Green™ 488 C5-maleimide was prepared in dimethyl sulfoxide to a stock concentration of 25mM. The 5'-hydroxyl terminus of the DNAzyme was labeled initially with ATPγS (Sigma, St. Louis, MO; 600 μM) using T4 Polynucleotide kinase (New England Biolabs, Ipswich, MA; 30U) followed by Oregon Green 488 maleimide (1mM) at 37°C as suggested by manufacture. The labeled DNAzyme was then purified by ethanol precipitation.

MMTV-PyMT Transgenic Mammary Tumor Model

The MMTV-PyMT transgenic mice express polyoma middle T antigen under the control of mouse mammary tumor virus long terminal repeat [58]. The MMTV-PyMT transgenic mouse model is a widely used preclinical model of estrogen and progesterone receptor negative luminal breast cancer with well-defined stages of progression and metastasis to lung [59, 60]. On a pure FVB/Nj strain background, all PyMT-positive females exhibit an average tumor latency of 58 days after birth, independent of pregnancy and will eventually develop mammary tumors in each of the ten mammary glands [61] (Figure 2-1), the time of tumor onset varies among individual glands [60]. More than 90% of mice develop pulmonary metastasis by 100 days [62]. More importantly, the mammary adenocarcinomas exhibit changes in biomarkers and expression patterns of various MMPs similar to those observed in human breast cancer patients [59, 63-67]. Therefore, this model was chosen to ascertain the role of AM9D as a pharmacologic inhibitor of MMP-9.

All animal experiments were conducted following approval by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee (IACUC). FVB/Nj female mice were obtained from Jackson Laboratory (Bar Harbor, ME) and crossed with PyMT positive FVB males. The offspring were genotyped by real-time PCR on a Roche LC 480 LightCycler using the following primers and universal probe library (UPL), probe #11 (Left primer: 5' AACCCGAGTTCTCCAACAG 3; Right primer: 5' TCAGCAAC ACAAGGATTTC 3') to identify MMTV-PyMT positive females. Female mice were palpated once a week beginning at approximately 4 weeks of age and palpable tumors were measured in two dimensions (longest diameter and shortest

Figure 2-1. Mouse mammary glands.

This is a basic diagram of the 10 mouse mammary glands and possible place of tumor development in the MMTV-PyMT transgenic mouse model. Reprinted with permission from Ward J. The Virtual Mouse Necropsy. Comparative Medicine Branch, NIAID, NIH. Accessed March 25, 2010. <http://icg.cpmc.columbia.edu/cattorette/Protocol/FilesInPdf/NCIVetp.pdf>.

width) with digital calipers. Tumor volume was calculated using the formula, tumor volume = $(\text{width}^2 \times \text{length}) / 2$. When each transgenic female developed at least three palpable tumors of dimensions of 3 mm x 5 mm, which typically occurred at 8 weeks of age, each tumor was injected intratumorally with 5 μ l PBS containing either 10 or 25 μ g of AM9D or control DNase using a Hamilton syringe mounted with a PT2, 26G needle. Tumors identified at approximately 8 weeks of age were injected once per week for a total of four weeks of therapy, and the site of intratumoral injection was varied to ensure that all areas of the tumor were exposed to the AM9D or control DNase. Palpable mammary tumors that arose after the first intratumoral treatment in other mammary glands of the same mice were left untreated. For each cohort, transgenic females with a combined number of at least 9 tumors of comparable size were utilized (AM9D, 10 μ g, n = 2 mice, 9 mammary tumors; AM9D, 25 μ g, n = 2 mice for a total of 10 mammary tumors and control DNase, 10 or 25 μ g, n = 4 mice, 12 mammary tumors). The above experiment was repeated using 25 μ g of either AM9D (2 mice; 12 tumors) or control DNase (2 mice; 9 tumors) and the results were included in the calculation of tumor endpoint weight reported.

Tumor growth was monitored weekly by caliper measurement. All animals were euthanized one week after the last DNase treatment (typically at 12 weeks of age). At necropsy, tumors were removed, final tumor dimensions were measured by calipers, and

the tumor wet weight was determined. Tumors were then either flash frozen in liquid nitrogen, or fixed in 4% paraformaldehyde overnight, followed by cryoprotection in 25% sucrose for several days. Cryoprotected tumors were then washed with 0.1% PBS prior to embedding in OCT compound and preparation of 8 micron frozen sections.

***In Situ* Hybridization**

The presence of DNazymes in mammary tumor slices was detected by *in situ* hybridization using 5' end fluorescently labeled anti-AM9D sense oligonucleotides (5'-CGACCTCAAtcgtttagtagccTGGcACCAC-3') as a probe. The tumor slices were incubated with 3 µg of probe in hybridization buffer consisting of 25% formamide, 2X SSC, 0.5 mg/ml Salmon Sperm DNA, 1X Denhart's solution, 100 mM DTT and 10% Dextran Sulfate, overnight at 42 °C. The slides were then washed twice with 2xSSC, 0.1% SDS for 5 min at RT followed by two washes with 2x SSC, 25% Formamide for 20 min at 45 °C, dried at RT, coverslipped and visualized by fluorescent and confocal microscopy. The nucleus was visualized by addition of DAPI/anti-fade to each slide.

Histology and Immunohistochemistry

Mammary tumor sections were stained with hematoxylin and Eosin Y (H&E; Fisher Scientific, Suwanee, GA). To block non-specific staining for immunohistochemistry, sections were treated with 2% BSA in 1X PBS/0.2% Triton X-100. Tumor vasculature was then visualized using rat anti-mouse CD31 antibody (1:50) (BD Biosciences, San Jose, California) and Alexa Fluor-594 goat anti-rat IgG (H+L) secondary antibody (Invitrogen, Carlsbad, CA). Stromal cells (myofibroblasts) were detected by incubating the sections with anti- α -smooth muscle actin antibody at 1:250 dilution (Sigma St. Louis, MO) overnight at 4°C and Alexa Fluor 488 goat anti-mouse IgG2a (Invitrogen, Carlsbad, CA) secondary antibody at 1:500 dilution, for 1 h at room temperature. MMP-9 protein was detected using a rabbit anti-mouse MMP-9 antibody at a 1:200 dilution (Santa Cruz) overnight at 4°C followed by Alexa Fluor-594 goat anti-rabbit IgG antibody (1:500) at room temperature for 1h. CD31 and caspase-3 stained sections were blindly reviewed and grouped depending on their degree of red (CD31) and green (caspase-3) positive staining. MMP-9 stained slides were quantified by taking z-stack images and using image J. Digital images were captured using a Bio-Rad Confocal Laser Scanning Microscope, using the Lasersharp 2000 software.

Statistical Analysis

All data are expressed as mean $\pm$ standard deviation or standard error. Data were analyzed with SSPS software (SigmaStat version 2.03) using one-way analysis of variance (ANOVA). Tumor growth over time among three groups was analyzed by two-way ANOVA using Prism software (Graphpad version 4.0b). In all cases, p values <0.05 were considered statistically significant.

RESULTS

MMP-9 Is Expressed in Mammary Tumors of MMTV-PyMT Mice

To confirm the presence of MMP-9 protein in late-stage mammary carcinomas, tumors were harvested from MMTV-PyMT transgenic females, at 12 weeks of age. Tumor sections were stained with antibodies to both α -smooth muscle actin (SMA), a marker for stromal myofibroblasts, and MMP-9. Immunohistochemical analysis demonstrated the presence of MMP-9 in stroma and in other areas, which could be tumor epithelium, recruited immune cells, and endothelium (Figure 2-2).

DNAzyme *In Vivo* Stability

Prior to testing AM9D for its effect on mammary tumor growth, the *in vivo* stability and cellular uptake of naked DNAzyme molecules was examined by intratumorally injecting tumor-bearing MMTV-PyMT transgenic female mice with fluorescently labeled AM9D (25 μ g) suspended in PBS. The animals were then sacrificed at different time intervals post AM9D injection, and mammary tumors were harvested, sectioned, and viewed under fluorescent microscope. Fluorescently labeled oligonucleotides could be easily detected in a diffuse pattern within the tumor for up to 14 days after injection (Figure 2-3). Moreover, the AM9D could also be detected in adjacent, non-injected mammary tumors of the same mouse (Figure 2-3b), indicating a wide distribution pattern than might be expected from intratumoral injection. Therefore, the DNAzymes are stable *in vivo* and can efficiently distribute within the injected tumor and to a nearby non-injected tumor.

Figure 2-2. MMP-9 in MMTV-PyMT mice mammary tumors.

Immunohistochemical staining for MMP-9 in mammary tumor sections. Tumors were resected from MMTV-PyMT mice and double stained with antibodies to α -smooth muscle actin to detect stromal cells (a) and MMP-9 (b). When channels were merged (c), these data show that MMP-9 is present in both stromal cells and in other areas. Magnification 200X. Scale bar in (c) is equivalent to 100 μ m.

Figure 2-3. Stability of DNAzyme in mammary tumors.

Mammary tumors were injected as described in the methods with fluorescently labeled AM9D and resected at either (a) 7 days, (b) 10 days, or (c) 14 days post-injection. (e) Mammary tumor injected with unlabeled DNAzyme was used as a negative control. Of note, (d) DNAzyme injected into the 2R tumor of a mouse was found to be distributed to an adjacent, non-injected mammary tumor, 3R, which had emerged after injections were first initiated. Scale bar in (d) is equivalent to 100 μm .

Effect of AM9D Treatment on Final Tumor Load

The efficacy of AM9D to reduce breast tumor volume in MMTV-PyMT transgenic mice was tested by directly injecting two concentrations (10 μ g or 25 μ g) of AM9D or control DNase into mammary tumors of transgenic females bearing at least 3 tumors per mouse, each at an early palpable size (~3 mm x 5 mm), once a week for 4 weeks. Tumor palpations were performed weekly to determine changes in tumor volume over time. The rate of tumor growth in tumors treated with 25 μ g of AM9D (n=12 tumors) was slower than that of the untreated tumors (n=7 tumors) or DNase control treated (n=9 tumors) tumors (Figure 2-4a). Although, the observed difference in tumor growth over time in control and AM9D treated groups was not significant at early stage of growth (8-11 weeks of age; 2-way ANOVA, $p>0.05$), the difference was statistically significant at 12 weeks of age (Figure 2-4), the study endpoint. The p values obtained for AM9D vs. control DNase treated and untreated tumors were $p<0.001$ and $p<0.01$, respectively. The final tumor volume of AM9D treated tumors was significantly reduced compared to that of the control DNase treated tumors (Figure 2-4b). In fact, administration of AM9D at 10 μ g was sufficient to reduce the size of the tumor by 39.5%, which increased to 50.1% when 25 μ g of AM9D was utilized ($p<0.01$ for statistical comparison of control to 10 μ g and to 25 μ g, one-way ANOVA).

Immunohistochemical analysis of the mammary tumors (Figure 2-5) confirmed that AM9D treatment successfully down-regulated MMP-9 protein expression. As shown in Figure 2-5b, AM9D treatment reduced MMP-9 expression by $66 \pm 11\%$ in tumor compared to the control DNase treatment (Figure 2-5a). In addition, *in situ* hybridization of the tumor tissue slices demonstrated the presence of DNase in tumor and the surrounding tissue (Figure 2-6). Taken together, these data show that AM9D efficiently decreases MMP-9 expression in tumors, which is responsible for the observed anti-tumor effects.

Effect of AM9D on Angiogenesis and Apoptosis

MMP-9 has been shown to play a role in tumor progression through increase of bioavailability of VEGF and other factors that promote angiogenesis [30]. To determine the mechanism of tumor volume reduction by AM9D, the tumor slices were stained for CD-31 and for caspase-3 to assess the effect of AM9D on angiogenesis and apoptosis, respectively. AM9D treatment substantially reduced the number of blood vessels in the tumor as demonstrated by the lack of robust CD-31 immunostaining in the AM9D treated group (Figure 2-7c) versus untreated (Figure 2-7a) or the control DNase treated (Figure 2-7b) groups. These data suggest that anti-angiogenic effect of AM9D delays tumor growth over time and tumor volume at our study endpoint. Moreover, our data also indicate that AM9D induces apoptosis in the tumor. This is based on the observation that only AM9D treated tumors contain a large number of caspase-3 positive cells (Figure 2-8). Therefore, AM9D therapy can simultaneously induce tumor cell apoptosis and decrease stromal cell neo-angiogenesis.

Figure 2-4. Effect of AM9D on the rate of tumor growth and final mean tumor volume.

(a) AM9D-treated tumors (■) grew at a slower rate than either untreated tumors (no injections, ▼) or tumors treated with control DNAzyme (▲). The difference between control DNAzyme treated and untreated tumors is not significant at 8-11 weeks of age ($p>0.05$), but is statistically significant at 12 weeks of age ($p<0.05$), the study endpoint. (b) Weekly intratumoral treatment of transgenic mice with 10 µg ($n=9$ tumors) or 25 µg AM9D ($n=21$ tumors) per tumor reduced mean tumor burden by 39.5% or 50.1%, respectively, when compared to tumors treated with control DNAzyme ($n=24$ tumors) ($p<0.01$ ANOVA).

Figure 2-5. Effect of AM9D on MMP-9 protein expression in MMTV-PyMT mammary tumors.

Mammary tumors treated with either 25 µg control DNAzyme or AM9D were stained with an anti-MMP-9 antibody. Mammary tumors treated with (b) AM9D for 4 weeks showed lower MMP-9 staining compared to (a) those treated with 25 µg control DNAzyme for 4 weeks. Images are shown at 200x magnification; scale bar is equivalent to 100 µm.

Figure 2-6. Fluorescent *in situ* hybridization.

In situ hybridization performed on 25 µg AM9D-treated mammary tumors at study endpoint. (a) Green signal indicates the presence of AM9D DNAzyme (arrows) and (b) DAPI was used to detect cell nuclei. (c) Images (a) and (b) merged. Magnification 400X.

Figure 2-7. AM9D reduces tumor vasculature.

Immunohistochemical staining for CD31 (red) to detect the vasculature in mammary tumors harvested from mice treated with 25 μ g of AM9D (c and e) or control DNAzyme (b and d). A subset of sections was also counterstained with DAPI (d and e). Untreated tumors (a) and tumors treated with control DNAzyme (b and d) contained more blood vessels than did tumors treated with AM9D (c and e). Images are shown at 200x (a-c) or 400x (d and e) magnification. Scale bar is equivalent to 100 μ m (a-c) and 50 μ m (d and e).

Figure 2-8. AM9D induces apoptosis.

Immunohistochemical staining of mammary tumors harvested from mice treated for four weeks with 25 μ g (a) control DNAzyme or (b) AM9D for caspase-3 indicate AM9D treatment (b) induces apoptosis in mammary tumors. Caspase 3 staining is shown in green and nuclei are stained blue with DAPI. Very few caspase-3 positive cells (green) were detected in tumors treated with control DNAzyme (a) compared to AM9D-treated tumors (b), where large regions stained positive for caspase-3. Scale bar is equivalent to 100 μ m.

DISCUSSION

In this study, we show for the first time, that the down regulation of MMP-9 in mammary tumors by a novel anti-MMP-9 DNzyme molecule results in a significant reduction in final tumor volume in the MMTV-PyMT transgenic mouse model of breast cancer. Down regulation of MMP-9 by AM9D was accompanied by a decrease in MMP-9 expression, decreased angiogenesis and increased apoptosis. Moreover, these effects were accomplished by intratumoral injection of naked DNzyme without the use of any carriers. Together, these data indicate that inhibition of MMP-9 expression by DNzyme has potential as a novel therapeutic modality to decrease growth and invasion of carcinoma cells in the clinical setting.

