Iterative, rational design of cyclic and bicyclic peptide inhibitors of matrix metalloproteinases 9 and 2

Matthew Davis

Advisor: Joshua Kritzer

Thesis Committee: Clay Bennett, Krishna Kumar

Abstract:

Matrix Metalloproteinases are zinc metalloproteinases involved in cancer progression as well as acute inflammation. Gelatinase A and B, or MMP-2 and MMP-9, are members of this family implicated in extracellular matrix remodeling and angiogenesis. The disulfide-bridged decapeptide CTT is a potent inhibitor of Gelatinases, and has been shown to selectively target tumor cells. This work focused on optimizing a head-to-tail cyclic peptide mimic of CTT, with the aim of improving serum stability as well as affinity to Gelatinases. This was performed via iterative design of a series of peptides testing certain conformational space. In this work, a stabilized cyclic peptide inhibitor of Gelatinases, cWGF9, was found (IC_{50}~49 μM). Subsequent series have elucidated its function and specific structural features. In addition, several bicyclic peptide inhibitors based on CTT have been found (IC_{50}~67 μM). These peptides have been shown to be more stable in serum and are specific for Gelatinases. Evidence suggests that inhibition of Gelatinases by these compounds may be different than chelation of the active-site zinc.

Introduction

Matrix Metalloproteinases are important therapeutic targets.

Matrix metalloproteinases (MMPs) are zinc-containing proteases that are implicated in cancer and inflammation.[1,2,3,4] MMPs cleave elements of the basement extracellular matrix, clearing out space for cell proliferation, angiogenesis, and metathesis.[5,6] This property has made MMPs a cancer target, as inhibition of proteases such as MMPs have halted cancer progression and have reduced inflammation in mouse models.[7,8] Gelatinase (MMP-2 and MMP-9) activity, specifically in matrix remodeling, has been tied to proliferation and tissue invasion of cancerous tumors, making these enzymes good targets for inhibition. [9,10,11]

Invasion of normal tissue by cancerous tumors requires significant remodeling of the extracellular matrix, the space surrounding cells. This matrix is made up of structural elements such as Gelatin, Collagen, and Fibronectin. MMPs contain a conserved active site consisting of a catalytic Zn^{2+} ion, hydrogen bonded to an activated water molecule. In catalysis, this water molecule will attack the carbonyl of a peptide bond.[12] There are multiple members of the MMP family that have specific functions in cancer proliferation and metastasis.

The predominant functional and first elucidated role of MMPs is in cell proliferation.[13,14] This process is dependent upon remodeling of the extracellular matrix. There is evidence that MMP-1, -2, -13, and-14 are implicated in ECM remodeling specific to the tumor microenvironment, but MMP-14 (Membrane-Type MMP-1) is central and rate-limiting to the process.[15,16] Cancer cell proliferation is driven in part by tumor-associated macrophages, which secrete MMP-2 and MMP-9 (Gelatinase A and B). These enzymes are important in mediating degradation of the extracellular membrane, and intravasation (metastatic movement into the blood vessels).[17,17,] These same processes are responsible for extravasation (diffusion of white blood cells from capillaries into the surrounding tissue) during

inflammatory conditions and wound healing. Gelatinases have also been implicated in mouse models of encephalitis, which involves leukocytes invading cerebral cells through the basement membrane surrounding the blood-brain barrier of mice. This process involves degradation of the ECM component dystroglycan, suggesting that these MMPs are involved in intravasation of tumor cells into the blood stream.[18] Protein-protein interaction events by MMPs can potentiate enzymatic activity. Interactions with the hemopexin domain of certain MMPs with structural proteins can localize MMPs to particular areas of the ECM, resulting in locally upregulated activity.[19] An example is the binding of MMP-2 to integrin $\alpha_v\beta_3$, which is crucial to mesenchymal cell invasive activity.[20]

MMPs simultaneously contribute to cancer progression through protealytically remodeling the ECM and activation of cell signaling events, especially in the tumor microenvironment. MMPs can affect growth signals such as the Transforming Growth Factor β (TGF-β) pathway. Mutations in the TGF-β pathway can result in avoidance of immune function[21,22] MMP-9 and -2 activate TGF-β through cleavage of its pro form. This process occurs first by compartmentalization of the Gelatinases by docking to the surface receptor CD44, which then cleaves and activates TGF-β.[17] MMP-2 and MMP-9 also cleave the ECM component latent TGF-β binding protein 1 (LTBP-1), solubilizing TGF-β and promoting proliferation of the tumor.[23,24]

There are several other signaling pathways affected by MMPs. The epidermal growth factor receptor (EGFR) pathway is a potent driver of cell proliferation, and mutations involved in this pathway are often associated with breast cancer and other malignant diseases.[25] Activation of latent EGF by ADAM (A Disintegrin and metalloproteinase domain-containing protein) results in upregulation of MMP-9. This protease then degrades E-Cadherin, a protein involved in cell-cell adhesion and differentiation.[26] This pathway may be a key player in ovarian cancer and metastasis. MMP-9 increases the bioavailability of the vascular endothelial growth factor (VEGF) by making sequestered VEGF in the ECM

available for its receptor VEGFR2.[27] This is a potent angiogenic switch that upregulates the recruitment of blood vessels.[28]

MMP-2, -9, and -14 are important regulators of angiogenesis and vasculogenesis, processes which involve the recruitment of local blood vessels and vasculogenic progenitor cells from the bone marrow.[29] Recruitment of blood vessels is a process that is highly dependent upon MMP action: heavily degraded basement membranes and impaired junctions between cells.[30] MMP-9 is also implicated in vasculogenesis, seen in mouse models.[31]

A metastatic tumor will travel through the bloodstream to a metastatic niche – an area where the metastases can survive in a normally tumor-inhospitable environment. The term is taken from the niche areas that support stem cells in healthy tissue.[32,33] In forming this metastatic niche, certain pathways are activated, including the NF-κB pathway, which activates MMP-9 expression.[34,35] Expression of MMP-9 most likely helps enable formation of metastatic niches by promotion of angiogenesis via the VEGF pathway as well as cleavage and activation of Kit-ligand, which recruits stem

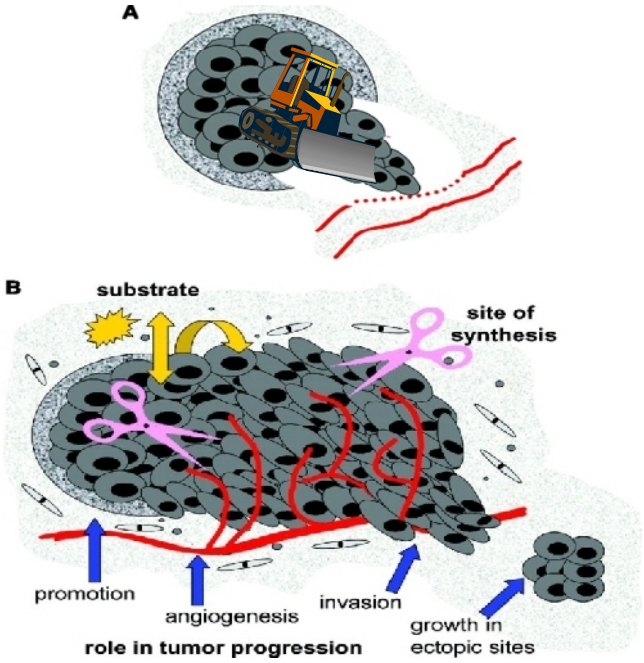


Figure 1: MMPs were traditionally thought of as simple proteases that model elements of the extracellular matrix in cancer progression (**A**) but have recently been implicated in many different cancer signaling pathways and also serve diverse cellular roles (**B**). The vastly different functions of the catalytically similar MMPs were the main reason for early drugs' failures in clinical trials.

and progenitor cells from bone marrow.[36]

MMPs are important therapeutic targets. With their function in proliferation, metastasis, angiogenesis, and signaling pathways, they are a high-value target for treatment of cancer. Specifically, MMP-2 and MMP-9, Gelatinases, are involved in a large number of these important processes, and represent potential targets for chemotherapeutic drugs.[37]

Inhibition of MMPs is traditionally mediated by Zinc-chelating groups

Early MMP drugs were largely unsuccessful. Early inhibitors used a hydroxamide group to tightly chelate the catalytic Zinc. While these early inhibitors were very effective *in vitro* and in cell and animal models, they largely failed in clinical studies.[38] Early drugs had very severe side effects including severe joint pain. This was due to the nature of the early inhibitors; Zinc-chelating hydroxamates were nonspecific MMP chelators. Broad-spectrum inhibition of MMPs results in inhibition of a number of essential cellular functions.[39] The only inhibitor currently on the market is a subantimicrobial-dose-doxycline (SDD), a locally-injected drug for periodontitis.[40,41] This drug is locally-injected, thus avoiding some of the severe side effects.