It is known that MMP-9 plays a key role in angiogenesis by releasing VEGF [30] and that its down regulation induces apoptosis by stimulating ERK pathway [32]. Martin et al. [68] have demonstrated that spontaneous lung metastases decreased by 80% in MMTV-PyMT MMP-9 null mice compared to MMTV-PyMT MMP-9 wild type mice. In addition, the lung tumors developed in MMTV-PyMT MMP-9 wild type mice are larger in size and are more highly vascular compared to those tumors that developed in MMTV-PyMT MMP-9 null mice. Thus, the data presented here is consistent with the above studies and demonstrate that down regulation of MMP-9 by AM9D also affects tumor growth via inhibiting angiogenesis and inducing apoptosis (Figures 2-7 and 2-8) as demonstrated by lack of CD31 staining and the enhanced presence of caspase3 in AM9D treated tumors.

Our results are consistent with those of Almholt, et al. [67] which showed that the broad-spectrum MMP inhibitor Galardin/GM6001, significantly reduced primary mammary tumor growth by 50% and reduced lung metastasis by more than 100-fold in the MMTV-PyMT model. However, contrary to broad-spectrum MMP inhibitors, including GM6001, AM9D treatment specifically down regulates MMP-9 without affecting the expression of other members of the MMP family (Figure 1-3b and 1-3c). As demonstrated by the extent of cytotoxicity of broad spectrum MMP inhibitors in prior clinical trials [41, 46, 47], total inhibition of MMPs is not practical, since various MMPs can exert both pro-tumorigenic and anti-tumorigenic properties [17]. In addition, MMPs are critical for normal physiological processes, such as bone growth and remodeling, ovulation, and wound healing [19]. Further, in comparison with GM6001 [67], the intratumoral injection of AM9D not only reduced the required frequency of therapy; it was equally efficient in reducing final tumor size. Once weekly, intratumoral injections of 25 μ g AM9D (1.25 mg/kg) was sufficient to reduce the size of these spontaneously developed tumors by 50% as compared to the 51% tumor reduction observed following daily administration of 100 mg/kg of GM6001. Thus, the high degree of specificity for targeting MMPs, the lack of *in vivo* toxicity and the increased efficacy of AM9D as compared to broad spectrum inhibitors should enhance the clinical response of solid tumors, including breast tumors, while evading the serious side effects experienced with systemic therapy based on broad-spectrum MMP inhibitors.

CHAPTER 3. DISTRIBUTION, CLEARANCE, AND CYTOTOXICITY OF DNAZYME

INTRODUCTION

Enzymatic DNA molecules have been used recently in cell based assays and preclinical animal studies to treat various cancers. We have shown that intratumoral injections of anti-MMP-9 DNAzyme (AM9D) reduced mammary tumor burden in MMTV-PyMT transgenic mice by 50% (Chapter 2). As described previously, DNAzymes designed to specifically target Aurora kinase A inhibited growth of human prostate cancer *in vivo* [52]. A DNAzyme targeting VEGF2 mRNA led to a 75% reduction in tumor size compared to saline treated control [53] and treatment of MDA-MB-231 xenograft solid tumor model with an EGR-1 mRNA specific DNAzyme (DzF) resulted in inhibition of tumor growth compared to saline treated control [69]. However, all of these preclinical studies of tumor growth were conducted via intratumoral injections. While intratumoral injections can illustrate the promise of DNAzymes relative to target specificity and therapeutic potential, it is impracticable for clinical trials in humans. Thus, successful systemic application of DNAzymes in pathophysiological and therapeutic studies requires prior demonstration of significant uptake of DNAzyme by the target tissues. Moreover, to optimize DNAzyme regimens, tissue distribution and clearance must also be studied *in vivo*.

In this body of work, we have shown, for the first time, that naked AM9D is safe when administered systemically to healthy FVB mice and distributes to all organs of healthy mice and the Mouse Mammary Tumor Virus-polyoma middle T antigen (MMTV-PyMT) transgenic mouse model of breast cancer.

MATERIALS AND METHODS

Synthesis of ³⁵S-radiolabeled Oligonucleotides

The DNAzyme molecules were labeled at the 5' end using ATPγ- ³⁵S (Perkin Elmer, Waltham, MA) and T4 polynucleotide kinase (Promega, Madison, WI). The ³⁵S-labeled DNAzyme ([³⁵S]-DNAzyme) was then purified using a centri-10 column (Applied Biosystems, Carlsbad, CA). [³⁵S]-DNAzyme was dissolved in sterile Phosphate Buffered Saline (PBS). The concentration of the solution was adjusted so that 100 μl administered to 20g mice resulted in a dose of 10 mg/kg of body weight (specific activity, 0.275 mci/kg). [³⁵S]-DNAzyme was mixed with unlabeled DNAzyme to obtain the desired concentration.

Animal Experiments

All animal studies were done in accordance with the guidelines established by the Animal Care and Use Committee at The University of Tennessee Health Science Center. Twenty-three 8 week-old female FVB mice weighing 20-25 g and 19 MMTV-PyMT transgenic mice (FVB strain) bearing mammary tumors of various sizes and weighing 17-22 g were used in this study. MMTV-PyMT transgenic mice develop tumors in 6-8 weeks of age. Mice bearing a range of early ($<500 \text{ mm}^3$) and late stage ($>750 \text{ mm}^3$) tumors were used.

Systemic Distribution

Normal Mice. [^{35}S]-DNAzyme, 100 μl (10 mg/kg; 0.275 mCi/kg) in PBS was administered into 23 healthy anesthetized mice via tail vein injection. Three animals were placed in metabolism cages, immediately following injection, for 7 days (168 h). The urine and feces were collected daily for total radioactivity determination from each of the three animals. All samples were collected and stored at -80°C until analysis. The remaining 20 mice were divided into 5 groups of 4 animals each and at 2, 6, 24, 48, and 72 h following intravenous administration of [^{35}S]-DNAzyme, one group of animals was sacrificed and liver, heart, lung, kidney, and spleen were removed for determination of total radioactivity. This experiment was repeated using 6 mice divided into 2 groups of 3 mice each. At 2 and 6 h following intravenous administration of [^{35}S]-DNAzyme, one group of animals was sacrificed and liver, heart, lung, kidney, spleen, intestines, brain, pancreas, and stomach were removed for determination of total radioactivity. Immediately prior to sacrifice, blood samples (0.5-1 ml) were collected from anesthetized mice via the retro-orbital vein.

MMTV-PyMT Mice. DNAzyme was administered as above into 19 tumor bearing mice and these were divided into 5 groups of 3 animals each and one group of 4 animals. At 2, 4, 8, 24, 48, and 72 h following [^{35}S]-DNAzyme administration, one group of animal was sacrificed. Tumor volumes were measured using calipers during the experiments, and after excision, wet weights were recorded. Tumors exceeding 1.37g were highly heterogeneous (i.e. necrotic areas and mixture of tumor consistency) and thus were not included in any statistical analysis of mammary tumors. Heart, lung, kidney, liver, spleen, and blood samples were removed for determination of total radioactivity.

Radioactive Sample Analysis

Organs were solubilized in at least 1 ml SOLVABLE according to manufacturer's instruction (Perkin Elmer) and incubated overnight at 50°C . Endogenous peroxidase activity was quenched with 10mM EDTA and 6% hydrogen peroxide for 1 h at 50°C . Blood samples were collected from each mouse and 200 μl of each sample was incubated with 1 ml SOLVABLE at 50°C for 1 h. The endogenous peroxidase activity in blood was quenched with EDTA and hydrogen peroxide, as above. Approximately 10 ml of

scintillation cocktail fluid was added to each sample and tissue radioactivity was measured using a Packard Tri-Carb 2900CA liquid scintillation analyzer. Counts per minute obtained from each sample were converted to microgram of DNAzyme equivalents and presented as the total μg DNAzyme per either weight or volume of tissue, blood, urine, or fecal output. Initial and final rates of clearance were calculated based on the slope of the line obtained from plotting μg DNAzyme per gram of tissue (ml blood) as a function of time (initial: 2-6 h for normal mice and 2-8 h for MMTV-PyMT mice, final: 24-72 h). All data were expressed as mean $\pm$ standard deviation of mean. The quenching effect of tissue on radioactive count was tested by adding appropriate amounts of [^{35}S]-DNAzyme to scintillation vials containing solubilized normal organs. No significant tissue quenching effect was observed.

Cytotoxicity

Healthy mice were intravenously injected with PBS or various concentrations (10 mg/kg, 32 mg/kg, and 75 mg/kg of body weight) of either AM9D or Scrambled DNAzyme. Five mice per dosage for a total of 35 mice were used. The mice were sacrificed 7 days after DNAzyme administration. Following dosing, animals were observed daily for signs of toxicity including trouble grooming, lack of food consumption, and any other signs of lethargy; none was observed. At necropsy, liver, heart, lung, kidney, and spleen were collected and fixed in 4% paraformaldehyde, impregnated with 25% sucrose that acts as a cryoprotectant, and were processed for histopathological evaluation (standard light microscopic examination of hematoxylin and eosin-stained tissue slides).

RESULTS

Kinetics of DNAzyme in Normal Mice

The systemic distribution of DNAzyme in mice and its rate of clearance were determined by injecting naked [^{35}S]-AM9D (10 mg/kg) in PBS via tail vein into healthy FVB mice and the amount of radioactivity present in blood and different organs was measured as a function of time. Figure 3-1a illustrates the tissue/blood distribution time profile of DNAzyme in mice. AM9D injected via tail vein in mice is distributed to all major organs; liver, heart, spleen, kidney, and lungs. Two hours after DNAzyme injection, approximately 10% of the total DNAzyme was found in major organs (Table 3-1). The order of percentage accumulation was: blood>liver>kidney>lung>spleen>heart. Blood contained a majority of DNAzyme with 6.3% of the total DNAzyme in the first 2 h and reduced to 2.1% after 6 h (Table 3-1). Studies revealed 1.8%, 1.1% and 0.4% of total DNAzyme injected was delivered to liver, kidney, and lung, respectively. Finally, spleen and heart contained 0.21% and 0.2% of total DNAzyme in

Figure 3-1. Distribution and elimination profile of [^{35}S]-DNAzyme in normal mice.

(a) Distribution profile for [^{35}S]-DNAzyme in tissue and blood. The tissue concentration of [^{35}S]-DNAzyme derived radioactivity is based on μg equivalent DNAzyme/g of organ and is the mean ($\pm$ stdev) of 3-4 mice for each tissue. Blood concentrations are the mean values from all samples collected at each time point throughout the experiment. The DNAzyme blood concentration was first corrected for total blood in mouse based on the animal's weight and a mean of blood volume of $79 \mu\text{l/g}$ body weight. Then, for calculation of $\mu\text{g DNAzyme/g}$ of blood, 1 ml blood was assumed to be equal to 1 g. (b) Elimination profile for [^{35}S]-DNAzyme. The urine and fecal concentrations are mean values calculated for the total output from three mice each day for a total of 7 days.

Table 3-1. Tissue distribution of [³⁵S]-DNAzyme in normal mice presented in average percentage of total DNAzyme.

Tissue	2 h	6 h	24 h	48 h	72 h	7 days
Liver	2.198 (1.59-2.55)	1.375 (.94-2.2)	0.281 (.16-.46)	0.141 (.12-.16)	0.108 (.05-.14)	0.185 (.09-.31)
Heart	0.205 (.15-.26)	0.144 (.06-.22)	0.042 (.03-.05)	0.022 (.01-.02)	0.016 (.01-.03)	0.005 (.003-.007)
Spleen	0.215 (.17-.26)	0.08 (.05-.14)	0.029 (.02-.04)	0.02 (.01-.02)	0.021 (.017-.023)	0.008 (.005-.01)
Kidney	1.132 (.90-1.5)	0.575 (.33-.84)	0.209 (.17-.24)	0.138 (.11-.16)	0.109 (.09-.12)	0.048 (.03-.07)
Lung	0.366 (.28-.51)	0.216 (.15-.30)	0.061 (.04-.07)	0.043 (.04-.05)	0.033 (.02-.04)	0.01 (.008-.01)
Blood	6.264 (3.0-11.4)	2.103 (.96-3.3)	0.305 (.16-.42)	0.143 (.08-.17)	0.102 (.09-.13)	0.068 (.05-.08)

Numbers in parentheses give the range of data.

2 h post injection, respectively. The majority of the DNAzyme was cleared from these organs within the first 24 h. Thirty-one percent of the delivered dosage of [³⁵S]-DNAzyme was cleared from the system via feces and urine over a period of 72 h.

This experiment was repeated for time points 2 and 6 hours after DNAzyme injections to assess distribution of DNAzyme to the following additional organs: brain, pancreas, stomach, and intestines. Similar to the above experiments, 100 µl of [³⁵S]-DNAzyme (10 mg/kg; 0.275 mci/kg) in PBS was administered into 6 healthy anesthetized mice via tail vein injection. The 6 mice were divided into 2 groups of 3 animals each and at 2 and 6h following intravenous administration of [³⁵S]-DNAzyme, one group of animals was sacrificed and liver, heart, lung, kidney, spleen, intestines, brain, pancreas, and stomach were removed for determination of total radioactivity (Figure 3-2). As shown in Figure 3-2, 2 and 6 h after injection, in addition to the organs previously described, DNAzyme can be found in brain (0.312 µg and 0.248 µg DNAzyme/g), pancreas (1.147 and 1.0 µg DNAzyme/g), intestines (11.11 and 11.42 µg DNAzyme/g), and stomach (11.92 and 7.41 µg DNAzyme/g). This data demonstrates that DNAzyme molecule is capable of passing the blood brain barrier and suggests that it can be used as a therapeutic agent against brain tumors, as well as tumors which affect the pancreas, liver, and stomach (gastrointestinal stromal tumor).

The rate of initial clearance (from 2-6 h) and final clearance (from 24-72 h) of DNAzyme from different organs were calculated based on the amount of DNAzyme remaining in tissues as a function of time (Table 3-2). The rate of initial clearance of DNAzyme from blood was faster than kidney followed by spleen>liver>heart>lung

Figure 3-2. Distribution profile of [³⁵S]-DNAzyme in additional organs of normal mice.

The tissue concentration of [³⁵S]-DNAzyme derived radioactivity is based on µg equivalent DNAzyme/g of organ and is the mean (+/- stdev) of organs isolated from 3 mice.

Table 3-2. Rate of [³⁵S]-DNAzyme initial and final clearance in normal mice.

Tissue	Initial clearance ^a μg DNAzyme/g tissue/h	Final clearance ^b μg DNAzyme/g tissue/h
Liver	0.281	0.0039
Heart	0.149	0.0052
Spleen	0.442	0.0023
Kidney	0.495	0.0074
Lung	0.143	0.0022
Blood	5.202	0.0123

^aInitial clearance is between 2-6 h.

^bFinal clearance is between 24-72h.