Modern Gelatinase inhibitors feature a greater degree of selectivity, but little variation in the method of inhibition. In many cases, the inhibitor is a hydroxamate or Zinc-chelating warhead attached to large scaffolding which serves to make surface contacts with other portions of MMP-2 or 9 that surround the active site. In particular, there are deep hydrophobic grooves (S1' and S2 specificity pockets) adjacent to the active site zinc ion, which can accommodate a large aromatic group.[42] New hydroxamate-based inhibitors feature a hydroxamate acid salt with large aromatic groups. Though many pharmaceutical companies have developed MMP inhibitors with greater selectivity than original broad-spectrum MMP inhibitors, most of the most specific new hydroxamate-drugs target four to six MMPs.

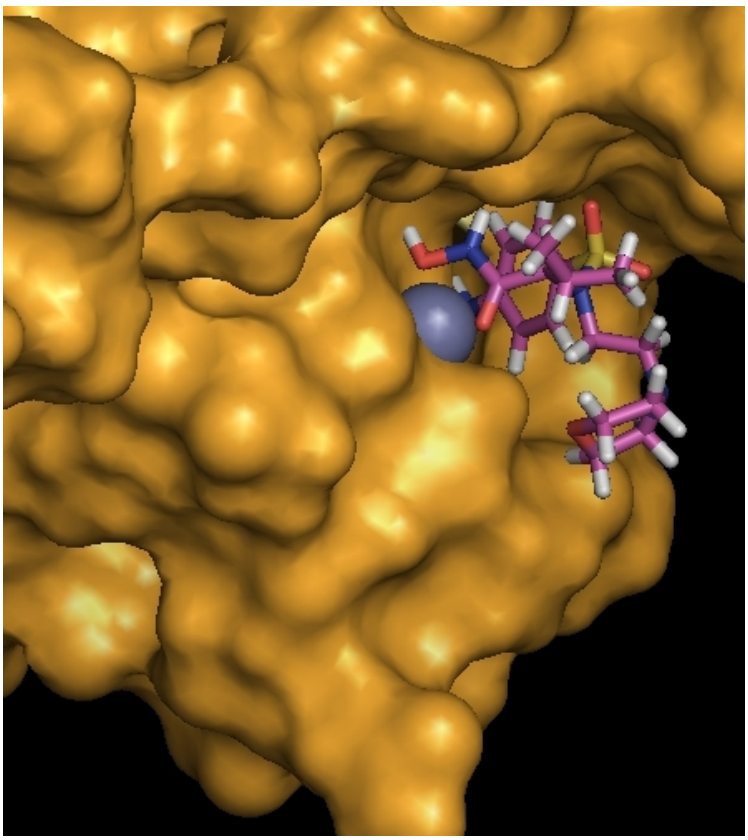


Figure 2: Schematic of MMP-2 catalytic domain complexed with a hydroxamate-based inhibitor (purple). Also pictured is the catalytic Zinc (grey), which is also complexed by three Histidines. Image is Pymol-generated from PDB ID 1HOV

There are a number of additional methods currently used to target MMPs specifically, though few are specific to Gelatinases. There is a phosphinic inhibitor of MMP-11 that is highly specific and potent. In addition, Sanofi-Aventis developed an inhibitor to MMP-13 using a pyrimidine motif to chelate the zinc. This approach is similar to that of Zoledronic Acid, a pyrimidine biphosphate compound that is mildly specific to Gelatinases. It was originally developed as osteoblast-mediated bone resorption inhibitor; Zoledronic Acid was shown to inhibit this process (Figure 4).[8]

Recently, a set of barbiturate-nitrate MMP-9 inhibitors were synthesized and tested. These molecules take advantage of the inhibitory activity of nitric oxide on MMP-9, and attach a barbiturate, which binds into the S1' specificity pocket.[4344] Another recently discovered Gelatinase inhibitor is SB-3CT, which is a mechanism-based inhibitor. This inhibitor takes advantage of a sulfirane functionality to

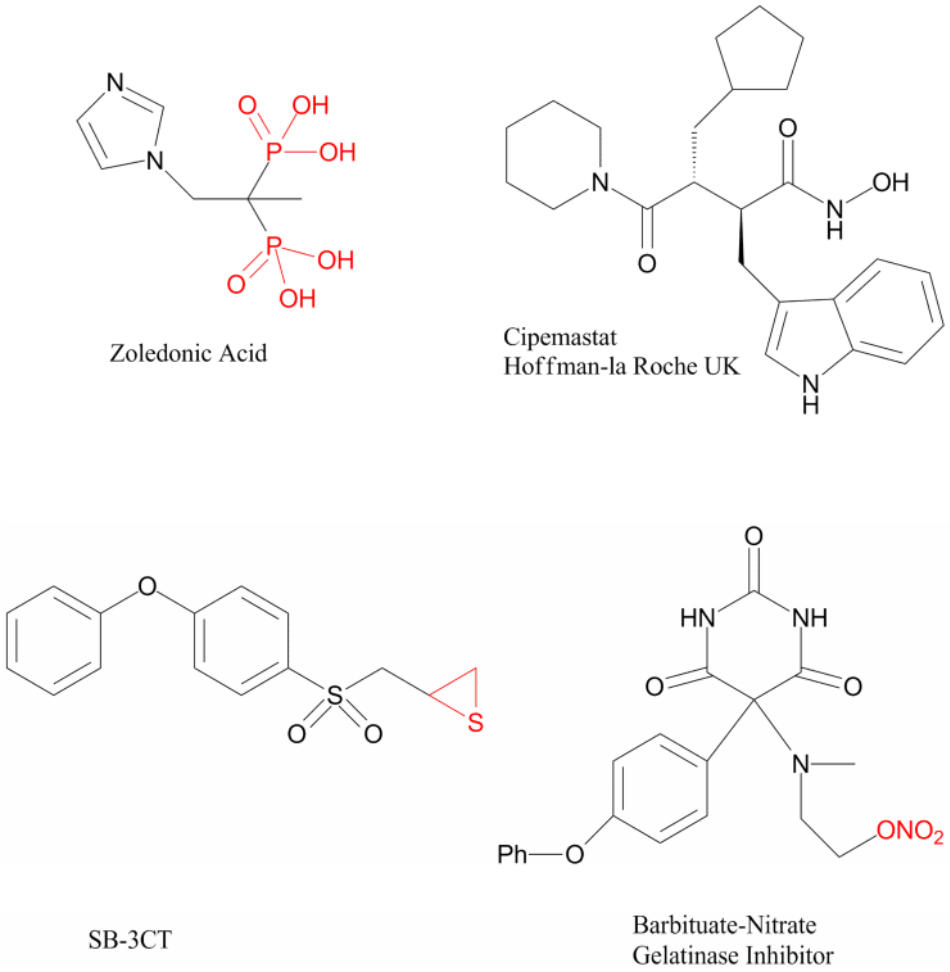


Figure 3: Inhibitors of Matrix Metalloproteinases. Zoledonic acid is a phosphinic inhibitor of Gelatinases. Cipemastat is a hydroxamate inhibitor of MMP-1. SB-3CT and the barbiturate-nitrate inhibitor are specific to Gelatinases as well.

both bind to the Zinc and to covalently bind onto a nearby glutamic acid which inactivates the active site. A biphenyl moiety of this inhibitor fits into the same hydrophobic cleft. Both of these inhibitors were shown to be specific for Gelatinases and had little to no activity against other MMPs.[45,46] In order to synthesize peptidic inhibitors of Gelatinases, these characteristics would have to be replicated.