(Table 3-2). The rates of initial and final clearance of DNAzyme from kidney were 0.495 μg DNAzyme/g of tissue/h and 0.0074 μg DNAzyme/g of tissue/h, respectively. The rate of initial clearance of AM9D from heart and lung are equivalent, however, DNAzyme is retained in lung longer as its rate of final clearance in lung is approximately 2-fold slower than that of the heart (Table 3-2).

Fecal excretion represented the major pathway of DNAzyme elimination (Figure 3-1b). The amount of DNAzyme excreted in feces (32%) is 3-fold greater than the amount released from urine (11%) over the 7-day period. As expected, DNAzyme excretion was time-dependent with the majority being eliminated in the first 72 h.

Kinetics of DNAzyme in Mammary Tumor bearing Mice

Female MMTV-PyMT transgenic mice bearing early to late stage mammary tumors were used to test the distribution of DNAzyme to mammary tumors. Naked [³⁵S]-DNAzyme in PBS was administered to tumor bearing animals via tail vein injections, animals were then sacrificed at different times and the amount of [³⁵S]-DNAzyme present in different organs, blood, and mammary tumors were determined as a function of time (Figure 3-3a and b). The order of percentages of [³⁵S]-DNAzyme accumulation in different organs of MMTV-PyMT mice was mammary tumor>liver≈blood>kidney>spleen>lung>heart. [³⁵S]-DNAzyme concentration in the mammary glands was markedly higher than in other tissues. In these mice, 4.1% of the total injected DNAzyme was accumulated in the mammary tumors in 2 h (Table 3-3). Mammary tumors of transgenic mice were of varying sizes and the amount

Figure 3-3. Distribution profile of [^{35}S]-DNAzyme in MMTV-PyMT mice.

(a) Transgenic mice developed on average 7 mammary tumors per mouse and the mean value of tumor for 3-4 mice were calculated. Small and medium size tumors displayed different accumulation and clearance profiles. For this reason, tumors weighing less than 1.37 g were divided into two categories, small (<0.7 g) and medium (≥ 0.7 g, ≤ 1.37 g). Tumors exceeding 1.37 g were excluded. (b) The tissue concentration of [^{35}S]-DNAzyme derived radioactivity is based on μg equivalent DNAzyme/g of organ and is the mean of 3-4 mice for each tissue. The blood samples were collected from all mice. The DNAzyme blood concentration was first corrected for total blood in mouse based on the animal's weight and a mean of blood volume of $79 \mu\text{l/g}$ body weight. Then, for calculation of μg DNAzyme/g of blood, 1 ml blood was assumed to be equal to 1 g.

Table 3-3. Tissue distribution of [³⁵S]-DNAzyme in MMTV-PyMT mice presented in average percentage^a of total DNAzyme.

Tissue	2 h	4 h	8 h	24 h	48 h	72 h
Liver	3.89 (2.7-5.76)	3.31 (3.2-3.5)	1.72 (1.5-1.9)	0.72 (.47-1.0)	0.46 (.419-.529)	0.31 (.215-.355)
Heart	0.33 (.27-.41)	0.24 (.17-.30)	0.17 (.11-.25)	0.05 (.03-.07)	0.018 (.012-.022)	0.009 (.007-.013)
Spleen	0.54 (.27-.88)	0.4 (.35-.46)	0.26 (.21-.32)	0.13 (.08-.24)	0.03 (.029-.038)	0.025 (.019-.028)
Kidney	1.27 (.83-1.9)	1.1 (.93-1.3)	0.63 (.54-.69)	0.3 (.03-.55)	0.15 (.13-.16)	0.14 (.011-.016)
Lung	0.38 (.18-.77)	0.28 (.18-.39)	0.184 (.1-.3)	0.07 (.04-.1)	0.051 (.046-.055)	0.029 (.022-.032)
Mammary Tumor ^b	4.1 (.06-9.2)	5.5 (.1-8.5)	5.2 (3.3-7.2)	1.3 (0.5-2.3)	0.049 (.011-.105)	0.038 (.020-.059)
Blood	3.52 (3.4-3.8)	2.97 (2.4-3.5)	1.64 (1.5-1.7)	0.69 (.52-.86)	0.216 (.168-.274)	0.115 (.080-.166)

^aNumbers in parentheses give the range of data.

^b Tumors larger than 1.37 g were excluded from calculations. Data range in mammary tumors is attributed to range of tumor sizes. The large range of data in organs, in some cases, is attributed to technical error of injection.

of DNAzyme accumulated in mammary tumors was proportional to the weight of the tumor burden (Figure 3-4).

Interestingly, the accumulated DNAzyme in the mammary tumor is initially cleared at a slower rate compared to the other organs, with rate of initial clearance (2-8 h post DNAzyme injection) of 0.068 μg DNAzyme/g tissue/h (Table 3-4). The mammary tumors characterized as small tumors (<0.7 g) continue accumulating DNAzyme 2-4 h post injection (Figure 3-3a). This corresponds with the initial clearance of DNAzyme from blood 2-4 h post DNAzyme injection (Figure 3-3b). Similar to DNAzyme distribution in normal mice, the percentages of the total DNAzyme accumulated in blood, liver, kidney, lung, spleen, and heart, 2 h post DNAzyme injection, was 3.5%, 3.9%, 1.3%, 0.4%, 0.5% and 0.3%, respectively (Table 3-3). However, the rate of initial clearance of DNAzyme from spleen was slower than blood but faster than any other organs tested followed by Liver $\approx$ Kidney>Heart>Lung>Mammary tumors (Table 3-4). The majority of the DNAzyme was eliminated from the organs and blood within the first 24 h (Figure 3-3b). The rate of initial clearance from liver and kidney was found to be the same (4.3 μg DNAzyme/g tissue/h) during the first 8 h of administration and slightly increased in kidney between 24-72 h. In heart, the rate of clearance in 2-8 h (initial) and 24-72 h (final) post administration was 2 and 3.4-folds faster than that in lung, respectively (Table 3-4).

Cytotoxicity of Intravenous Injection of AM9D in Healthy Mice

Safety of DNAzyme delivered intravenously in healthy FVB mice was determined using single injection of escalating concentrations of AM9D or control DNAzyme (10 mg/kg, 32 mg/kg, and 75 mg/kg of body weight) in PBS. PBS was used as a control. Seven days post DNAzyme injection, mice were sacrificed and liver, spleen, lung, heart, and kidney were isolated and analyzed for cytotoxicity by H&E staining (Figure 3-5). All tissues were evaluated (in a blinded manner) for evidence of capillary leakage, acute cellular damage including necrosis or apoptosis, acute inflammation and mitotic activity by a pathologist. No evidence of acute cytotoxicity, mitogenesis, cell death, local or widespread, was observed in the organs examined.

DISCUSSION

These results indicate that DNAzyme oligonucleotides could be delivered intravenously to all organs without the use of lipids or other carrier molecules, as naked DNAzyme was injected, and that the [^{35}S]-AM9D was present in various organs including mammary tumor tissues and organs of high incidence for metastasis (i.e. lung). In addition, mammary tumors can take up the DNAzyme faster and retain it longer than any other organs. Together, these data suggest that DNAzymes can be useful as a therapeutic agent to treat human patients with breast cancer or any other solid tumors.

Figure 3-4. Distribution of [³⁵S]-DNAzyme in MMTV-PyMT mammary tumors.

Total tumor burden was measured in grams and the total µg DNAzyme found in tumors were calculated. DNAzyme injected into mice bearing tumors of varying sizes show a proportional correlation between tumor size (all tumors combined per mouse) and DNAzyme distribution ($Y=1.4045x-0.4159$, $R^2=0.7764$).

Table 3-4. Rate of [³⁵S]-DNAzyme initial and final clearance in mice with mammary tumors.

Tissue	Initial clearance ^a µg DNAzyme/g tissue/h	Final clearance ^b µg DNAzyme/g tissue/h
Liver	0.434	0.0052
Heart	0.277	0.0082
Spleen	0.653	0.0278
Kidney	0.426	0.0063
Lung	0.134	0.0024
Mammary Tumor ^c	0.068	0.0128
Blood	1.507	0.0569

^a Initial clearance is between 2-8 h.

^b Final clearance is between 24-72 h.

^c Tumors larger than 1.37 g were excluded from calculations.

Figure 3-5. Pathology of organs from mice injected with AM9D, control DNAzyme, or PBS.

Mice injected with PBS, or various concentrations of control or AM9D showed no difference in organ pathology. The tissues displayed were extracted from mice that received the highest dose (75 mg/kg of AM9D and control DNAzyme; 400x magnification).

Recently, DNazymes have emerged as novel, highly selective inhibitors of gene expression. Although they have yet to be used in clinical trials, reports of studies demonstrating the efficacy of DNzyme based cancer therapies are beginning to emerge [50]. However, thus far, all of the preclinical studies testing the effect of DNzyme on tumor growth were via intratumoral or subcutaneous injections. In spite of the fact that tumor growth is an important factor in cancer progression, metastasis is ultimately the determining factor for cancer survival for many types of cancer [70-72]. Thus, while intratumoral injections can show promise as to the DNazymes specificity to its target and future therapeutic capabilities, it is not feasible in humans. To advance the therapeutic potential of DNzyme toward clinical trial, it is necessary to design an anti-metastatic DNzyme and demonstrate its anti-metastatic potential based on its systemic distribution, safety and efficacy.

Expression of MMP-9, by tumor and stroma cells has been shown in breast cancer patients [73] and is one of the most important factors for metastatic behavior of tumor cells [23, 54, 74]. MMP-9 degrades type IV collagen, one of the most abundant collagens in the ECM [11, 75] and is involved in cell proliferation, angiogenesis, and apoptosis [9, 10, 30]. Increasing evidence suggests that MMPs contribute to the formation of a microenvironment that promotes tumor growth during early stages of tumorigenesis. Our observation that AM9D treatment decreased MMP-9 production (Figure 1-3c and 2-4) and reduced invasive behavior of human MDA-MB-231 cells (Figure 1-3d) suggested that AM9D has a great potential as an anti tumorigenic/metastatic agent. Thus, to utilize anti-MMP-9 DNzyme (AM9D) as a therapeutic agent in breast cancer preclinical and clinical studies, healthy and MMTV-PyMT transgenic mouse model bearing early to late stage tumors were used to test the distribution of AM9D to different organs and mammary tumors. The order of percentage accumulation of DNzyme in healthy mice in different organs was: blood>liver>kidney>lung>spleen>heart. In addition to these organs, [³⁵S]-DNzyme was also distributed to brain, intestines, stomach, and pancreas (Figure 3-2). Approximately, 43% of the delivered dosage of [³⁵S]-DNzyme was cleared from the system via feces and urine over a period of 72 h (Figure 3-1b). This data is consistent with Agrawal et al.'s observation that 40% of intravenously administered anti-sense phosphorothioate oligonucleotides complementary to HIV RNA in mice, is excreted in urine and 16% in feces within the first 48 h [76]. Contrary to these data, however, we observed a higher excretion of DNzyme in feces than in urine (Figure 3-1b).

Interestingly, when [³⁵S]-DNzyme was administered to MMTV-PyMT transgenic mice the amount of DNzyme accumulated in mammary tumors 2 h post injection was 0.6% and 0.2% higher than in either blood or liver, respectively, and its rate of initial clearance from mammary tissue was slower than the other organs (Tables 3-3 and 3-4). The amount of DNzyme accumulated in mammary tumors was proportional to the tumor burden (Figure 3-4), suggesting a favorable therapeutic outcome. This is expected considering that mammary tumors can be highly vascularized [77].

DNzyme administered systemically is safe and at 75 mg/kg of body weight caused no observable mortality or organ toxicity in mice. Pathological studies did not

reveal any evidence of acute toxicity, mitogenesis, or cell death, local or widespread in any organs examined (Figure 3-5), contrary to those described for other oligonucleotides [78]. Several investigators have reported hepatocyte toxicity and necrosis [76, 78] and renal tubular structural perturbations [76] with oligonucleotide administration as low as 20 mg/kg [78] and as high as 30 mg/kg/day over 14 days (total 420 mg/kg) [76]. This however, was not observed in this study. It should be noted that the delivered dosage of AM9D for this study (75 mg/kg) was much higher than those used in the other DNAzyme studies [78], however, lower than some studies which showed hepatocyte toxicity [76].

In conclusion, systemic distribution and safety/toxicity studies revealed that the naked AM9D is non-toxic when systemically administered, and has favorable pharmacological properties with respect to intravenous administration and tissue distribution. Most importantly, mammary tumors can take up the naked DNAzyme. The amount of DNAzyme accumulates in the mammary gland is higher and its rate of clearance is slower than any other organs tested. These factors may permit the development of DNAzyme as a cancer therapeutic agent with much improved toxicity profiles as compared with conventional drugs.

CHAPTER 4. AM9D TREATMENT OF EXPERIMENTAL METASTASIS

INTRODUCTION

The role of MMP-9 in cancer metastasis has been widely researched. The treatment of DU145 prostate cancer cells with curcumin, an anti-metastatic derivative from tumeric, has been shown to decrease the levels of MMP-2 and -9 and lead to a decrease in tumor volume [79]. Curcumin treatment has also been shown to alter the invasive and metastatic properties of MCF10A human breast epithelial cells [80] and decrease metastasis to lung in a human breast cancer xenograft model [81] by decreasing the levels of MMP-9 and COX-2. According to Eiseler, et al., highly invasive breast cancer human tissue samples, regardless of stage and progesterone or estrogen dependence, were shown to have reduced Protein Kinase D1 (PKD1) expression in greater than 95% of the tumor samples compared to normal breast tissue [82]. Furthermore, the invasiveness of MDA-MB-231 cells was reduced by the re-expression of PKD1, which was shown to negatively regulate MMP-9, the mainly expressed MMP in MDA-MB-231 cells. [82]. Thus, PKD1 regulates breast cancer cell invasion through regulation of the expression of MMP-9. Furthermore, Chen et al. provided evidence that the presence of increased levels of MMP-9 in integrin $\alpha 1$ -null mice enhanced the number of tumors on lung but these tumors were smaller compared to lung tumors in WT counterparts [83]. They also showed that inhibition of MMP-9 by doxycyclin in these $\alpha 1$ -null mice led to reduced number of lung colonies but bigger tumors were observed. This was because reduced MMP-9 plasma levels led to decreased generation of angiostatin, an anti-angiogenic molecule. Thus, while an MMP-9 inhibitor may be useful in treating tumor cell migration, invasion, and metastasis it may adversely increase growth and vascularization of tumors intravenously introduced to lung.

In previous Chapters of this body of work we have shown that, in accordance with the findings of Eiseler, et al. [82], down-regulation of MMP-9 by AM9D decreases the invasive potential of MDA-MB-231 human breast cancer cells. We have also shown the presence of MMP-9 in MMTV-PyMT mammary tumors (Figure 2-2) and presented evidence that MMP-9 expression is significantly lower in AM9D treated tumors compared to control (Figure 2-5), leading to a reduction in final tumor load (Figure 2-4). Furthermore, AM9D when injected via the tail vein of normal and MMTV-PyMT mice was shown to be distributed to all major organs including lung and was tested to be safe up to 75 mg/kg of body weight (1500 μ g/100 μ l of PBS).

MMP-9 is involved in tumor cell extravasation and colonization [84-87]. Thus, down-regulation of MMP-9 by intravenous administration of AM9D was expected to inhibit the tumor cells from participating in extravasation and seeding of the tumor cells in the lungs. Consequently, the number of lung colonies will be reduced and further lead to increased survival of mice compared to control treated counterparts.

Using an experimental lung metastasis animal model, we have shown that both AM9D and control DNzyme treatment were equally effective in inhibiting early stage

experimental metastasis. However, mice treated with these two cohorts did not show statistically significant overall increased survival.

MATERIALS AND METHODS

Establishing eIF1 alpha-Luciferase2 Mammary Tumor Epithelial Cells (luc-MTECs)¹

Several late stage mammary tumors (>500 mm³) were isolated from *Hif1a* DF; PyMT+ females. Tumors were chopped with scalpels then with razor blades and the paste digested with type III collagenase (1 mg/ml, Worthington Biochemical, Lakewood, NJ) in RPMI media containing 5% FBS (5 ml/g tissue) for 2 hours at 37°C. Cells were pelleted at 1100 rpm and then washed 4 times with digestion buffer. Cells were plated with F12 medium containing 5 µg/ml insulin, 2 µg/ml hydrocortisone, 5 ng/ml EGF, 50 µg/ml gentamycin, 100 U penicillin/streptomycin and 10% FCS, as described by Rijnkels, M., et al. [88]. After 48-72 h, the plating medium was switched to complete mammary cell growth medium (DMEM/F12, 5% FBS, 5 mg/ml insulin, 10 ng/ml EGF). A subset of MTECs was subsequently weaned to reduced growth factor media (DMEM/F12 + 2% FBS or DMEM/F12 + 0.5% FBS). For sub-cultivation, cells were rinsed twice with Puck's A saline, then incubated for up to 60 min at 37°C in a 3:1 solution of dispase II/0.025% trypsin reconstituted in Puck's A saline. No EDTA was utilized for subcultivating cells, as we found that treating cells with trypsin-EDTA changed tumor cell morphology from an epithelial (cuboidal) to a mesenchymal (spindle) appearance. The luciferase expressing MTEC cells were generated by transducing monolayer cultured WT cells with pEILP (eIF1alpha-Luciferase2-IRES-puromycin, Junming Yue, UTHSC Viral Vector Core) lentivirus particles at 10 m.o.i. For 48 h hours, followed by washing and immediate selection with puromycin to establish pools of stable transfectants. All cells were passaged fewer than 30 times and media was routinely tested for mycoplasma using the MycoAlert kit (Lonza, Basel, Switzerland).

Animals

All animal studies were performed in accordance with the guidelines established by the Animal Care and Use Committee at The University of Tennessee Health Science Center. Fifty-six 8-week-old female FVB mice were intravenously injected with luc-MTECs (1x10⁶ cells) resuspended in 100 µl of sterile PBS containing 0.9 mM CaCl₂ and 0.493 mM MgCl₂. The injected animals were then divided into two groups. Group I contained 30 mice and group II contained 26 mice.

¹ Manuscript in preparation: Schawb, L., Peacock, D., Cushing, R., Majumdar, D., Ingels, J. and Seagroves, T., Hypoxia Inducible Factor-1a Promotes Primary Tumor Growth and Tumor-Initiating Cell Activity in the MMTV-PyMT Model of Breast Cancer.

Group 1. To determine the effect of AM9D treatment on early stage of metastasis, mice in this group were divided into 3 sub-groups of 10 mice each. On day 1-post MTEC injections the mice received weekly injections of PBS (100 μ l; subgroup 1a), AM9D (1500 μ g/100 μ l PBS; subgroup 1b) or control DNase (1500 μ g/100 μ l PBS; subgroup 1c). All animals were anesthetized with isoflurane before each injection. Mice received a total of 3 treatments on days 1, 7, and 14 post tumor cells injections and were euthanized one week after the last DNase treatment (Day 21). Mice were weighed weekly and observed for any signs of lethargy (i.e. weight loss, trouble breathing, not grooming) during treatment. Xenogen Bio-imager was used to monitor the development of lung nodules on days 14 and 21 post tumor cell inoculation. The luciferase positive cells were identified in lungs on day 21-post tumor inoculation at which time treatments were stopped and animals were sacrificed. Their lungs were inflated with 4% paraformaldehyde by cannulation of the trachea, excised, and photographed for any visual macro metastasis. Lungs were then fixed in 4% paraformaldehyde overnight. Paraffin embedded, 4 micron frozen sections were prepared and analyzed by Dr. Stanley Kosanke (Pathologist, University of Oklahoma). The lung sections were analyzed for the presence of macro and micro lung metastasis. At necropsy, liver, heart, brain, kidney, and spleen were collected and fixed in 4% paraformaldehyde, impregnated with 25% sucrose that acts as a cryoprotectant, and were processed for histopathological evaluation (standard light microscopic examination of hematoxylin and eosin-stained tissue slides).

Group 2. To determine the effect of AM9D treatment on survival of the animals with lung nodules, the MTEC inoculated mice were divided into 3 subgroups of 8-9 mice each. The mice in this group received weekly injections of PBS (100 μ l; subgroup 2a; n=8), AM9D (1500 μ g/100 μ l PBS; subgroup 2b; n=9) or control DNase (1500 μ g/100 μ l PBS; subgroup 2c; n=9) starting from the first day post MTEC injection. Mice were weighed weekly and monitored for any signs of lethargy (i.e. weight loss, trouble breathing, not grooming). The animals were then sacrificed when they lost ~20% body weight, or if they had trouble breathing, were panting and not grooming. The maximum treatment received by the last surviving animals was 9 injections and these animals were euthanized one week after the last treatment. Lungs were excised, photographed, weighed and fixed in 4% paraformaldehyde, as stated above.

RESULTS AND DISCUSSION

Effect of AM9D Treatment on Early Stage Experimental Metastasis

The efficacy of AM9D treatment in inhibiting and/or reducing early stage experimental metastasis was tested by treating luciferase labeled MTEC (luc-MTEC) inoculated FVB mice with PBS, 1500 μ g of either AM9D or control DNase, once a week for three weeks. The animals were monitored weekly for the presence of luc-MTEC in lung by Xenogen. At the earliest detection of MTEC in the lung (twenty one days after the initial treatment), animals were sacrificed. Luc-MTEC injected via tail vein of the

animals were able to metastasize to and colonize in the lungs (Figure 4-1). The average number of gross macro metastases observed in AM9D (1.1 ± 1.5) and control DNAzyme treated animals (1.6 ± 3) were, however lower than that of PBS treated groups (20.25 ± 31.3 ; Figure 4-2). This data suggests that AM9D and control DNAzyme treatment were equally effective in inhibiting metastasis and lung colonization. However, we cannot ignore the large standard deviation in the PBS control cohort due to the variability of 0 to more than 70 surface nodules observed at this stage of experimental metastasis. The analysis of macro metastasis in sectioned lung tissue revealed that AM9D therapy retarded lung metastasis by 56% compared to PBS, however, control DNAzyme treatment was 72% and 88% more effective in reducing lung macro metastasis than both AM9D and PBS treatments, respectively (Figure 4-2b).

The analysis of micro metastasis in sectioned lung tissues also revealed that control DNAzyme therapy retarded lung micro metastasis by 34% and 24% compared to AM9D and PBS treatment, respectively (Figure 4-2c). As mentioned above, a few mice in each group did not show any signs of gross macro metastasis (Figure 4-1a). However, analysis of lung tissue slices revealed that majority of the animals developed lung nodules, irrespective of lack of gross lung macro metastasis. The percentages of control DNAzyme, AM9D, and PBS treated animals that did not show any gross macro metastasis were 57%, 50%, and 37%, respectively. These data suggest that AM9D and control DNAzyme therapy are equally affective in reducing lung metastasis. These data also suggest that although AM9D down-regulates MMP-9 protein production *in vitro* and *in vivo*, both AM9D and control DNAzyme may have a sequence-related off target effect.

Bacterial DNA containing unmethylated CpG dideoxynucleotide sequence motifs have been shown to stimulate immune cells and this stimulatory effect can be mimicked

Figure 4-1. Representative lungs images extracted from two mice in the PBS treated group at early stage experimental metastasis.

PBS treated mice had (a) as few as no macromets to (b) greater than 70 macromets as visualized by gross tissue analysis. Arrows in (b) are pointing to macromets.

by synthetic CpG containing oligodeoxynucleotide (CpG-ODN) [89]. CpG motifs have been shown in literature to activate Toll-like receptor 9 (TLR9) and lead to the activation of macrophages, dendritic cells, and B cells [89, 90]. This activation is important for the treatment of cancer. CpG monotherapy is sufficient for inducing T-cell-mediated rejection of established tumors [91]. For these reasons, synthetic CpG-ODNs are currently in human clinical trials for the treatment of cancer [89]. In addition, CpG's that contain a purine two bases ahead of or a pyrimidine two bases after the CG nucleotides have been shown to be more stimulatory compared to CpG-ODNs that do not [90]. The sequences of AM9D and the control DNAzyme each contain two CpG motifs making them possible functional CpG-ODNs (Figure 4-3). The presence of macrophages and dendritic cells in lungs of animals treated with AM9D and control DNAzyme should attest to this hypothesis. Macrophages and dendritic cells were not observed in lungs 7 days after injection, however, lungs should be observed at a shorter time after injection.

AM9D was well tolerated in this early stage experimental metastasis mouse model. Body weights of animals in all groups were similar throughout the 3 weeks of treatment and at sacrifice (Figure 4-4). No obvious adverse effects, including swelling of joints and difficulty in walking, were observed in DNAzyme-treated mice. Furthermore, analysis of organs from mice exposed to weekly injections of AM9D or control DNAzyme for 10 weeks showed no toxic effects (Figure 4-5).

Effect of Long Term AM9D Treatment on Lung Tumor Load in Experimental Metastasis and on Survival

The efficacy of long term AM9D therapy to reduce lung tumor load and increase survival was tested by intravenously injecting PBS or 1500 µg of AM9D or control DNAzyme into 8 week old female FVB mice previously injected with 1×10^6 luc-MTECs, once a week until mice showed signs of lethargy (~20% weight loss, trouble breathing/panting, and/or not grooming). The animals were then sacrificed; their lungs were excised, weighed, and photographed. Mice were observed bi-weekly to determine changes in health and weighed weekly (Figure 4-6a). The lungs of all animals sacrificed due illness were completely covered with macro metastasis and no differences between the wet weight of lungs extracted from AM9D and control treated animals was observed (Figure 4-6b). Lungs in the PBS treated cohort showed a slightly elevated lung tumor burden (calculated by dividing lung weight by body weight; Figure 4-6c) compared to control DNAzyme and AM9D treated mice. Mice in the AM9D treated group displayed increased signs of progression-free survival between analysis points 34 to 42 days, however there was no significant extended survival compared to the control treated groups (log rank test, $p > 0.05$; Figure 4-7). This data is consistent with the results obtained from Marimastat clinical trial studies demonstrating that progression-free survival significantly increased in the patients with inoperable gastric cancer using Marimastat, but overall survival did not improve. In subgroups of patients with prior chemotherapy and of patients without distant metastases, overall survival was significantly better in the Marimastat-treated group [45]. These data suggest that AM9D

AM9D: 5'- GTGGTGCCAGGCTAGCTACAA**CG**ATTGAG**GT**CG -3'

Control: 5'- CTAGTCAG**CG**GCTAGCTACAA**CG**ATAAGCTGCT -3'

Figure 4-3. CpG regions in AM9D and control DNzyme.

AM9D and control DNzyme both contain two CpG regions (red). In each of these sequences one of the CpG regions has a pyrimidine 2 spaces after (orange). In the AM9D sequence there is a purine 2 spaces before the CpG region (blue).

Figure 4-4. Mouse body weights measured during experimental metastasis

The body weights of all the experimental animals were maintained during the course of the treatment (3 weeks) and no difference in body weights was observed between PBS (green), AM9D (blue) or control DNzyme (red) treatment groups.

Figure 4-5. Analysis of mice organ pathology.

Mice intravenously injected with luc-MTEC and treated with once weekly injections of 1500 μ g of either AM9D or control DNAzyme showed no difference in organ pathology. Representative images of various organs of AM9D and control DNAzyme treated mice shown (400x magnification).

Figure 4-6. Effect of various treatments on mice lung tumor burden.

(a) Mice treated with once weekly injections of PBS or 1500 μ g of either AM9D or control DNase were weighed on their final day of survival. Mice lungs were weighed upon excision (b) and their total lung tumor burden (c) was calculated. Weights of mice and lungs and calculation of lung tumor burden did not show a significant difference between AM9D, control DNase, and PBS treated groups ($p > 0.05$; error bars show standard deviation).

Figure 4-7. Survival curve.

Survival curve of mice injected with 1×10^6 luc-MTEC and treated with weekly injections of 100 μ l PBS (green), 1500 μ g AM9D (blue) or control DNAzyme (red). Log Rank Test $p=0.5$.

can be used as an adjuvant to primary surgery in order to blunt tumor growth and treat metastasis.

The lack of effect of long term AM9D treatment on tumor growth and overall survival in this experimental metastasis model may be due to time of AM9D delivery, frequency of delivery, mouse strain, pro-tumorigenic effects of MMP-9 downregulation, or a combination of these effects.

Time of delivery and/or frequency of delivery of AM9D may be responsible for the lack of significant decreased tumor burden in AM9D treated mice compared to control cohorts. Shibue, et al., have shown that D2 cells extravasate into lungs between 3-48 h after tail vein injection [92]. If MMTV-PyMT MTECs mimic this behavior, DNAzyme treatment may have been administered too late to block extravasation and colonization. Chen, et al., showed that treatment of CT26 (colon carcinoma cells) inoculated mice with 40 mg/kg/day of doxycyclin starting immediately or 2-weeks after tumor cell injections resulted in fewer but larger and more vascularized lung tumors. On the other hand, when therapy started at the time of tumor injections, mice had fewer lung tumors than mice treated 2-weeks after injection of colon carcinoma cells [83]. This suggests that the time and/or the frequency of the treatment have significant effect on development of lung experimental metastasis. In both early stage metastasis and long term AM9D treatment experimental groups, DNAzyme treatment began one day after injection of MTEC tumor cells and continued once weekly. The data presented in Chapter 3 (Distribution, Clearance, and Cytotoxicity of DNAzyme) indicated that DNAzyme administered intravenously is cleared from the lung within 24 h, but has an extended clearance from mammary tumors. Therefore, while weekly intratumoral injections may have been effective in reducing tumor growth (Figure 2-4), it may not be

sufficient for treatment of lung metastasis. If the observed lack of significant effect of AM9D therapy on survival of animals with lung metastasis is due to time of initial treatment or frequency of drug delivery, future experiments with DNAzyme treatment should begin in advance to or at the same time as tumor cell inoculations and delivered more frequently. We have observed, however that bi weekly injections (100 µl/injection), via tail vein results in hardening of the tail and necrosis. Therefore, the use of a different avenue of DNAzyme delivery is a necessity. Jugular vein may be the most efficient route for delivery of AM9D. This would allow for daily administration of 75 mg/kg AM9D, which would result in a total delivery of 525 mg DNAzyme/kg/week.