Most of the inhibitors discussed have similar modes of binding to MMPs. These binding mechanisms, while with greater selectivity for Gelatinases, do feature some degree of promiscuity. Next generation MMP therapeutics will feature specific non-catalytic binding to these proteases. We feel that peptidomimetics have the potential to be those next-generation inhibitors. There are very few

peptidic or peptidomimetic-based inhibitors of metalloproteases. The only existing are either broad-spectrum or target TNF-Converting Enzyme (TACE), a zinc protease, and leave out the MMP family. Stabilized peptides or peptidomimetic inhibitors have several distinct advantages: degradation products of peptides are well tolerated and peptides can mimic biological topology very well. This makes them potentially very effective inhibitors.

CTT is a peptide Gelatinase inhibitor that may represent a different mode of MMP inhibition

In a phage display experiment using disulfide-bridged cyclic peptides against MMP-9, several binding motifs were enriched: a CXXHWGFXXC, CLRSGXGC, and CXPXC. Of these, the best inhibitors were of the form CXXHWGFXXC. The most effective of the CXXHWGFXXC series was the cyclic decapeptide CTTHWGFTLC, or CTT, which contained the recognition motif HWGF. CTT was able to inhibit tumor growth in cell models, and phage displaying CTT were seen to home to angiogenic blood vessels *in vivo*. CTT, when injected into mice intravenously, was seen to home to tumors as well, and

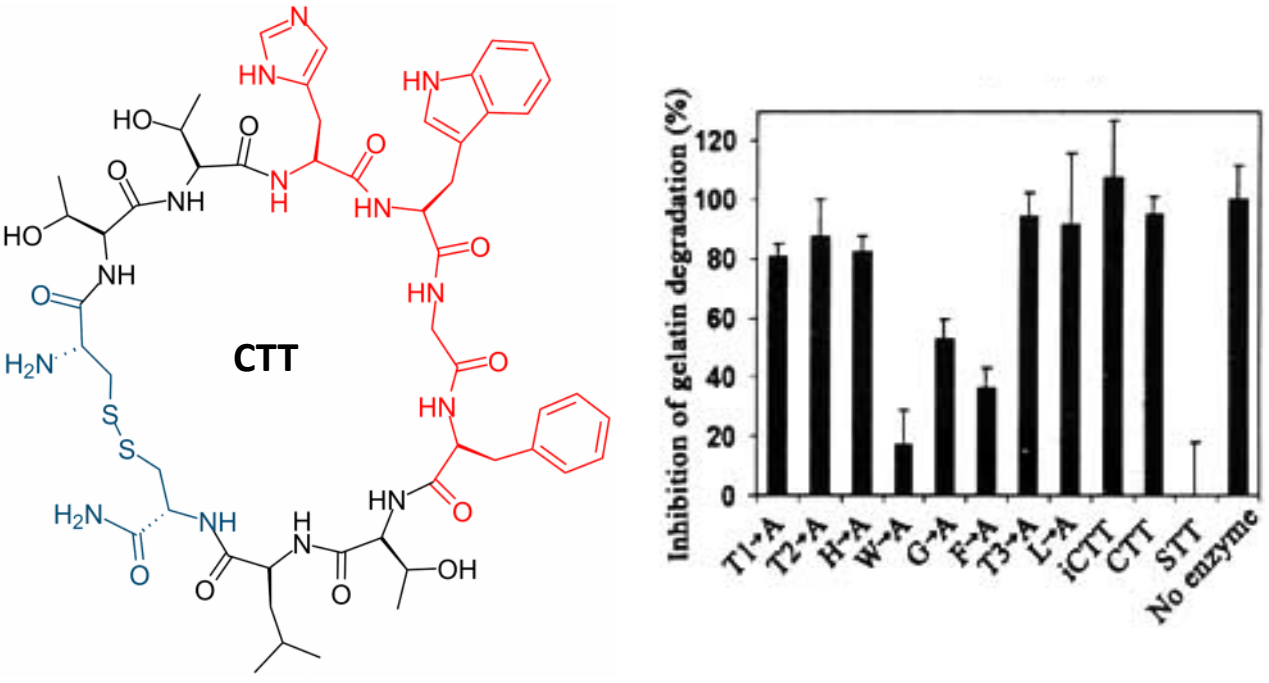


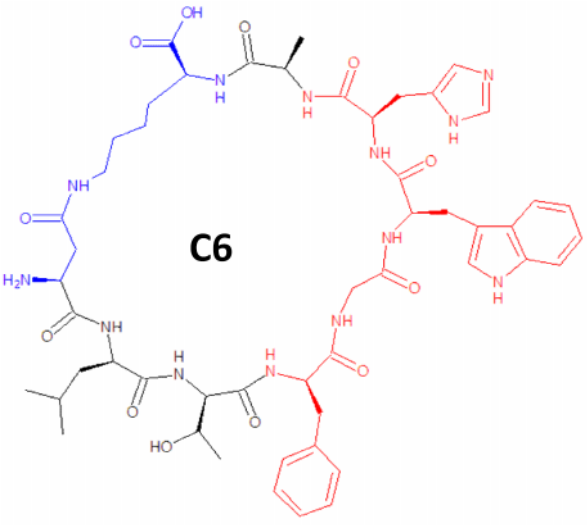
Figure 4: CTT is a disulfide bridged Gelatinase inhibitor (blue) which is active against MMPs and has the conserved motif HWGF (red). Cyclization is essential for function, as the uncyclized analogue STTHWGFTLS was an ineffective Gelatinase inhibitor.

shrink tumor size. The peptide is selective for MMP-2 and MMP-9, and does not inhibit a variety of related MMPs. It has a reported IC_{50} of roughly 30 μM in a collagen degradation assay.[47]

Many important structural features arose from SAR studies of this peptide. Replacement of the cysteines with serines in the peptide STT yielded an inactive, linear version of CTT. This shows that cyclization is essential for the function of CTT. Alanine scans by intein expression showed that while the WGF motif is essential for inhibiting MMP-9 and MMP-2, the Histidine is not important for function. This result suggests an alternate mode of MMP inhibition, independent of chelation of the zinc.

Incorporation of the non-natural amino acid 5-fluorotryptophan was shown to inhibit cell invasion *in vivo* six times better than natural CTT, and was significantly more serum-stable. However, there is no quantitative data on the *in vitro* efficacy of this unnatural analogue relative to CTT.[48] Intein splicing peptide expression was also used to incorporate Green Fluorescent Protein (GFP) to the end of CTT in a separate paper. Here, CTT was expressed as a histidine tagged C-terminal fusion protein with GFP, and was purified using nickel-affinity chromatography. It was shown to bind to cells expressing MMP-9, while an inactive GFP fusion was shown to not bind. These results suggest that CTT can be used as an imaging agent, and can tolerate fusion to large proteins.[49]

CTT is a hydrophobic peptide, and has been shown to associate with a lipid bilayer. Augmented emission anisotropy and reduced collisional quenching by I^- of the Tryptophan of CTT in unilamellar phosphatidylcholine/phosphatidylethanolamine liposomes showed that CTT associated with liposomes. CTT was able to increase the cellular uptake of Rhodamine-B containing liposomes 3- to 4-fold in Gelatinase-expressing cells. In addition, 4-fold augmented cell death of leukemia and sarcoma cells was seen by the CTT-enhanced delivery of chemotherapeutics. This has interesting implications for the inhibitory potential of CTT, and suggests that CTT, as an effective binding agent of cell membranes, can act as a homing agent to metastatic sites in a liposome-delivered cancer drug.[50] There is ample evidence



Physicochemical properties of peptidyl MMP-2 inhibitors

MMP-2 inhibitor	MMP-2 Inhibition IC_{50} (μM)
c(CTTHWGFTLC)NH_2 (**C1**)[b]	30.30
c(ATTHWGFTLD)NH_2 (**C2**)[c]	10.87
c(ATTHWGFTL-β-Ala) (**C3**)[d]	83.90
c(ATAHWGFTLD)NH_2 (**C4**)[c]	9.28
c(KTAHWGFTLD)NH_2 (**C5**)[c]	7.97
c(KAHWGFTLD)NH_2 (**C6**)[b]	7.56
c(KHGLTWFAD)NH_2 (**C7**)[b]	N/A
Cy5.5-c(CTTHWGFTLC)NH_2 (Cy5.5-**C1**)	N/A
Cy5.5-c(KAHWGFTLD)NH_2 (Cy5.5-**C6**)	N/A
Cy5.5-c(KHGLTWFAD)NH_2 (Cy5.5-**C7**)	N/A

Figure 5: Inhibition of MMP-2 by C6, a sidechain-cross-linked analogue of CTT. This peptide was able to home to tumor cells and be imaged, and shows the potential for stabilized CTT analogues.

that CTT can home to tumor cells, so this potential as a liposome-soluble chemotherapy agent is promising.