As mentioned above, Chen, et al., showed that intravenous administration of 40 mg/kg/day of doxycyclin into animals inoculated with CT26 (colon carcinoma cells) immediately or 2-weeks after tumor cell injections resulted in fewer but larger and more vascularized lung tumors. The increased vascularization was attributed to decreased production of angiostatin by MMP-9 [83]. Thus, MMP-9 inhibitors may have a null or pro-tumorigenic effect when administered intravenously. If anti-MMP-9 DNAzyme (AM9D) therapy leads to (1) decreased MMP-9 protein, (2) decreased anti-angiogenic factors, and (3) subsequent increased vascularization, then it is expected that AM9D treated animals develop larger lung tumors and have a decreased survival rate than the control DNAzyme and PBS treated cohorts. This is contrary to our observation that the AM9D treated mice showed increased signs of progression-free survival between analysis points 34 to 42 days compared to control cohorts (Figure 4-7). Hence, while AM9D did not show extended survival compared to control treated animals, it also did not show detrimental effects such as larger tumors or decreased survival. Thereby, the therapeutic effect of AM9D on experimental metastasis should continue to be evaluated with several adjustments in treatment regimen.

Another possibility for the observed lack of significant outcome of AM9D therapy on lung experimental metastasis may be related to the mouse strain used in these studies. Martin et al. showed that the treatment outcome of a gelatinase inhibitor, SB-3CT, on experimental metastasis is strain dependent [68]. In their experiments, luciferase-labeled 17L3C cells (MTEC from MMTV-PyMT mice) were injected via tail vein into C57BL/6 and FVB/N mice followed by daily injections of 50 mg/kg of the drug SB-3CT or vehicle for 14 days. They found that SB-3CT therapy resulted in a significant decrease in tumor growth in C57BL/6 mice, while mice on a FVB/N background showed enhanced tumor growth according to luciferase signal upon treatment. The mice utilized in our experimental metastasis model are FVB/N, the same strain that showed enhanced tumor growth when treated with a gelatinase inhibitor in the Martin, et al. publication [68]. These data suggest that the mice with different genetic backgrounds should be used to assess the significance of genetics on efficacy of AM9D in experimental metastasis.

In Chapter 2, I have shown that AM9D is able to reduce tumor growth by up to 50%. In order to determine if AM9D can be used in the treatment of breast cancer metastasis, the determining factor in breast cancer patient survival, the aforementioned experiments (including changes in time and frequency of treatment) must be performed.

CHAPTER 5. MMP-9 TRESSPASSES ON MMP-1'S TURF

INTRODUCTION

High expression levels of a variety of Matrix Metalloproteinases (MMPs), including MMP-1 and MMP-9 have been reported to be associated with progression and poor prognosis of breast cancer [93-95]. MMP-1 and -9 degrade type I and type IV collagen, respectively, two of the most abundant collagens in the ECM [11], making both enzymes important in cancer development, proliferation, and angiogenesis through their involvement in ECM degradation and cytokine release and activation [10, 11]. With the use of a transgenic multistage carcinogenic mouse model, MMP-9 has been shown to be up regulated in angiogenic islets, leading to an increase in the bioavailability of VEGF [30]. MMP-1 has been shown to be one of the most highly over expressed genes in bone metastasis [7]. In addition, researchers have shown that the presence of MMP-1 in the stromal-tumor microenvironment plays a role in altering the behavior of tumor cells by binding to and activating PAR1 and facilitating invasion [5, 96]. We have shown that down regulation of MMP-9 by AM9D inhibits the invasion of MDA-MB-231 cells (Chapter 1, Figure 1-3d). Thus, inhibition of MMP-1 and -9 expression can be used as a therapeutic modality to decrease growth and invasion of tumor cells.

Although MMP-1 and -9 have different substrate specificity, they are both composed of three major domains: NH₂-terminal pro-domain, central catalytic domain, and COOH-terminal hemopexin-like domain connected to catalytic domain via a proline-rich hinge region (Figures 1-1 and 5-1). The hinge region of MMP-9 is extended and is similar to Type V collagen. During protein activation, the pro-domain is cleaved [97]. MMP-9 has an additional domain known as the gelatin binding domain composed of 3 homology fibronectin-type II modules of 58 amino acids inserted into the catalytic domain (Figure 5-1b) [98]. We have shown that this domain is responsible for the type V collagen activity of the enzyme [40, 99]. The carboxyl-terminal domain of MMP-9 is involved in anchoring the enzyme to CD44 [100-102] and binds to heparin [103]. Although, the role and significance of individual domains in substrate recognition by MMP-9 has been defined, the enzyme reaction mechanism of MMP-9 has yet to be determined.

Latent MMP-9 is a 92 kDa protein that upon activation is converted to an 82 kDa protein that auto-catalytically process to a 68 kDa (fully processed) species [104]. Many cell lines studied contain both 82 kDa and 68 kDa proteins [102, 104]. In an attempt to delineate the complete role of MMP-9 in breast cancer, we isolated and purified the 92 kDa latent MMP-9 from a highly invasive human breast cancer cell line, MDA-MB-231 and demonstrated that it can be fully processed to a 68 kDa protein upon activation. The amino terminal amino acid analysis of the 68 kDa protein revealed that it has the same NH₃-termini, Met⁷⁵, as the 82 kDa protein, indicating that 68 kDa protein is a carboxyl terminal truncated form of the enzyme. Further characterization of the 68 kDa protein revealed that the enzyme in addition to gelatin and types V and XI collagens, similar to MMP-1, was also able to cleave native triple helical type I collagen. As mentioned

Figure 5-1. Schematic diagram of MMP-1 and -9.

The enzymes share pro-domain (grey), a catalytic domain (yellow and red), a hinge region domain (black jagged line), and a c-terminal hemopexin-like domain (dark blue). The catalytic domain of all MMPs contain a Zn binding active site (AS) region (red). The hinge region domain is located between the catalytic and c-terminal hemopexin-like domain (a and b). MMP-9 contains an extended hinge region, which is often referred to as the type V collagen-like domain (blue oval) due to its Proline rich composition. The catalytic domain of MMP-9 contains 3 homology fibronectin-like repeats, shown in light green (b).

previously, the 82 kDa protein does not cleave type I collagen as a substrate. These data suggest that MMP-9 plays a dual role in tumor cell invasion; by cleaving type IV collagen, it exposes an HUIV26 cryptic site that is important for angiogenesis and tumor growth [31] and by cleaving type I collagen, the most abundant collagen of the ECM it aids MMP-1 in increasing the proliferation and invasive potential of tumor cells. This dual role that MMP-9 plays in tumorigenesis makes it an even more attractive anti-cancer target. To derive a therapeutic efficient inhibitor specific for MMP-9, it is important to investigate MMP-9 mechanism of substrate specificity.

In this Chapter, the mechanism of type I collagenolysis of 68 kDa MMP-9 was investigated by deletion and alanine scanning site directed mutagenesis techniques. These studies revealed that presence or the lack of interaction between the side chains of Glu⁴¹⁵ in the active site and Glu⁴⁴⁷ and/or Arg⁴⁵¹ located in the hinge region determines specificity of MMP-9 toward type I collagen substrate. The carboxyl-terminal truncated 68 kDa species lacks both Glu⁴⁴⁷ and Arg⁴⁵¹ thus, its active site is flexible and capable of accommodating and consequently cleaving type I collagen. On the other hand, the interaction between Glu⁴¹⁵ and Glu⁴⁴⁷ and/or Arg⁴⁵¹ makes the active site of 82 kDa MMP-9 rigid, preventing the enzyme from cleaving type I collagen as a substrate.

MATERIALS AND METHODS

Mutagenesis

MMP-9 Δ 444-502. Type-V like hinge domain (amino acid residues 444-502) was deleted from the full-length MMP-9 cDNA (pETNG) by the polymerase chain reaction (PCR) procedure. The pETNG expression system containing full length MMP-9 cDNA [75] was used as a template for two separate PCR reactions. The 5' primer, 5'-AAG GAG ATA TAC ATA TGC GGG CGA AAC TCC TAG GAA TAG-3' and 3' primer, 5'-GG CAC AGT AGT GGC ACC ATA GAG GTG CCG GAT GCC-3' were used to amplify a 1.55kb region of the pETNG corresponding to nucleotides 43 to 1593 and the 5' primer, 5'-GGC ATC CGG CAC CTC TAT GGT / GCC ACT ACT GTG CC-3' and 3' primer, 5'-TCG AGG TCG ACG GTA TCG ATA AGC TTG ATA TCG AAT-3' were used to generate a ~0.85 kb PCR product corresponding to nucleotides 1381 to 2400 (1401-1575 looped out) of the MMP-9 sequence. The PCR products were sequenced to verify that the desired deletion mutation is the only mutation generated by PCR. The 1.55 kb and 0.85 kb PCR products were used to generate 2.3 kb MMP-9 cDNA lacking Type-V like hinge domain (nucleotides 1401-1575) by PCR using 5'-AAG GAG ATA TAC ATA TGC GGG CGA AAC TCC TAG GAA TAG-3' and, 5'-TCG AGG TCG ACG GTA TCG ATA AGC TTG ATA TCG AAT-3' as 5' and 3' primers, respectively. The 2.3 kb PCR product was digested with restriction enzymes XbaI and SalI and the 2.2 kb XbaI/SalI cDNA fragment was ligated to NheI/SalI fragment of the expression vector pET12C for 1 h at room temperature using T4 DNA Ligase (Promega, Madison, WI) to generate pET Δ 444-502.

Alanine Scanning Site-Directed Mutations in $\Delta\text{CT}^{(1-456)}$. The following 3' mutagenic primers were used to substitute Arg⁴⁴⁵ (nucleotides 1405-07), Glu⁴⁴⁷ (nucleotides 1411-13), Glu⁴⁴⁹ (nucleotides 1417-19) or Arg⁴⁵¹ (nucleotides 1423-25) with Ala (substituted nucleotide(s) underlined) and generate a stop codon at position 446 (nucleotide 1439) in MMP-9 molecule using pETNG as template: R445A, 5'-GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG CCG TGG CTC AGG TTC AGG GGC AGG ACC ATA-3'; E447A, 5'-CAG GTC GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG CCG TGG CTC AGG TGC AGG GCG AGG-3'; E449A, 5'-CAG GTC GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG CCG TGG CGC AGG TTC AGG-3'; R451A, 5'-CAG GTC GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG CGC TGG CTC AGG-3'. All of the above primers were complimentary to nucleotides 1401-1439 in NG and contained a unique XbaI restriction site (TCT AGA) and a STOP codon (TAG) at the 3'end. The 5' primer used in all of the above PCR reactions was 5'-AAG GAG ATA TAC ATA TGC GGG CGA AAC TCC TAG GAA TAG-3'. All the PCR products were sequenced by Dideoxy method [99] and were found to be free of any secondary mutations. The resulting PCR products were digested with XbaI and SalI and ligated to NheI /SalI fragment of pET-12C expression vector.

E415G in $\Delta\text{CT}^{(1-456)}$. This mutant was generated using two PCR reactions and pETNG as a template. The first PCR used 5' primer, 5'-CAT TCC TCA GTG CCG GGG GCG CTC ATG TAC CC-3' and 3' primer, 5'-CAG GTC GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG-3' to generate a 160 bp PCR fragment containing nucleotides 1300 to 1440. The above 5' primer contained a unique XbaI restriction site (TCT AGA) and a STOP codon (TAG) at the 3'end. This 160bp fragment was used as a 3'primer for the second round of PCR using pETNG as a template and 5'-AAG GAG ATA TAC ATA TGC GGG CGA AAC TCC TAG GAA TAG-3' as 5' primer. This generated a 1.4 kb PCR product (nucleotides 43-1440), which was digested with restriction enzymes NdeI and XbaI and ligated to NdeI-NheI fragment of pET-12c expression vector as described above.

Double Mutant. The $\Delta\text{CT}^{(1-456)}$ E415G/E447A mutant was generated using the previously published Gel. B/E415G mutant in pET-12c as a template [105]. The 5' primer, 5'-AAG GAG ATA TAC ATA TGC GGG CGA AAC TCC TAG GAA TAG-3' and 3' primer, 5'-CAG GTC GAC TCT AGA CTA TTA GGT GGT GGT TGG AGG CCG TGG CTC AGG TGC AGG GCG AGG-3', were used to generate Glu to Ala mutation at position 447 (nucleotides 1341-1343) and a stop codon at position 456 (nucleotide 1439) in MMP-9 E415G mutant protein resulting in $\Delta\text{CT}^{(1-456)}$ truncated mutant with Glu⁴¹⁵ to Gly and Glu⁴⁴⁷ to Ala substitutions. The PCR product was digested with XbaI and SalI and ligated with NheI and SalI fragment of pET-12c expression vector (Novagen, Rockland, MA).

Enzyme Purification. Plasmids encoding for wild-type and mutant enzymes were introduced into *E. coli* strain BL21(DE3)(pLysS), the cells bearing the plasmids were induced by IPTG and the latent recombinant proteins were purified on gelatin-agarose affinity columns as described previously [75]. Purified enzymes were dialyzed against

50mM Tris-HCl, pH 7.5, 5mM CaCl₂, 150mM NaCl, and 2mM ZnCl₂ and stored at -20 °C until use.

Gelatin Zymography

The activity of the purified enzymes toward gelatin was assessed by running proteins on a non-reducing SDS/Polyacrylamide gel (8%) containing 1 mg/ml gelatin. Protein samples were mixed with the SDS/PAGE sample buffer without reducing agents and were loaded without being boiled. After electrophoresis, the gels were washed twice with 50 mM Tris-HCl, 5 mM CaCl₂, 1 mM ZnCl₂, 1% Triton X-100, and 0.02% NaN₃ at 25 °C and incubated in the same buffer for 16 h at 37 °C. The gels were stained with Coomassie Brilliant Blue. Gelatin zymography depicts MMPs as negatively stained bands of gelatinolytic activity.

Determination of K_{cat}/K_m Values for the Fluorogenic Peptide Substrate Mca-PLGL(Dpa)AR-NH₂.

The latent enzymes were activated by 1 mM APMA at 37°C for 14-16 h. The concentrations of the active enzymes, [E]_T, were determined by active-site titration with the MMP inhibitor GM6001 as described previously [106]. The K_{cat}/K_m values for the fluorogenic peptide substrate were then obtained by assaying the enzymes at 23°C using 1.5 μM peptide substrate as previously described [99]. Assays were conducted under the condition where $K_m \gg [S]$, and K_{cat}/K_m values were calculated from the following equation: $K_{cat}/K_m = v_o / [E]_T[S]$.

Kinetic Analysis of Type I Collagenolytic Activity

To determine the K_{cat}/K_m values of the MMP-9 mutant enzymes for type I collagen, enzymes (0.02 μM) were mixed with various concentrations of bovine type I collagen (1.1, 1.65, 2.2, and 2.75 μM) in assay buffer that was composed of 50 mM Tris, pH 7.5, 5 mM CaCl₂, 150 mM NaCl, and 2 μM ZnCl₂. Reactions were allowed to proceed at 27 °C for 6 h before being quenched by the presence of 10 mM EDTA. Reaction mixtures were boiled for 5 min and electrophoresed on 8% SDS-PAGE. Proteins bands were visualized by Coomassie Brilliant Blue staining. The integrated intensities of the α1(I) and α2(I) collagen bands were determined by using an Alpha Imager 2000 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA). The difference between the integrated intensities of the α1(I) collagen band in the presence and in the absence of the enzyme was used to calculate the catalytic activity of the enzyme as described previously [107].

Analysis of Gelatinolytic Activity of the Enzymes

The gelatinolytic activity of enzymes was determined using ^{14}C -labeled gelatin as described previously [40, 108, 109]. Specific activity was calculated by dividing the amount of gelatin degraded per hour by the nanomoles of enzyme present in the assay. The assays were performed in triplicates and the results were averaged.

RESULTS

Expression, Purification, and Activation of Deletion Mutant Enzymes

The major aim of this study was to identify the structural elements that are sufficient to endow MMP-9 with type I collagenolytic activity. Since the fully processed carboxyl-terminal truncated MMP-9 (68 kDa protein) was shown to be able to cleave type I collagen, a series of MMP-9 deletion mutant enzymes (Figure 5-2) were designed to address the role and significance of the hemopexin domain and hinge region in type I collagen substrate specificity of MMP-9. The first deletion mutant, $\Delta\text{CT}^{(1-456)}$ consisted of complete deletion of the hemopexin domain (amino acid 503-706) and partial deletion of the hinge region (amino acid 457-502). $\Delta\text{CT}^{(1-443)}$ consisted of complete deletion of both the hemopexin domain and the hinge region (amino acids 444-706). The $\Delta 444-502$ mutant is comprised of MMP-9 molecule lacking only the hinge region, amino acid residues 444-502. All mutant enzymes were expressed in *E. coli* as latent enzymes and purified on gelatin agarose affinity column as described previously [75].

Characterization of Deletion Mutants MMP-9

To assess whether deletion mutants were correctly folded and retained general proteolytic activity, they were activated with APMA and the catalytic efficiencies of MMP-9, $\Delta\text{CT}^{(1-456)}$, $\Delta\text{CT}^{(1-443)}$ and MMP-9 $\Delta 444-502$ enzymes toward gelatin and a general MMP fluorogenic peptide substrate, Mca-PLGL(Dpa)AR-NH₂, were determined. As demonstrated in Figure 5-3 and Table 5-1, all activated mutant enzymes were able to cleave gelatin. However, only the $\Delta 444-502$ mutant enzyme with intact hemopexin domain had similar gelatinolytic activity as the wild type MMP-9. All other deletion mutations resulted in a decreased catalytic efficiency of the enzyme toward gelatin substrate. $\Delta\text{CT}^{(1-456)}$ and $\Delta\text{CT}^{(1-443)}$ mutants had 3.2-fold and 3.6-fold lower activity toward gelatin compared to WT MMP-9, respectively (Table 5-1). This is consistent with Roeb, et al., finding that MMP-9 hemopexin domain has a high affinity for gelatin [103]. Therefore it is not surprising that $\Delta\text{CT}^{(1-443)}$ and $\Delta\text{CT}^{(1-456)}$, both lacking the hemopexin domain, had lower activity toward gelatin substrate while, specific activity of MMP-9 hinge region deleted mutant ($\Delta 444-502$) for gelatin was similar to that of the wild type enzyme.

Figure 5-2. MMP-9 recombinant deletion mutants.

Full-length MMP-9 is comprised of 706 amino acids and does not use type I collagen as a substrate. Δ 444-502 mutant is comprised of MMP-9 molecule lacking hinge region, amino acid residues 444-502. Δ CT⁽¹⁻⁴⁵⁶⁾ recombinant mutant lacks amino acid residues 457-706 corresponding to the hemopexin domain and partial hinge region. In Δ CT⁽¹⁻⁴⁴³⁾ recombinant mutant both hemopexin domain and hinge region were deleted (amino acid residues 444-706).

Figure 5-3. Gelatin- gel zymography of purified mutant enzymes.

(a) Lanes 1, 3, and 5 show the latent form of WT MMP-9, $\Delta\text{CT}^{(1-456)}$ and $\Delta\text{CT}^{(1-443)}$, respectively. Lanes 2, 4, and 6 shows the active form of WT MMP-9, $\Delta\text{CT}^{(1-456)}$ and $\Delta\text{CT}^{(1-443)}$, respectively. (b) Lanes 1 and 2 show the latent and active forms of $\Delta 444-502$, respectively.

Table 5-1. Proteolytic activity of deletion mutant enzymes

Enzyme μM^{-1}	Kcat/Km (McaPLGLDpaAR) $\text{mM}^{-1}\text{S}^{-1}$	Specific Activity for Gelatin $\mu\text{g/h/nmol}$	Kcat Type I collagen h^{-1}	Km Type I collagen μM^{-1}	Kcat/Km Type I collagen $\text{h}^{-1} \mu\text{M}^{-1}$
MMP-1	14.17 ± 0.5	272 ± 9	146	5.36	27.23
MMP-9	585 ± 30.9	$187,669 \pm 11,921$	ND	ND	ND
$\Delta 444-502$	145.33 ± 2.58	$187,157 \pm 224$	33.03	8.9	3.71
$\Delta\text{CT}^{(1-456)}$	128.9 ± 14.1	$58,959 \pm 359$	ND	ND	ND
$\Delta\text{CT}^{(1-443)}$	613 ± 2.1	$52,272 \pm 5149$	3.7	2.1	1.8

ND=Not Determined

As shown in Table 5-1, the catalytic efficiency (K_{cat}/K_m) of both $\Delta CT^{(1-456)}$ and $\Delta 444-502$ toward the Mca-PLGL(Dpa)AR-NH₂ were 4.5 and 4 times lower than that of the wild type enzyme, respectively. The $\Delta CT^{(1-443)}$ mutant, however, had similar K_{cat}/K_m for the peptide substrate to wild type MMP-9 ($585 \pm 30.9 \text{ mM}^{-1} \text{ S}^{-1}$). These findings suggest that presence of amino acid residues 444-456 in the hinge region plays a role in general proteolytic activity of the enzyme.

As mentioned previously, contrary to 82 kDa MMP-9, the 68 kDa active MMP-9 cleaves type I collagen, suggesting a role for carboxyl terminal domain and hinge region in type I collagenolytic activity of the enzyme. Therefore, the ability of deletion mutants to cleave type I collagen was assessed. As demonstrated in Table 5-1, only deletion mutants lacking the entire hinge region with ($\Delta 444-502$) or without ($\Delta CT^{(1-443)}$) the carboxyl terminal domain were able to cleave type I collagen. $\Delta CT^{(1-443)}$, in addition to gelatin and type V collagen (data not shown), was able to use triple helical type I collagen as a substrate (Table 5-1) with K_{cat}/K_m value of $1.8 \text{ h}^{-1} \mu\text{M}^{-1}$. This value is 15.1 times lower than the value obtained for MMP-1 ($27.23 \text{ h}^{-1} \mu\text{M}^{-1}$). Interestingly, contrary to this enzyme, $\Delta CT^{(1-456)}$ does not cleave type I collagen, suggesting that presence of amino acids 444-456 in the $\Delta CT^{(1-456)}$ molecule hinders the enzyme from utilizing type I collagen as a substrate. This hypothesis was confirmed by the observation that complete deletion of the hinge region from the full length MMP-9 ($\Delta 444-502$) endowed the enzyme with type I collagenolytic activity. The catalytic efficiency of the $\Delta 444-502$ mutant toward type I collagen substrate was $3.71 \text{ h}^{-1} \mu\text{M}^{-1}$ (Table 5-1). This value is 2 times higher than the value obtained for $\Delta CT^{(1-443)}$. This may be due to the hemopexin domain binding to and helping to present type I collagen to the AS in $\Delta 444-502$ molecule.

Glu⁴⁴⁷ and Arg⁴⁵¹ Residues in the Hinge Region Play a Role in Type I Collagenolytic Activity of MMP-9

Analysis of activity of $\Delta CT^{(1-456)}$ and $\Delta CT^{(1-443)}$ enzymes toward type I collagen substrate suggested that the 13 amino acid residues, ⁴⁴⁴PRPEPEPRPPTT⁴⁵⁶ in the hinge region of MMP-9 determines the substrate specificity of the enzyme (Figure 5-4, Table 5-2). The role of specific amino acid residues in this region of the MMP-9 molecule in its type I collagenolytic activity was evaluated by alanine scanning site directed mutagenesis. The charged amino acid residues, Arg⁴⁴⁵, Glu⁴⁴⁷, Glu⁴⁴⁹ and Arg⁴⁵¹ in $\Delta CT^{(1-456)}$ (Figure 5-4) were individually substituted with Ala and the effect of each mutation on substrate specificity of the enzyme was evaluated. All site directed mutant enzymes were expressed in *E. coli*, purified, and their enzymatic activities were determined following activation by APMA.

Gelatin gel zymography showed that upon activation with APMA, the 45 kDa unglycosylated latent R445A, E447A, E449A, and R451A $\Delta CT^{(1-456)}$ mutant enzymes were converted to 37 kDa active species (Figure 5-5) as expected.

Figure 5-4. Alanine mutations in $\Delta\text{CT}^{(1-456)}$.

Schematic diagram of $\Delta\text{CT}^{(1-456)}$, showing the amino acid residues in the hinge region involved in type I collagenolytic activity of the enzyme. The amino acids in red signify the amino acids individually mutated to Ala.

Table 5-2. Proteolytic activity of the Ala mutants

Enzyme μM^{-1}	Kcat/Km (McaPLGLDpaAR) $\text{mM}^{-1}\text{S}^{-1}$	Specific Activity for Gelatin $\mu\text{g/h/nmol}$	Kcat Type I collagen h^{-1}	Km Type I collagen μM^{-1}	Kcat/Km Type I collagen $\text{h}^{-1} \mu\text{M}^{-1}$
MMP-1	14.17 ± 0.5	272 ± 9	146	5.36	27.23
$\Delta\text{CT}^{(1-456)}$	128.9 ± 14.1	$58,959 \pm 359$	ND	ND	ND
R445A	328.1 ± 11.9	$71,154 \pm 174$	ND	ND	ND
E447A	85.95 ± 1.5	$10,239 \pm 130$	0.418	0.157	2.66
E449A	275.37 ± 16.8	$149,680 \pm 19,758$	ND	ND	ND
R451A	135.1 ± 10.26	$248,706 \pm 13,896$	0.641	0.236	2.71

ND=Not Determined

Figure 5-5. Gelatin- gel zymography of purified site directed mutant enzymes.

Lanes 1, 3, 5 and 7 correspond to the latent forms and lanes 2, 4, 6 and 8 correspond to the active forms of $\Delta\text{CT}^{(1-456)}$ mutant enzymes R445A, E447A, E449A, and R451A , respectively.

The catalytic efficiencies of all of the mutant enzymes toward the peptide substrate Mca-PLGL(Dpa)AR-NH₂ and gelatin were then determined at 23 °C and 37 °C, respectively. As shown in Table 5-2, all of the Ala substitutions changed catalytic efficiency and specific activity of the $\Delta\text{CT}^{(1-456)}$ enzyme for peptide substrate and gelatin, respectively. While, R451A substitution did not have a significant effect on Kcat/Km value of the $\Delta\text{CT}^{(1-456)}$ for the synthetic substrate , the catalytic efficiency of R445A and E449A for the substrate were found to be 2.6 and 2.3 times higher than that of $\Delta\text{CT}^{(1-456)}$, respectively. The observed differences between catalytic efficiency of $\Delta\text{CT}^{(1-456)}$ and the Ala mutant enzymes for synthetic substrate could be due to higher catalytic activity (Kcat) and/or higher affinity of the mutant enzymes for the synthetic substrate. The E447A mutant had 1.5 times lower Kcat/Km value for the synthetic substrate than $\Delta\text{CT}^{(1-456)}$.

All of the alanine mutations also changed specific activity of $\Delta\text{CT}^{(1-456)}$ enzyme for gelatin substrate. While the specific activities of R445A, E449A, and R451A mutants for gelatin substrate were 1.2, 2.5, and 4.2-fold higher than that of $\Delta\text{CT}^{(1-456)}$, respectively, introduction of Ala mutation at position 447 in $\Delta\text{CT}^{(1-456)}$ reduced gelatinolytic activity of the enzyme by 5.7-fold (Table 5-2). These data suggest that amino acid residues at positions 447, 449, and 451 are involved in general proteolytic activity of MMP-9.

The effect of Ala mutations in type I collagenolytic activity of $\Delta\text{CT}^{(1-456)}$ was assessed using soluble type I collagen as a substrate. As shown in Table 5-2, substitution of Glu⁴⁴⁷ and Arg⁴⁵¹ with Ala endowed $\Delta\text{CT}^{(1-456)}$ with type I collagenolytic activity. E447A and R451A (Table 5-2) were able to cleave type I collagen less efficiently than $\Delta\text{CT}^{(1-456)}$ mutant enzyme (3.71 h⁻¹μM⁻¹; Table 5-1). The Kcat/Km of E447A and R451A

mutants were 2.66 and 2.71 h⁻¹ μM⁻¹, respectively (Table 5-2). These data indicate that the presence of Glu⁴⁴⁷ and Arg⁴⁵¹ residues in MMP-9 hinders the enzymes from cleaving type I collagen. We have previously shown that presence of Glu⁴¹⁵ in the active site of MMP-9 also prevents the enzyme from using type I collagen as a substrate [105]. This was based on the observation that substituting Glu⁴¹⁵ in the active site of MMP-9 with Gly, an amino acid counter part in MMP-1, provided the enzyme with type I collagenolytic activity [105]. These data together suggests that interaction between the side chain of Glu⁴⁴⁷ and/or Arg⁴⁵¹ with Glu⁴¹⁵ make the active site of MMP-9 rigid and capable of accommodating specific substrates, i.e. gelatin and type V collagen. Removal of the side chain of either of these amino acid residues or a complete deletion of the hinge region as in Δ444-502 and ΔCT⁽¹⁻⁴⁴³⁾ mutant enzymes makes the structure of the active site more flexible and capable of cleaving both types I and V collagen.

Interaction Between Glu⁴¹⁵ and Glu⁴⁴⁷ and/or Arg⁴⁵¹ Dictates Substrate Specificity of MMP-9

To test the above hypothesis we introduced Glu⁴¹⁵ to Gly mutation into ΔCT⁽¹⁻⁴⁵⁶⁾ and E447A/ΔCT⁽¹⁻⁴⁵⁶⁾ enzymes (Figure 5-6) and studied the effect of mutations on type I collagenolytic activity of the enzymes. If interaction between the side chains of Glu⁴¹⁵ and Glu⁴⁴⁷ dictates substrate specificity of MMP-9, then removal of the side chain of either amino acids from ΔCT⁽¹⁻⁴⁵⁶⁾ should result in an enzyme capable of cleaving type I collagen in addition to gelatin and synthetic substrate. Moreover, introduction of E415G mutation in E447A/ΔCT⁽¹⁻⁴⁵⁶⁾ should, in principal, increase the activity of the enzyme toward type I collagen.

As shown in Figure 5-6 and Table 5-3, the latent E415G/ΔCT⁽¹⁻⁴⁵⁶⁾ (E415G) and E415G/E447A/ΔCT⁽¹⁻⁴⁵⁶⁾ (DM) are 47 kDa proteins that after activation by APMA are

Figure 5-6. Schematic diagram and gelatinolytic activities of E415G and DM.

The figure to the left is schematic diagrams of ΔCT⁽¹⁻⁴⁵⁶⁾ showing the position of Glu⁴¹⁵ and Glu⁴⁴⁷ in the protein molecule. To the right is a gelatin gel zymography depicting molecular weights of latent (lanes 1 and 3) and active (lanes 2 and 4) forms of E415G/ΔCT⁽¹⁻⁴⁵⁶⁾ and E415G/E447A/ΔCT⁽¹⁻⁴⁵⁶⁾ (DM) mutant enzymes, respectively.