CTT has been shown to be a potent inhibitor of tongue Squamous Cell Carcinoma (SCC). It was able to fully inhibit all Gelatinase activity. In addition, the group utilized a version of CTT which incorporated a hydrophilic tail (CTT2), which was shown to have similar inhibitory activity, but dissolve in water rather than hydrophobic media. In addition, this change resulted in lower cytotoxicity, and the authors suggest that the lower cytotoxicity and *in vivo* data gathered suggest that CTT2 may home to tumors better than CTT.[51,52]

CTT analogues have also been investigated as tumor imaging agents. In a study, the lactam-cyclized peptide (KAHWGFTLD)NH_2, cyclized between the ε-amine of the lysine and the sidechain of the aspartate, exhibited a fourfold increase in activity. These peptides were then coupled to Cy5.5, a fluorescent group that can be attached to the C-terminus via a peptide coupling. This fluorescent CTT analogue was able to bind to overexpressed Gelatinases and image proliferative tissue. These results

support the tumor-homing properties of CTT-like peptides, and also suggest that stabilization of the amide backbone can improve affinity to Gelatinases.[53]

The Goal of this Project is to develop novel constrained peptide MMP inhibitors

The aforementioned protein-protein interactions of Matrix Metalloproteinases offer a rich new target for inhibition. Modern MMP drugs require a significantly greater degree of selectivity, so inhibitors that can target protein-protein interactions would be a large step forward in the development of effective MMP therapeutics. Using the yeast two-hybrid system, a method where protein-protein interactions can be screened in *S. Cerevisiae*, we planned on finding MMP protein/protein interactions. Finding an MMP interaction in vivo would then open up the project to counter-selection strategies to screen for a cyclic peptide inhibitor of that protein-protein interaction. These cyclic peptides would be synthesized via our Split-Intein Circular Ligation of Peptides and Proteins (SICCLOPS) method to create large, diverse libraries of cyclic peptides to screen *in vivo*. The goal of this project is to find a potent inhibitor of an MMP protein-protein interaction that would be highly stable and very selective.

The yeast two-hybrid method has been used before to demonstrate activation of MMP-2 by an MMP-14/Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1) complex, so there is evidence that MMPs could work in a yeast two-hybrid format. In addition, the SICCLOPS cyclic peptide library has previously been demonstrated to be effective at quickly generating cyclic peptide inhibitors of high-value protein targets. MMPs were chosen as a target for this library because they are a high-value therapeutic target, and their protein-protein interactome is not very well understood. In addition, we plan to test the hemopexin-domain interactions of MMPs, an area which should be stable in the yeast two-hybrid, and is the primary interaction domain of MMPs.

CTT is a peptide that may have a novel mode of inhibiting Gelatinases. The fact that its recognition motif contains no strong Zinc chelators suggests that it inhibits MMP-9 through hydrophobic interactions, and represents an exciting new way to target these proteins. There is significant evidence

that not only is CTT a selective inhibitor of Gelatinases, but replacement of its disulfide bridge - a relatively unstable moiety in serum – with a stabilized inhibitor can be beneficial to inhibitory activity. The goal of this project is the design head to tail, lactam cyclized and bicyclized peptide inhibitors of MMP-2 and MMP-9 based off of CTT. This strategy will be carried out by a several-pronged approach consisting of multiple rounds of iterative design. First, the disulfide bridge in the original peptide will be taken out in favor of a lactam bridge, which will offer stabilization and protease-resistance *in vivo*. This linker will be optimized in a variety of ways in order to best put the peptide in an active conformation to inhibit Gelatinases. Then, the peptide will be bicyclized, and the bicyclic linker method, length, and stereochemistry will be optimized in order to best lock the CTT analogue in its active conformation.

CTT may exhibit a different mode of MMP inhibition. The fact that the Histidine is not essential for function suggests that there is no real effective chelating group. This goes against the trend of common MMP inhibitor molecules. Stabilization of CTT though cyclization and bicyclization will improve its rigidity, and help to pay the entropy penalty for binding to the metalloprotease up front, rather than forcing the molecule to sample a large amount of conformation space before inhibition. This higher-affinity, rigid inhibitor will be easier to characterize structurally. From there, it is a long term goal to elucidate the mode of binding of this class of MMP inhibitor.

Previous Work: Probing MMP Protein-Protein Interactions Using the Yeast Two-Hybrid:

Introduction:

Exploring MMP protein-protein interactions was accomplished using the Yeast Two-Hybrid system. In this system, two proteins are fused to either end of a transcription factor: the DNA binding (DB) and activation domains (AD).[54] If these two proteins interact, the function of the transcription factor will be restored, and transcription of the gene specified by the transcription factor will result. The yeast system is set up such that reconstitution of the GAL4 Promoter will result in transcription of reporter genes.[55] (Figure A)

There were a number of MMP constructs made for this study, many to study specific processes. For example, the extracellular matrix metalloproteinase inducer (EMMPRIN) protein has been shown to stimulate MMP production *in vivo* by interactions with proteins such as Cyclophylin A and elements of the extracellular matrix. It may also interact with MMP-1 as a direct modulator of activity. The Tissue Inhibitors of Matrix Metalloproteinases (TIMP) proteins are endogenous inhibitors that form 1:1

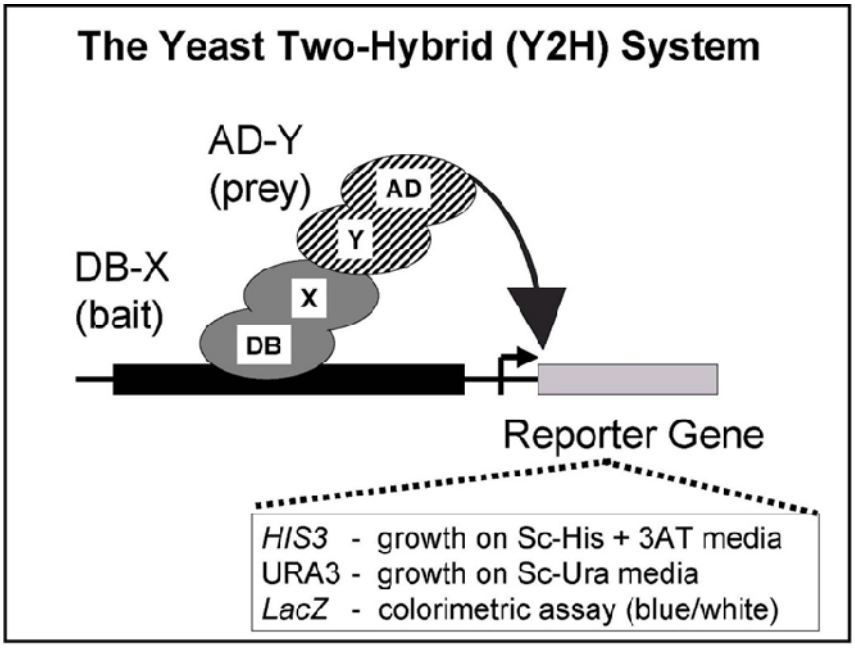


Figure A: Schematic of the Yeast Two-Hybrid system. Two proteins are fused to the Activation (AD) and DNA-Binding (DB) domains of a transcription factor. Interaction of the proteins results in transcription of the reporter gene, which results in growth on Histidine or Uracil deficient media. Taken from Reference 55.

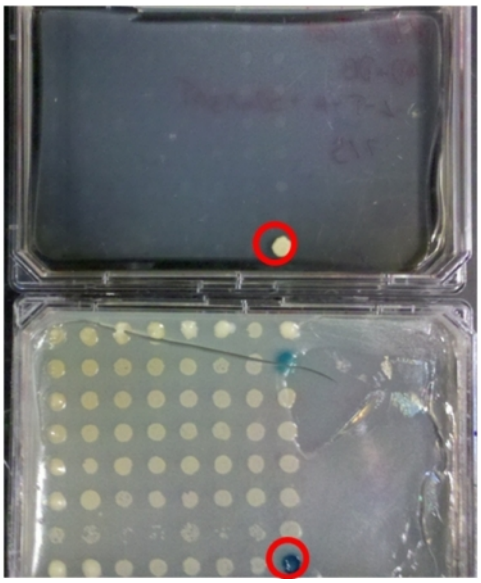


Figure B: Example of positive results in the yeast two hybrid system. Fos/Jun protein-protein interaction is circled in red. (Above) plating of potential interactions on Uracil-deficient media, where negative controls do not grow, and Fos/Jun interaction grows. (Below): X-Gal colony lift assay, where Fos/Jun interaction turns blue and other interactions remain colorless.

stoichiometric complexes with active metalloproteinases through a conserved binding ridge.[56] The MMP-TIMP interaction has previously been seen in yeast two-hybrid, and inhibition of this interaction with stabilized cyclic peptides could offer an interesting probe for the function of the MMP-TIMP interaction.[57] Also, research performed by Kuliopolis et al suggests that MMP-1 can cleave the protease-activated domain-1 (PAR-1), to initiate a signaling cascade related to thrombogenesis. [58,59] These potential interactions, along with several MMP truncation constructs, were examined to find potential interactions.

Methods:

In the yeast two-hybrid system, the GAL4 transcription factor is reconstituted in interacting systems. This transcription factor activates transcription for Histidine and Uracil synthesis, as well as α-galactosidase. Therefore, if two proteins fused to these transcription factors interact, yeast should be able to grow on Histidine or Uracil-deficient media. Additionally, colonies with interacting proteins will

Construct	Description	Interactions of interest
EMMPRIN	Full Length	MMP-1
TIMP-1	Full Length	MMP-9
TIMP-2	Full Length	MMP-2/MMP-14
mMMP1b	Catalytic Only	EMMPRIN, TIMP
mMMP1e	Catalytic-Hemopexin	EMMPRIN, TIMP
hMMP1a	Full Length	EMMPRIN
hMMP1b	Catalytic Only	"
hMMP1c	Pro-Catalytic	"
hMMP1d	Hemopexin	"
hMMP1e	Catalytic-Hemopexin	"
MMP2a	Full Length	TIMP-1, TIMP-2
MMP2b	Catalytic Only	"
MMP2c	Pro-Catalytic	"
MMP2d	Hemopexin	"
MMP2e	Catalytic-Hemopexin	"
MMP9a	Full Length	TIMP-1
MMP9b	Catalytic Only	"
MMP9c	Pro-Catalytic	"
MMP9d	Hemopexin	"
MMP9e	Catalytic-Hemopexin	"
MMP14a	Full Length	TIMP-1, TIMP-2
MMP14b	Catalytic Only	"
MMP14c	Pro-Catalytic	"
MMP14d	Hemopexin	"
MMP14e	Catalytic-Hemopexin	"
PAR1	EC Domain 1	MMP-1
PAR2	EC Domain 1	"
PAR3	EC Domain 1	"
PAR4	EC Domain 1	"

Table 1: Constructs for use in Yeast Two-Hybrid studies. All constructs were AD and DB fusions.

turn blue when plated with X-α-Gal (5-Bromo-4-Chloro-3-indolyl a-D-galactopyranoside), a chromogenic substrate of α-galactosidase. (See Figure B)

Once a protein-protein interaction was found, the strain could be transformed with the SICLOPPS cyclic peptide library. Selection of cyclic peptide inhibitors of these interactions is a simple process. The yeast is plated media containing 5-Fluoroorotic acid (5-FOA) and Uracil. 5-FOA processed by yeast results in production of 5-flurouracil a suicide inhibitor that is toxic to the yeast. Yeast that cannot produce uracil on its own will survive, while yeast which has an active GAL4 transcription factor will not survive. Therefore, cyclic peptides that inhibit this interaction will result in growth on 5-FOA

plates. Therefore, growth of an interacting strain on 5-FOA plates represents a potential inhibitor of MMP protein-protein interactions.

Results:

Twenty nine cassettes containing MMPs, TIMPs, or other related interacting partners, were constructed and put into AD and DB yeast two-hybrid vectors using Gateway cloning technology. (See Table 1) Vectors containing AD fusions also contained a Tryptophan auxotrophic marker, while those with DB fusions contained a Leucine auxotrophic marker, to ensure that yeast transformed with these vectors would survive on nutrient-deficient media. All constructs were transformed into Mav103 and Mav203 strains and mated against all other constructs in a large grid to screen for all potential protein-protein interactions. This was performed by first growing a mixture of the two yeast strains in YPD (Yeast Extract Peptone Dextrose) overnight, then plating on Leucine/Tryptophan deficient SD media to

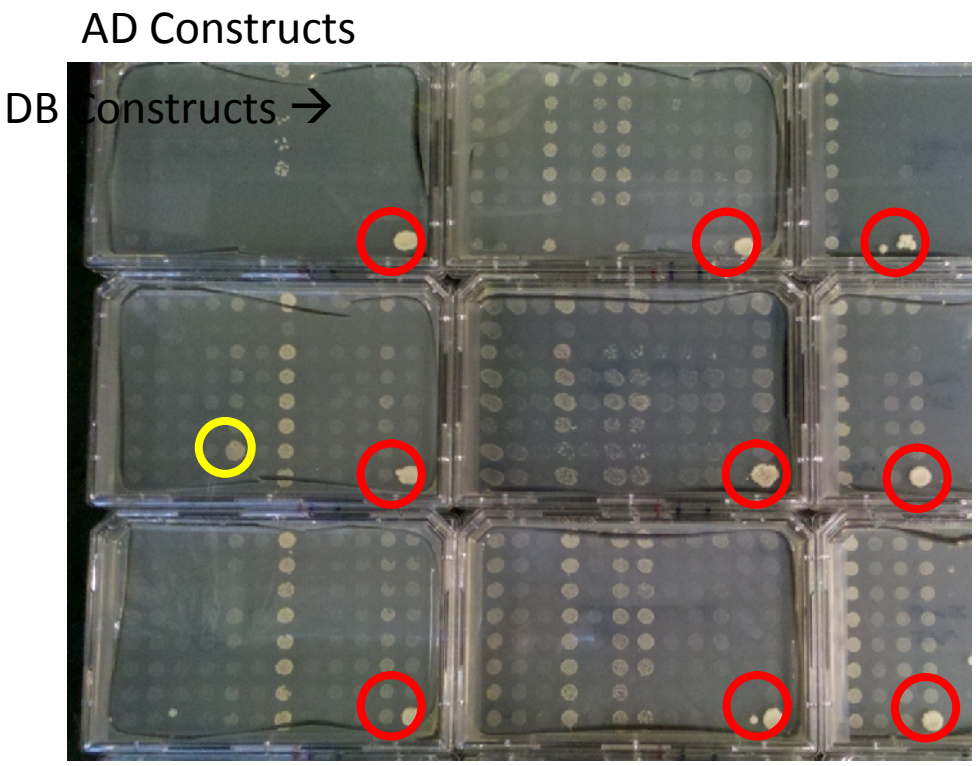


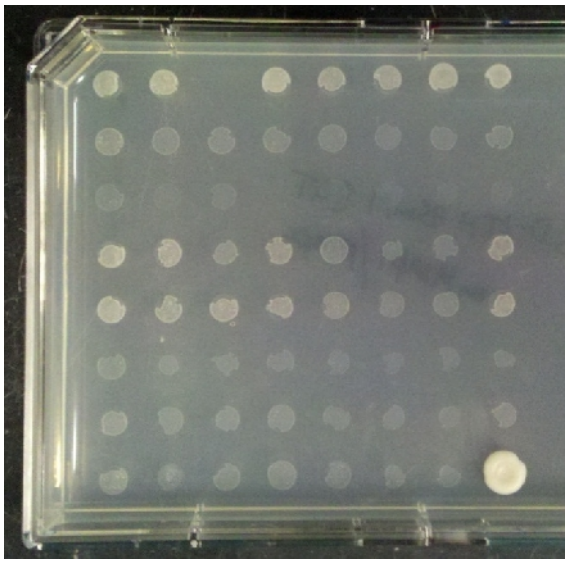
Figure C: Results of mating of MMP and MMP-related compound constructs. All compounds were tested as AD and DB fusions. In red is the Fos/Jun positive control on each SD-His-Leu-Trp+25 mM 3AT plate, and in yellow is the lone interaction seen, between the catalytic domain of human MMP-9 and full length mouse MMP-1.

select for diploid yeast (See Figure C).