Table 5-3. E415G and DM proteolytic activity.

Enzyme μM^{-1}	Kcat/Km (McaPLGLDpaAR) $\text{mM}^{-1}\text{S}^{-1}$	Specific Activity for Gelatin $\mu\text{g/h/nmol}$	Kcat Type I collagen h^{-1}	Km Type I collagen μM^{-1}	Kcat/Km Type I collagen $\text{h}^{-1} \mu\text{M}^{-1}$
MMP-1	14.17 ± 0.5	272 ± 9	146	5.36	27.23
$\Delta\text{CT}^{(1-456)}$	128.9 ± 14.1	$58,959 \pm 359$	ND	ND	ND
E415G	130.63 ± 0.53	$56,779 \pm 2,956$	8.041	2.3	3.5
DM	29.52 ± 0.6	$39,864 \pm 984$	11.89	1.94	6.1

ND=Not Determined

converted to 37 kDa proteins capable of cleaving gelatin and using Mca-PLGL(Dpa)AR-NH₂ as a substrate. The catalytic efficiency and specific activity of E415G for the synthetic substrate and gelatin were equal to those of $\Delta\text{CT}^{(1-456)}$, respectively (Table 5-3). Contrary to E415G, the catalytic efficiency of DM mutant enzyme for the synthetic substrate and its specific activity for gelatin were 4.4 and 1.4-fold lower than those of $\Delta\text{CT}^{(1-456)}$ (Table 5-3). This data confirms the previous observation that amino acid residue at position 447 is involved in general proteolytic activity of MMP-9.

The catalytic efficiency (Kcat/Km) of E415G and DM enzymes for type I collagen was determined at 27°C (Table 5-3). We found that similar to previously described Gel.B/E415G mutant [105], mutation of Glu⁴¹⁵ to Gly in $\Delta\text{CT}^{(1-456)}$ confers the enzyme with type I collagenolytic activity at an efficiency of $3.5 \text{ h}^{-1} \mu\text{M}^{-1}$ (Table 5-3). The double mutant had a catalytic efficiency of $6.1 \text{ h}^{-1} \mu\text{M}^{-1}$, which is 1.7- and 2.3-fold higher compared to individual E415G and E447A mutants, respectively (Table 5-2) and is equal to the efficiency of single mutants combined. This additive effect of double mutations on the rate of cleavage of type I collagen by $\Delta\text{CT}^{(1-456)}$ proves our hypothesis that the interaction between Glu⁴¹⁵ and Glu⁴⁴⁷ is the major determinant in collagen substrate specificity. The interaction(s) between the side chains of Glu⁴⁴⁷ and Glu⁴¹⁵ makes the active site rigid, preventing the active site from accommodating type I collagen molecule. Breaking this interaction by either replacing, Glu⁴⁴⁷ or Arg⁴⁵¹ to Ala makes the active site flexible and capable of cleaving type I collagen.

DISCUSSION

In this report we demonstrate, for the first time, that the ability of 68 kDa MMP-9 to cleave type I collagen comes from its active site ability to adopt a conformation that mediates collagen binding and catalysis and this process is dependent on the lack of interaction between a Glu residue at position 415 located in the AS region of the enzyme and Glu⁴⁷⁷ residue located in the hinge region. The removal of amino acid side chain at

positions 415 and/or 447 provides flexibility necessary for the enzyme active site to change conformation upon binding collagen, leading to catalysis. This is based on the observation that only MMP-9 mutants lacking Glu⁴¹⁵ (as in E415G), Glu⁴⁴⁷ (as in E447A, Δ CT⁽¹⁻⁴⁴³⁾, and Δ 444-502 mutants), or their combination (as in E447A/E415G/ Δ CT⁽¹⁻⁴⁵⁶⁾) were able to use type I collagen as a substrate. The carboxyl terminal deletion mutant (Δ CT⁽¹⁻⁴⁵⁶⁾) and full length MMP-9 although had high activity toward the peptide substrate and gelatin, were not able to cleave type I collagen. Δ CT⁽¹⁻⁴⁵⁶⁾ was by all indications stable, properly folded, and able to be processed by APMA to a 37 kDa active species. An amino acid sequence alignment revealed that the amino acid residues 444-456 (⁴⁴⁴PRPEPEPRPPTTT⁴⁵⁶) is responsible for the observed difference in the type I collagenolytic activity of Δ CT⁽¹⁻⁴⁵⁶⁾ and Δ CT⁽¹⁻⁴⁴³⁾ mutant enzymes (Figure 5-2 and 5-4).

We have previously demonstrated that MMP-9 mutant (Gel.B/E415G), in which Glu⁴¹⁵ located in its active site is substituted by Gly, its corresponding residue in all type I collagen cleaving MMPs, is able to cleave native triple helical type I collagen [105]. These data together suggested that the interaction between Glu⁴¹⁵ and an amino acid residue(s) in the sequence ⁴⁴⁴PRPEPEPRPPTTT⁴⁵⁶ make the active site of MMP-9 rigid, preventing the enzyme from binding to and cleaving type I collagen.

This was confirmed by the observation that the replacement of Glu⁴⁴⁷ and Arg⁴⁵¹ in Δ CT⁽¹⁻⁴⁵⁶⁾ with Ala (E447A and R451A) endowed the enzyme with type I collagenolytic activity (Table 5-2). These mutants retained peptide substrate and gelatinolytic activity, indicating that the general proteolytic activity of Δ CT⁽¹⁻⁴⁵⁶⁾ was not compromised by these substitutions. The effect of E447A and R451A mutation on kinetic parameters of Δ CT⁽¹⁻⁴⁵⁶⁾ for type I collagen was similar to the replacement of Glu⁴¹⁵ with Gly in Δ CT⁽¹⁻⁴⁵⁶⁾ (Table 5-3) and removal of the entire hinge region of MMP-9 (Δ 444-502; Table 5-1). As shown in the diagram below (Figure 5-7), presence of the 13 amino acids in full-length MMP-9 and Δ CT⁽¹⁻⁴⁵⁶⁾ makes the AS of these enzymes rigid and blocks type I collagen from entering the AS. Further processing of 82 kDa by COOH-terminal truncation, to 68 kDa enzyme and deletion of the remaining hinge region in Δ CT⁽¹⁻⁴⁴³⁾ removed the Glu⁴¹⁵ and Glu⁴⁴⁷ interaction and made the AS less rigid. This allowed type I collagen to enter the AS of Δ CT⁽¹⁻⁴⁴³⁾ and be cleaved with a Kcat/Km of 1.8 h⁻¹ μ M⁻¹ (Figure 5-7; bottom left). Substitution of Glu⁴¹⁵ with Gly (Figure 5-7; top right), Glu⁴⁴⁷ (Figure 5-7; bottom right) or Arg⁴⁵¹ with Ala also affected the interaction of the hinge region with AS Glu⁴¹⁵ by breaking the side chain ionic interactions. This made the active site less rigid and capable of cleaving type I collagen with a Kcat/Km of 3.5, 2.66 and 2.71 h⁻¹ μ M⁻¹, respectively. These data indicated that the interaction of Glu⁴¹⁵ and Glu⁴⁷⁷ in MMP-9 dictates substrate specificity of the enzyme.

This was substantiated by substituting the Glu⁴¹⁵ residue with Gly and Glu⁴⁷⁷ with Ala in Δ CT⁽¹⁻⁴⁵⁶⁾. The catalytic activity (Kcat) of the double mutant E415G/E477A/ Δ CT⁽¹⁻⁴⁵⁶⁾ toward type I collagen was 1.5 and 28.4 folds higher than that of E415G and E477A mutants, respectively (Table 5-2 and 5-3). The affinity of the double mutant for type I collagen (Km), however, was similar to that for E415G mutant. Generation of the E415G/E447A/ Δ CT⁽¹⁻⁴⁵⁶⁾ (DM) lead to an additive effect on type I collagen catalytic efficiency. The Kcat/Km of 6.1 h⁻¹ μ M⁻¹ was approximately 2 times

Figure 5-7. Schematic diagram of MMP-9 mutants with type I collagenolytic activity.

This diagram shows full length MMP-9 without any mutations (upper left) and the rigidity of the AS caused by the interaction between Glu⁴¹⁵ and Glu⁴⁴⁷, which inhibits type I collagen (green ball) from entering the AS. When Glu⁴¹⁵ is mutated to Gly (upper right), the hinge region is completely deleted (lower left), or Glu⁴⁴⁷ is mutated to Ala (lower right) the interaction between Glu⁴¹⁵ and Glu⁴⁴⁷ is broken and the enzyme gains type I activity.

higher compared to E415G, E447A, and R451A single mutations in $\Delta\text{CT}^{(1-456)}$.

According to the X-ray crystal structures of MMP-9 catalytic domain [105] and full length MMP-1 [110], Glu⁴¹⁵ is located in a turn at the bottom of the AS where the polypeptide chain turns back towards the center of the enzyme and Glu⁴⁴⁷ and Arg⁴⁵¹ are located at the beginning of the hinge region (Figure 5-7). Considering that Glu⁴¹⁵ is not positioned along the enzyme/substrate interface, it is unlikely that it or a substituting residue would directly interact with the collagen substrate. The significance of this residue in type I collagenolysis is probably related to its direct/indirect interaction with Glu⁴⁴⁷/Arg⁴⁵¹ affecting the conformation and flexibility of the AS of the enzyme. Thus, it is conceivable that any substitution at these positions have a general effect on the mobility of the AS. The observation that the $\Delta\text{CT}^{(1-443)}$, E415G, E477A R451A and $\Delta 444-502$ mutant enzymes are capable of cleaving type I collagen supports this notion and suggests that the flexibility provided by the lack of Glu⁴¹⁵-Glu⁴⁴⁷ interaction is essential for type I collagenolytic activity. Consistent with this hypothesis is also the fact that full length MMP-9 and $\Delta\text{CT}^{(1-456)}$ mutant enzymes cannot cleave native type I collagen substrate (Table 5-1). Thus, it is reasonable to conclude that the interaction of Glu⁴¹⁵ with the hinge region makes the active site rigid, preventing MMP-9 from cleaving type I collagen. Disruption of this interaction, on the other hand, provides the flexibility necessary for enzyme active site to change conformation and be able to bind and cleave collagen substrate.

These data indicate that therapeutic studies which target only MMP-1 may not be very effective due to the cleavage of type I collagen by the fully processed MMP-9. Thus, this novel role identified for the type V collagen-like hinge region of MMP-9 in cleavage of type I collagen aids in understanding the relationship between the structure and function of MMPs and the involvement of different exocites in the substrate specificity. This can lead to discovery of novel and targeted therapeutic agents.

CHAPTER 6. SUMMARY

Increasing evidence suggests that MMPs contribute to the formation of a microenvironment that promotes tumor growth during early stages of tumorigenesis. MMP-9 is one of the most important factors for metastatic behavior of tumor cells [23, 54, 74]. MMP-9 expression has been shown to be upregulated in breast tumors isolated from cancer patients [73]. MMP-9 degrades type IV collagen, one of the most abundant collagens in the ECM [11, 75] and is involved in cell proliferation, angiogenesis, and apoptosis [9, 10, 30]. Our observation that AM9D specifically decreased MMP-9 mRNA and protein production (Figure 1-3b and c) and reduced invasive behavior of human MDA-MB-231 cells (Figure 1-3d) suggested that AM9D has potential as an anti tumorigenic/metastatic agent.

MMTV-PyMT transgenic mice were used to test the effect of AM9D on tumor growth *in vivo*. This model was chosen because of its similarity to human breast cancer and its metastatic potential. AM9D treatment of 10 µg and 25 µg/tumor/week for a total of four weeks showed a 39.5 and 50% reduction in final tumor load. Immunohistochemical analysis of tumor tissues showed that AM9D treatment reduced MMP-9 production in tumor cells by 66% compared to control DNAzyme treatment. In addition, AM9D treatment decreased vasculature and induced apoptosis in mammary tumors as demonstrated by CD31 and Caspase-3 immunostaining, respectively. These results showed promise as to the use of AM9D to treat tumor growth *in vivo*. However, there were several limitations in this study. First, the tumors of the MMTV-PyMT model developed at different time points and only the earlier developed tumors being treated with AM9D or control DNAzyme. This led to termination of treatment after 4 injections. Second, due to the development of the large size mammary tumors during the course of the treatment, the mice were sacrificed prematurely, inhibiting our ability to fully assess the effect of AM9D on spontaneous lung metastasis. Thus, experimental metastasis was utilized to determine the effect of AM9D on metastasis.

To utilize AM9D as a therapeutic treatment in experimental metastasis, AM9D needed to first be evaluated for its distribution, clearance and safety in normal and MMTV-PyMT mice. Healthy FVB and MMTV-PyMT mice were injected with naked [³⁵S]-AM9D (10 mg/kg) in PBS via tail vein. [³⁵S]-DNAzyme was distributed to all major organs in mice, including liver, kidney, lung, spleen, heart, pancreas, intestines, stomach, and brain (Figure 3-2). In normal mice the order of percentage accumulation was: blood>liver>kidney>lung>spleen>heart. Studies revealed 1.8%, 1.1% and 0.4% of total DNAzyme injected was delivered to liver, kidney, and lung, respectively (Table 3-1). In MMTV-PyMT mice bearing small to large mammary tumors, the order of percentages of [³⁵S]-DNAzyme accumulation in different organs was mammary tumor>liver≈blood>kidney>spleen>lung>heart. In these mice, 4.1% of the total injected DNAzyme was accumulated in the mammary tumors in 2 h (Table 3-3) and the amount of DNAzyme accumulated in mammary tumors was proportional to the total weight of tumor burden (Figure 3-4). Interestingly, the accumulated DNAzyme in the mammary tumor is initially cleared at a slower rate compared to the other organs. The

percentages of total DNAzyme accumulated in various organs in MMTV-PyMT mice were similar to normal mice (Table 3-1 and 3-3). In normal and MMTV-PyMT mice the majority of the DNAzyme was cleared from these organs within the first 24 h. Thirty-one percent of the delivered dosage of [³⁵S]-DNAzyme was cleared from the system of normal mice via feces and urine over a period of 72 h (Figure 3-1b). The safety of DNAzyme was tested by a single injection of AM9D or control DNAzyme in various concentrations (10 mg/kg, 32 mg/kg, and 75 mg/kg of body weight) and 100 µl PBS into normal FVB mice. Systemic distribution and safety/toxicity studies revealed that the naked AM9D is non-toxic when systemically administered, and has favorable pharmacological properties with respect to intravenous administration and tissue distribution. Most importantly, mammary tumors can take up the naked DNAzyme. The amount of DNAzyme accumulates in the mammary gland is higher and its rate of clearance is slower than any other organs tested. Taken together, these data show the potential of DNAzymes to be distributed to organs susceptible to tumors (brain, pancreas, stomach, etc.), taken up by tumors, and display extended half-life in tumors.