Mating was successful for a large number of the potential interactions. The positive control for the yeast two-hybrid was the Fos/Jun interaction – two Leucine zipper transcription factors that have a strong and modular interaction compatible with the yeast two-hybrid. After mating, the yeast was suspended in water and plated onto Leucine/Tryptophan/Uracil and Leucine/Tryptophan/Histidine deficient media. After three days, the plates were examined. Any colonies that grew were potential MMP protein-protein interactions.

Initial screens yielded the lone result of an interaction between human MMP-9 and activated mouse MMP-1. (Figure C) However, further testing showed that this interaction was a false positive – it was not able to be reproduced on Histidine deficient plates, and never grew on Uracil-deficient plates (Figure D). Unfortunately, no MMP interactions were observed in a yeast two-hybrid context.

The absence of any significant interaction between MMPs in the yeast two-hybrid system is likely for several reasons. Most importantly, the interactions must take place in the nucleus in order for



SD-LTH+25mM

Figure D: The results of the first screen could not be replicated on either histidine or uracil deficient media (not pictured) – the interaction between mouse MMP-1 and human MMP-9 was a false positive.

transcription to occur. Matrix Metalloproteinases are extracellular proteins, so were unlikely to be folded in a similar way in the cell's nucleus. Also, being a reducing environment, the nucleus precludes formation of disulfide bonds, which would negatively affect the structure of MMPs, TIMPs, and PAR extracellular domains. Experience dictates that only roughly 20% of the interactome is compatible with the yeast two-hybrid, so the difficulty with MMPs is understandable. Because a usable strain was not found, this particular project was abandoned, and a design route was undertaken to design synthetic cyclic peptide inhibitors of MMPs.

Materials

Solid-Phase Peptide Synthesis

All peptide synthesis reagents such as Fmoc-protected natural and unnatural amino acids were purchased from Anaspec, Inc. or EMD Chemicals. Specialized amino acids and resins were obtained through Chem Impex International. Solvents and chemicals were purchased through Sigma Aldrich Inc. An Aaptec Solid Phase Peptide Synthesizer was utilized to synthesize the series of peptides, along with a CEM microwave synthesizer. Glass reaction vessels were obtained from ChemGlass, and other glassware via Sigma Aldrich or VWR. Reverse-Phase High Performance Liquid Chromatography was performed using a Varian Pro-Star system (Agilent). Water was purified using a Millipore purifier, and Acetonitrile was bought from Sigma Aldrich. A preparatory C8 column was used in purification.

MMP Assays

All buffer materials were obtained from Sigma Aldrich, and a Millipore HQ water purifier was used to make the buffers. Enzymes, substrates, and enzyme kits were obtained from Anaspec, Inc. Fluorescence intensity readings were taken using a Tecan Plate Reader.

Serum Stability:

10x PBS and human serum were obtained via Sigma Aldrich. Reverse Phase High Performance Liquid Chromatography was performed using a Varian Pro-Star system (Agilent). Water was purified using a millipore purifier, and Acetonitrile was bought from Sigma Aldrich. An analytical C18 column was used to analyze the results.

Methods

Peptide Synthesis:

Rink Resin Loading:

Synthesis of CTT cyclic peptide derivatives has been carried out via Fluorenylmethyloxycarbonyl chloride (Fmoc) Solid Phase Peptide Synthesis (SPPS). Rink resin is an amide resin often used with SPPS, which results in an amide functionality at the C-terminus after cleavage. We used this resin to synthesize cyclic peptides entirely on-resin with Asparagine linked in two different ways (See table 2). First residue attachment is accomplished via a peptide coupling of a C-terminal (Fmoc-Asp-OAll) or sidechain allyl-protected Aspartate residue (Fmoc-Asp(OAll)-OH) onto a rink resin. Sidechain-protected Aspartate is used to create cyclic peptides linked through Aspartate's side chain with a carboxamide group rather than a C-terminus, whereas C-terminally protected Aspartate is used to create head-to-tail cyclic peptides with an asparagine sidechain. This was carried out in minimal DMF, with 2 equivalents of the amino acid, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), and N-Hydroxybenzotriazole (HOBt). The coupling was activated using six equivalents of diisopropylethylamine (DIPEA). The resin was reacted with these reagents for 35 minutes, then is washed with DMF and dried using DCM. After the loading was determined, the resin was swollen in DMF and capped using a 1:1:3 mixture of DIPEA, acetic anhydride, and DMF. Afterwards, the resin was dried using DCM and stored in a desiccator until later use.

The resin loading was measured by incubating milligrams of loaded resin in 2.0 mL of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) for 35 minutes in triplicate. This was then diluted to 10mL in acetonitrile, then 2mL was added to 23mL of acetonitrile. This was then measured in a UV-Vis spectrophotometer at 304 nm. The absorbance values were then converted to resin loading values using equation (1).[60]

(1) $$Loading\left(\frac{meq}{g}\right) = \frac{16.4 \times \Delta Abs}{mg\ resin}$$

Wang Resin Loading:

Wang resin is a hydroxyl resin, so carboxylic acid groups are what result upon cleavage from the resin. This resin was used in synthesis of peptide which were not sidechain linked so could not be cyclized on-resin as with peptides that required a carboxylic acid rather than a carboxamide functionality after cleavage if they were cyclized on-resin (See table 2). The attachment of the first amino acid is an anhydride formation of the acid followed by coupling onto the resin using dimethylaminopyradine (DMAP). This is done by reacting ten equivalents of Fmoc-protected amino acid with 5 equivalents of N,N-dicyclohexylcarbodiimide (DCC) in 15 mL of DCM. This is stirred under an argon atmosphere and at 40C. This mixture will concentrate into a slurry, which is dissolved in 5mL of DMF. While this is happening, Wang resin is swelled for 30 minutes. The anhydride solution is agitated with the Wang resin along with 2 equivalents of DMAP. After 45 minutes of coupling, the resin is washed with DMF and DCM and dried, and the resin loading test was applied to determine resin loading.

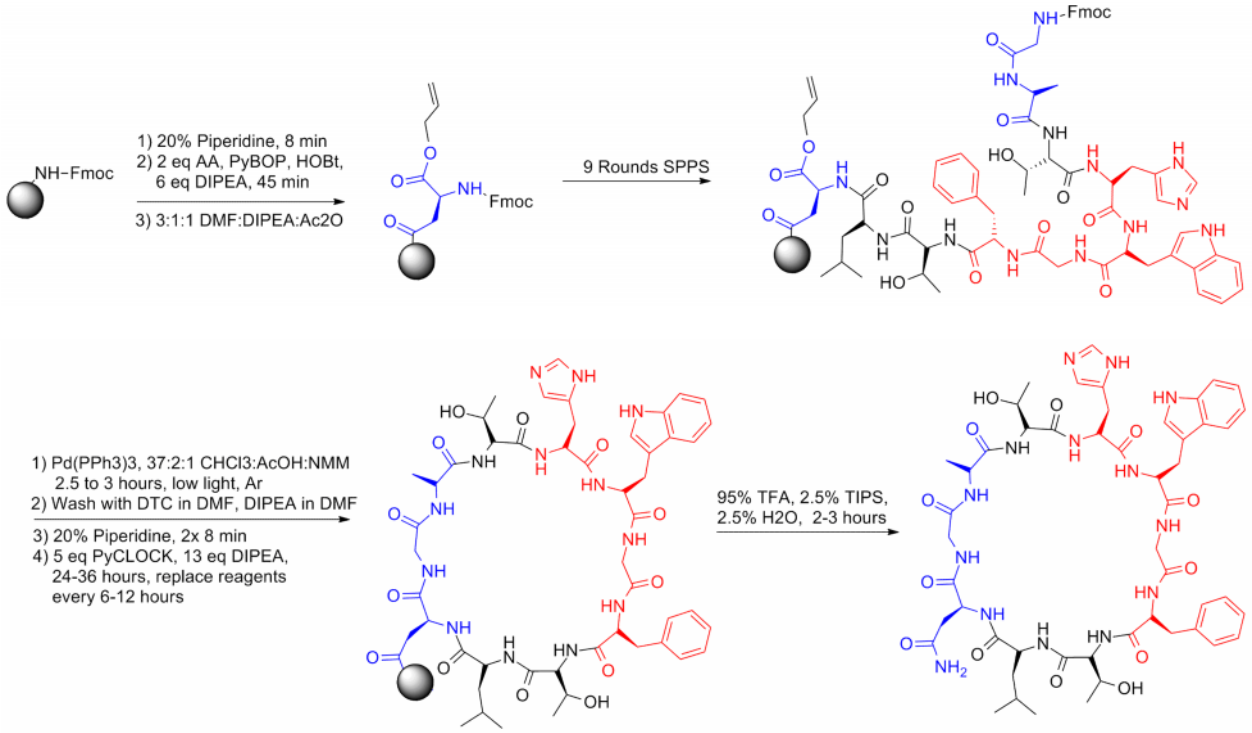
Peptide Synthesis:

Peptide synthesis was accomplished with successive couplings and deprotections of Fmoc solid phase peptide synthesis (SPPS) using a robotic peptide synthesizer. The automated synthesizer was used to synthesize multiple peptides at once in parallel (see table 2). The Aaptec synthesizer used could be programed to couple, deprotect, and wash in a DMF format for up to 16 peptides at once. The deprotection consists of adding 5 mL of 20% piperidine in DMF, and a ten minute mixing period. Coupling consists of addition of 1.0 mL of 0.4 M O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 1.0 mL of 0.4 M Fmoc-protected amino acid in N-methylpyrrolidine (NMP), and 0.5 mL of DMF 0.5 mL of DIPEA. The reagents are then mixed for 35 minutes in the peptide synthesizer. The coupling step is repeated twice to ensure complete attachment of the residue. After each step, the resin is washed in DMF using a wash protocol.

SPPS has also been done using a CEM microwave peptide synthesizer. This method helps to rapidly synthesize peptide scaffolds using a microwave to expedite reactions. The microwave synthesizer was used to synthesize individual peptides rapidly rather than several in parallel. Deprotections were carried out in 7.0 mL of 20% piperidine in DMF for three minutes at 65^0C. Couplings are performed with 5 equivalents of the Fmoc-protected amino acid, PyBOP, and HOBt, as well as 13 equivalents of DIPEA in 4-6 mL of DMF for five minutes at 65^0C with a four minute cool-down step. The resin is washed with DMF in between coupling and deprotection cycles.

Cyclization of Linear Peptides:

Cyclic peptides that were sidechain-protected with allyl groups were synthesized in a straight chain, then deprotected on both ends and cyclized on resin (Scheme 1). Deallylations are performed using Tetrakis(triphenylphosphine)palladium$^{(0)}$ ($Pd(P(Ph_3))_4$) catalyst. Three equivalents of catalyst were added to the allyl-protected resin, in a solution of 37:2:1 chloroform:acetic acid:N-methylmorpholine. 5



Scheme 1: Synthesis of cyclic peptides on-resin. Allyl-protected amino acid is loaded onto a resin. After building the linear chain, the allyl group is deprotected with Pd(Tetrakis) and cyclized on-resin with PyCLOCK. The resulting cyclic peptide is cleaved off the resin with TFA.

mL of this mixture is added to the resin and bubbled with Argon for thirty minutes, then placed on an orbital shaker for 2-3 hours. After reacting, the resin was washed with 25 mg of diisodithiocarbiodiimide in 10 mL DMF as well as 10% DIPEA in DMF. The N-terminus was then deprotected using 20% piperidine, and washed with DMF. The swollen resin is then cyclized

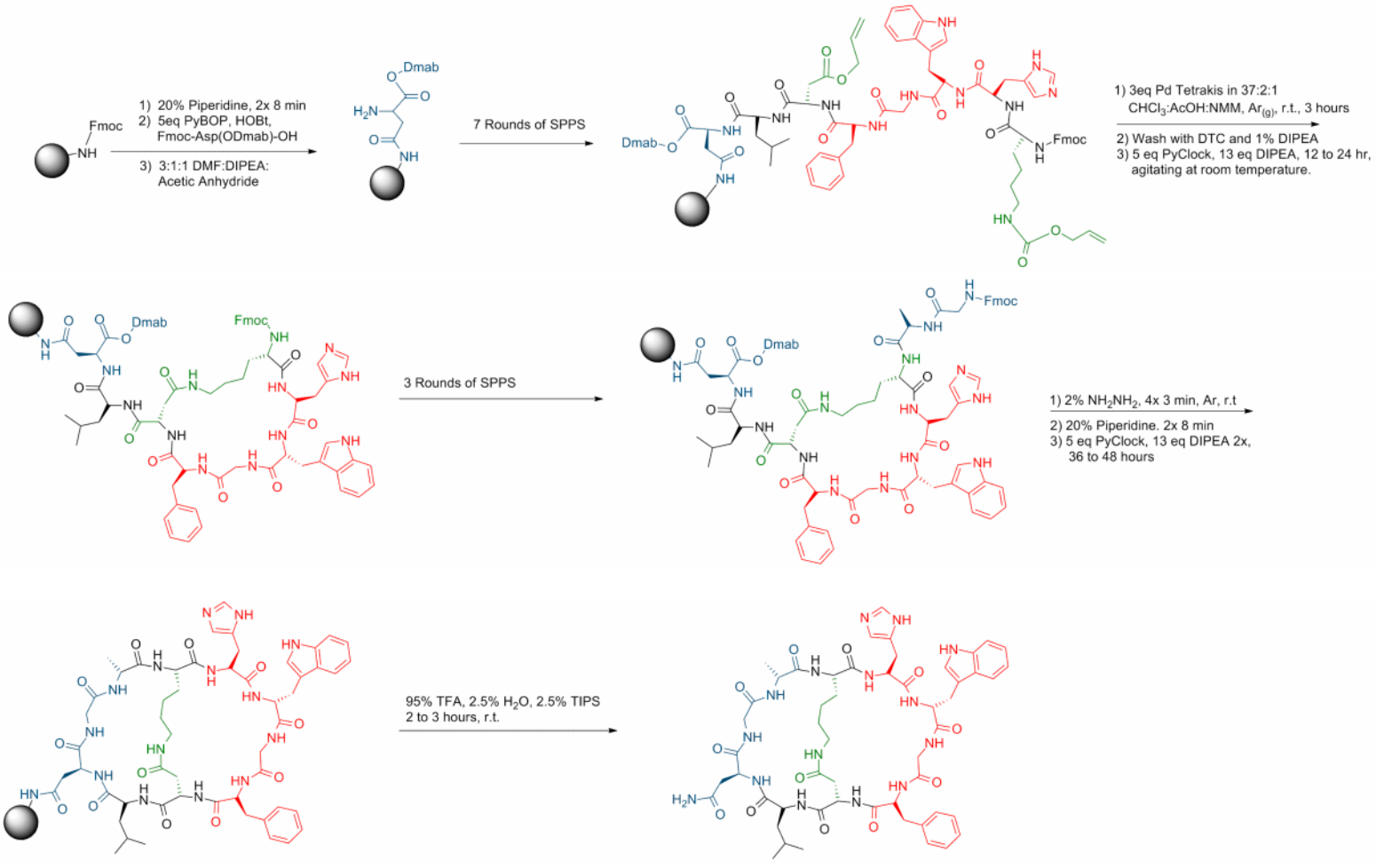
Cyclization proceeds via a number of methods. Most commonly, the deprotected peptide on-resin is mixed with 5 equivalents of PyCLOCK/HOBt-Cl and 13 equivalents of DIPEA for 12-24 hours. This has been performed both on an orbital shaker (replacing reagents every 8-12 hours) and by using the automated peptide synthesizer, programming replacement of 1.0 mL 0.4M PyCLOCK, 1.0 mL DMF, and 0.5 mL DIPEA every 6 hours for a total cyclization time of 18 hours. Cyclization has also been accomplished using a microwave peptide synthesizer with a total coupling time of 20 minutes at 65^0C. Generally, the first cyclization is accomplished at a high yield if performed on-resin, and ratios of cyclcodimer for this series is low (<5%, by analytical HPLC).