AM9D and control DNAzyme both showed evidence of a possible sequence related effect on metastasis when used as therapy for early stage experimental metastases. This may be due to AM9D and control DNAzyme containing CpG regions. CpG monotherapy has been shown to induce T-cell-mediated rejection of established tumors [91]. Assessment of long term lung tumor burden and survival suggests that AM9D treated mice have an advantage of progression-free survival over mice treated with control DNAzyme or PBS between days 32-42, however, all cohorts were eventually overcome by an equal volume of lung tumor burden (Figure 4-4) and there was no advantage of overall survival in either groups. This lack of effect maybe due to time of AM9D delivery, frequency of delivery, mouse strain, pro-tumorigenic effects of MMP-9 downregulation, or a combination of these effects. These are all concerns that must be investigated to confirm the anti-metastatic effect of AM9D.

Finally, in this body of work we showed that MMP-9, through its cleavage of type I collagen, might play a more important role in tumorigenesis than once believed. We have demonstrated that 68 kDa truncated fully processed MMP-9 in addition to type IV collagen, is capable of cleaving type I collagen. Kinetic analysis of truncated MMP-9 demonstrated that amino acid sequence 444-456 in the hinge region of MMP-9 is responsible for substrate specificity of the enzyme. The absence of this region in $\Delta\text{CT}^{(1-443)}$ and $\Delta 444-502$ garnered these enzymes with type I collagenolytic activity. Further analysis of amino acid sequence 444-456 identified the two amino acids Glu⁴⁴⁷ and Arg⁴⁵¹ as being directly involved in substrate recognition by the enzyme. Substitution of Glu⁴¹⁵ with Gly and/or replacement of Glu⁴⁴⁷ and Arg⁴⁵¹ with Ala in $\Delta\text{CT}^{(1-456)}$ made the enzyme capable of using type I collagen as a substrate. The catalytic efficiency of the double mutant (E415G/E447A/ $\Delta\text{CT}^{(1-456)}$) toward type I collagen was 2-fold higher than either E415G or E447A mutant, indicating that interaction of Glu⁴¹⁵ with Glu⁴⁴⁷ dictates substrate specificity of the enzyme. Lack of this interaction makes the active site flexible, allowing the enzyme to cleave both type IV and I collagen, the two most important components of the ECM. This observation is important because it further illuminates the role of MMP-9 in tumorigenesis and metastases.

CHAPTER 7. AM9D: FROM PRECLINIC TO CLINIC

AM9D FOR BREAST CANCER PATIENTS

There are several lines of evidence that suggest MMP-9 levels in serum and tissue could be a sensitive biomarker for tumor aggressiveness and metastasis in breast cancer patients [111-115]. Moses, et al. first described that the analysis of urinary MMP-9 may be a useful determinant for disease status in a variety of human cancers, including breast cancer [113]. In this study, urine specimens were obtained from patients prior to surgical or other therapeutic intervention and all breast cancer urine specimens were obtained from patients with metastatic disease. One hundred percent of these specimens were positive for MMP-9 compared to only 24% of normal control breast tissue [113]. Pellikainen, et al. evaluated the expression of MMP-9 in estrogen receptor positive (ER+/HER2) breast cancer patients and discovered positive stromal MMP-9 expression predicted decreased progression-free survival and decreased overall survival [116]. Furthermore, Wu, et al. measured serum and tissue levels of MMP-9 in breast cancer patients, benign breast disease patients, and healthy controls and correlated the levels with patients' clinicopathologic characteristics [112]. Sixty-three and 77% of the breast cancer patients displayed high MMP-9 serum levels and tissue staining, respectively, that positively correlated with lymph node metastasis [112].

As described earlier (Chapter 1), breast cancer is divided into 3 subtypes: luminal A (ER or PR⁺/HER2⁻), luminal B (ER or PR⁺/HER2⁺), and triple negative (ER⁻/PR⁻/HER2⁻). Interestingly, MMP-9 serum levels were equally increased in HER2⁺ and HER2⁻ patients, as well as, ER⁺ and ER⁻ breast cancer patients [112]. Triple negative breast cancer accounts for approximately 15% of breast cancer cases and is marked with high relapse rates, metastasis, and early death [117]. In a comparison of breast cancer patients with triple-negative or HER2 overexpression, the triple-negative patients experienced more lymph node metastasis compared to those with overexpressed HER2 [118]. MMP-9 expression was positively correlated with lymph node metastasis in breast cancer patients [112]. Additionally, the 5 year disease-free survival in patients with triple-negative was much shorter than HER2-expressing patients [118]. The lack of standard therapy and poor prognosis are causes of the high mortality [117]. Targeting MMP-9 that has been shown to be expressed in hormone positive and negative breast cancer patients, could play an important role in tumor treatment and reduce metastasis to lymph nodes and other metastatic sites of breast cancer patients. We have shown the distribution of MMP-9 DNzyme (AM9D) to all major organs, including lung, brain and liver (Chapter 3, Figure 3-2). To this end, AM9D will be a useful therapeutic treatment for hormone positive and negative breast cancer patients at the early stages of tumor growth and metastasis.

AM9D AS AN ADJUVANT TO SURGERY

MMPs play a role in the normal physiology and pathology of wound healing. When tissue suffers injury cell proliferation increases, inflammatory cells infiltrate the wounded area and release MMPs, including MMP-9, while the tissue regenerates [119]. A first line treatment for breast tumor is surgical excision. Zhao, et al., demonstrated that surgical excision of an implanted MDA-MB-245 mammary tumor in mice led to increased serum expression of MMP-9 on days 1, 3, 19, 26, 33, and 40 after surgical site wounding compared to non-surgical tumor bearing control cohorts [115]. This increased MMP-9 expression was positively correlated with the increased tumor volume in the surgery group between weeks 4-6 compared to the control group. Furthermore, elevated host serum MMP-9 levels, as a result of surgery wound, was positively correlated to metastatic lung tumor burden at the study endpoint [115]. To my knowledge, no studies have been performed on breast cancer patients to determine the levels of MMP-9 serum expression post surgical incision. However, if MMP-9 serum expressions in patients mimic those of preclinical studies, AM9D would play an important therapeutic role as an adjuvant to breast tumor surgery to abate tumor metastasis and surgery-stimulated growth at the site of excision.

FUTURE AM9D PRECLINICAL STUDIES

MMPs are considered promising targets for breast cancer therapy because of their major involvement in malignant pathologies and metastasis to distant organs [120]. The preclinical studies outlined in this body of work further display the therapeutic potential of downregulating MMP-9 expression by an MMP-9 specific catalytic DNA molecule. While we show the efficacy of AM9D on reducing tumor growth (Chapter 2), its efficiency on inhibition of metastasis needs to be further explored. We displayed that a majority of DNAzyme is cleared from major organs, including lung, in the first 24 h (Chapter 3, Figures 3-1 and 3-3). The chronic cytotoxicity experiments for DNAzyme were performed with weekly injections of DNAzyme for 9 weeks (Chapter 4, Figure 4-5). To further explore AM9D efficacy on the treatment of metastasis, a therapeutically efficient regimen must be determined. To accomplish this goal DNAzyme injections could be increased from weekly to daily injections over a period of 3 weeks for survival analysis upon early stage metastasis and until mice are overcome by tumor burden. Performing daily injections of 75 mg/kg AM9D for a period of 3 weeks would expose animals to a total of 1,575 mg/kg DNAzyme, which would require re-evaluation of mice for cytotoxicity. This would be a 7-fold increase from the original 225 mg/kg injected in previous experiments and should escalate DNAzyme accumulation in major organs.

If multiple injections prove to be too cumbersome, DNAzyme can be packaged into nanoparticles as an alternate delivery approach. Designing nanoparticles has the added effect of targeting two metastatic sites, such as lung and liver, simultaneously [121]. Targeted delivery is a promising method for the development of efficient and safe therapeutic molecules for oncology [122]. Rapid vasculature development in tumor results in larger than normal pore size within vessel walls, which allows nanoparticles to

more easily extravasate into tumor tissue than normal tissue [123]. Tumor tissues also contain a deficiently developed lymphatic system that limits drainage of nanoparticles. Therefore, nanoparticles can improve the accumulation of DNAzyme within the tumor through their enhanced permeability and retention capabilities [123]. Similar to our observations of DNAzyme clearance (Chapter 3), Bellocq, et al. showed the majority of systemically administered DNAzyme cleared from organs after 24 h in mice. However, they found that injecting DNAzyme packaged with transferrin-modified polyplexes nanoparticle resulted in slower clearance of DNAzyme compared to DNAzyme packaged with non-modified polyplexes [121]. Transferrin receptor (Tf-R) is often upregulated in rapidly growing cells; furthermore, lung and liver both have high Tf-R expression [121]. Packaging our AM9D with transferrin-modified polyplexes would require additional functional and pharmacokinetic studies; however, this could decrease non-specific distribution throughout the body, decrease the observed rapid excretion, and increase accumulation thereby eliminating the need for daily injections of AM9D.

In lieu of the emerging organ toxicities and side effects of broad spectrum inhibitors, including tyrosine kinase inhibitors [124], we cannot ignore the possibility of AM9D side effects surfacing. While we showed no evidence of organ toxicity in mice injected with DNAzyme (Chapter 3), we negated to observe normal mammary tissue for the presence of toxicity in these studies. MMP-9 has been shown to be present in normal breast tissue [125]. To my knowledge, there are no studies outlining a clear role of MMP-9 in normal breast tissue. Therefore, the down regulation of MMP-9 in normal breast tissue by AM9D may lead to potential side effects. Any possible toxicities may be found through the investigation of MMP-9 in normal breast tissue in the absence and presence of AM9D.

AM9D opens the door to the development of effective anti-MMP therapies. AM9D could be used as a therapy for hormone positive and negative breast cancer patients as an adjuvant to surgery and in combination with other chemotherapeutic modalities. Overall, AM9D shows promise as a therapeutic modality for breast cancer treatment and warrants further investigation.

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APPENDIX. DEVELOPMENT OF AM9D MATERIALS AND METHODS

DNAZYME

Several DNAzyme molecules were synthesized by Integrated DNA Technology (Coralville, IA). All the DNAzymes consisted of nine oligonucleotides on each arms complementary to different regions of MMP-9 mRNA flanking the nucleotide 10-23 catalytic core [49, 126]. Sequences of DNAzymes targeting mRNA of MMP-9 were, AM9D-1: 5'-AGCCAGCAggctagctacaacgaCAGGAGCAC-3'; AM9D-2: 5'-AAGGGTGGAggctagctacaacgaTGGCGCTGT-3'; AM9D-3 5'-ATAGCGGTAggctagctacaacgaAGGTATTCC-3'; AM9D-4: 5'-CCCCAGAGAggctagctacaacgaTTCGACTCT-3'; AM9D-5: 5'-GTGGTGCCAggctagctacaacgaTTGAGGTCG-3'. Scrambled DNAzyme (CTAGTCAGCggctagctacaacgaTAAGCTGCT), where the arms flanking the 10-23 catalytic core were composed of random sequence and sense control DNAzymes containing sense oligonucleotide sequences corresponding to each DNAzyme in both arms were used as negative controls. Nucleotides in lower case represent the 10-23 catalytic domain and the corresponding MMP-9 sequences are in capital. Where indicated, DNAzymes were fluorescently labeled at 5' end with Oregon Green™ 488 maleimide as suggested by the manufacture (Molecular Probes, Eugene, OR). The labeled oligonucleotides were purified with the MERmaid Kit (BIO 101, La Jolla, CA).

***IN VITRO* TRANSCRIPTION AND CLEAVAGE ANALYSIS OF MMP-9 RNA BY DNAZYMES**

The 764-nucleotide MMP-9 RNA transcript was prepared by *in vitro* transcription (T3 polymerase) of pBSNG4.1 [75] digested with BsmI restriction enzyme. DNAzymes (12.5 µM) were incubated with the RNA substrate (0.48 µM) in 50 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂ at 37 °C for 2 h. The reactions were stopped with 90% formamide in TBE and the reaction products were separated on a 4% polyacrylamide-urea gel. The RNA products were stained with Ethidium Bromide and their intensities were determined using an Alpha Imager 2000 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA).

CELL TRANSFECTION

The COS-7 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATTC, Manassas, VA). COS-7 cells were grown in Dulbecco's modified Eagles medium (DMEM), pH 7.4, containing 10% fetal bovine serum (FBS), 100 i.u./ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 10% CO₂. The COS-7 NG was generated by stably transfecting COS-7 cells with pcDNA3-MMP-9. Sub-confluent COS-7 NG cells (60-70%) were incubated in serum free medium and transiently transfected with Oregon Green™ 488 maleimide labeled

DNAzyme (AM9D-5; 24 µg) using DOTAP transfection reagent according to the manufacturer's instruction (Roche, Mannheim, Germany). After 24 h incubation at 37 °C in serum free medium, cells were collected, sorted, and the transfected cells were isolated for further analysis. MDA-MB-231 human breast cancer cells (ATCC; Manassas, VA) were plated onto 10 cm dishes in DMEM media supplemented with 10% fetal bovine serum (FBS) and allowed to grow to 80-90% confluence at 37 °C with 5% CO₂. The cells were then serum-starved for 4 hours prior to transient transfection with Oregon Green™ 488-maleimide -labeled AM9D-5 or control DNAzyme (24 µg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After a 18 h incubation at 37 °C in serum free medium, transfected cells were sorted for the presence of Oregon Green on a BD Biosciences Aria cytometer and positive cells were collected for further analysis.

ANALYSIS OF MMP-9 MRNA AND PROTEIN PRODUCTION IN AM9D TRANSFECTED CELLS

The amount of MMP-9 mRNA and its enzyme activity in the DNAzyme transfected COS-7 NG cells were measured by reverse transcription-polymerase chain reaction (RT-PCR) and gelatin gel zymography [75], respectively. Total RNA from the transfected COS-7 NG cells was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with random hexamer primers (Promega, Madison, WI) using MMLV-RT enzyme (Invitrogen, Carlsbad, CA). The amount of MMP-9 mRNA present in the RNA samples was quantified by PCR using specific MMP-9 primers (forward; 5'-GCAGGAATGCGGCTCTGG-3' and reverse primer; 5'-CCCGTCGAAGGGATACC-3'). β-actin mRNA was also amplified as an internal control, with forward; 5'-CAAGAGATGGCCACGGCGGCT-3' and reverse; 5'-TCCTTCTGCATCCTGTCAGCA-3' Primers. The PCR products were subjected to 1% agarose gel and visualized by Ethidium Bromide staining. MMP-9 expression was quantified by an alpha imager 2000 documentation and analysis system (Alpha Innotech corporation San Leandro, CA).

CELL INVASION ASSAY

MDA-MB-231 cells were transfected with fluorescently labeled AM9D-5 or control DNAzyme for 18 h in serum free DMEM medium as above. The fluorescent positive cells was identified by flow cytometry, isolated and seeded in ECMatrix™ invasion chambers (Millipore, Billerica, MA). After 24 h incubation at 37 °C with 5% CO₂, the number of cells that migrated through the ECM layer and attached to the polycarbonate membrane was quantified spectrophotometrically at 560 nm according to the manufacturer's protocol. All assays were conducted in triplicate and the values were averaged.

VITA

Miranda Alvina Marie Hallett was born in 1983 in Trenton, NJ. She is the daughter of Steve and Alvina Hallett. She was raised in Yardley, PA with her four older brothers, Steve, Jr., Jamal, Rashad, and Khaleef. After graduating Pennsbury High School with honors, Miranda attended Spelman College in Atlanta, GA. Miranda graduated in 2006 with a Bachelors of Science degree in Chemistry. Immediately following graduation, she then entered into the Integrated Program in Biomedical Sciences at UTHSC.