In-solution cyclization with peptides not attached to the resin by a sidechain proceeds via reaction with 2 equivalents of PyCLOCK/HOBt-Cl and 6 equivalents of DIPEA for 8 hours at room temperature. This reaction occurs after cleavage of the linear peptide off the resin and purification using RP-HPLC. The peptide is diluted to 2 mM or less in the cyclization solution in order to limit cyclodimer formation. After cyclization, the peptide is concentrated using a rotovap and purified using RP-HPLC.

Cyclization via formation of a disulfide bridge was done in the synthesis of CTT. The crude, ether precipitated peptide (see below) was diluted first into 1.5 mL of DMSO, then into 200mL of a 0.02 M sodium phosphate buffer (pH 8.6). The solution is then air-oxidized overnight, and subsequently concentrated to 4-6 mL on a rotovap and purified on an RP-HPLC with water with 0.1% TFA and Acetonitrile with 0.1% TFA as the mobile phases. Peptides were purified on a 5-60% acetonitrile gradient over 30 minutes.

Peptide Cleavage and Preparation for Assays:

Peptide cleavage as well as global deprotection is carried out using Trifluoroacetic Acid (TFA). For sulfur-containing peptides, a cleavage cocktail of 94% TFA, 2.5% water, 2.5% ethanedithiol (TIPS), 1.0% triisopropylsilane (TIPS) was used. For peptides that do not contain sulfur, the cleavage cocktail contained95% TFA, 2.5% water, and 2.5% TIPS. 10 mL of cleavage cocktail was used per 100 µM synthesis, and the cleavage was carried out for 2 to 4 hours. Water and TIPS are scavengers for carbocations, and EDT is a scavenger for sulfur protecting groups. TFA is used to cleave all acid-labile elements of the peptide chain. Peptides were purified using a Galaxie RP-HPLC with water with 0.1%



Scheme 2: Synthesis of bicyclic CTT analogues. Dmab-protected amino acid is loaded on to the resin. Then, the linear chain is synthesized with two allyl-protected amino acids. After Palladium deprotection, the peptide is cross-linked. Then, the peptide is completed and deprotected with hydrazine and piperidine, followed by a 36-48 hour macrocyclization on-resin.

TFA and Acetonitrile with 0.1% TFA as the mobile phases. Peptides were purified on a 5-60% acetonitrile gradient over 30 minutes.

Fractions for all peptides are freeze-dried into 2 mL eppendorf tubes, and reconstituted in minimal DMSO. The concentrations of the peptides were taken by measuring the UV-Vis spectrum of a 1:100 dilution of the peptide in water at 280. cWGF-series peptides had an approximate molar absorptivity of 5500 $M^{-1}cm^{-1}$. The peptides are then diluted to 10mM in pure DMSO. These are the stock solutions used for the MMP Quenching assay.

Bicyclic Peptide Synthesis

Bicyclic peptides are synthesized using a similar method, with the linear chain assembled on an Aapptec automated peptide synthesizer (Scheme 2). Resin loading is the same procedure as above, but instead Dmab-protected aspartate is used in the synthesis in place of Allyl-protection. The Dmab group is hydrazine-labile and stable to palladium deprotection.[61] The two sidechain residues to be cross-linked are protected by Allyl (carboxy) and Alloc (amine) functionalities in lactam-bicyclized peptides. Amino acids used for these cross-linkages are Fmoc-Lys(Alloc)-OH, Fmoc-Asp(OAll)-OH, and Fmoc-Glu(OAll)-OH.

After the initial eight residues are synthesized, the peptide is removed from the synthesizer using DCM and dried. Then, it is treated with Pd-Tetrakis in the manner discussed above in a glass RV. The peptide is then transferred back into the peptide synthesizer and is coupled for 24 hours using 1.5 mL DMF, 1.5 mL of 0.4 M PyCLOCK, and 0.5 mL DIPEA replacing reagents every 6 hours. After cyclization, the linear chain is completed with three more rounds of SPPS on the automated synthesizer.

The carboxy-protecting Dmab group on these cyclic peptides is taken off using Hydrazine (2% in DMF, 4 times for 3 min). The peptide is then macrocyclized for 48 hours in the peptide synthesizer, using PyCLOCK and replacing regents every six hours. The peptide is cleaved and purified using the same methods as for cyclic peptides, on RP-HPLC in preparatory mode.

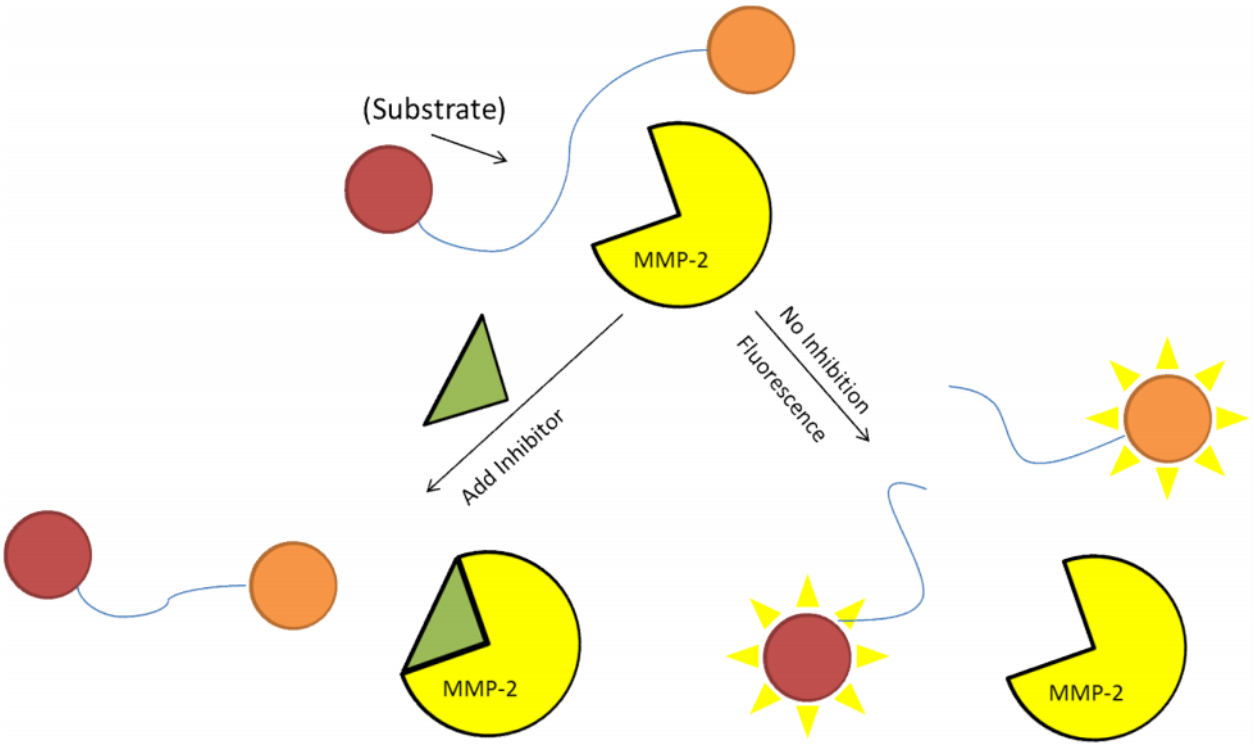
Peptide	First Amino Acid, Resin	Synthesis of Linear Chain	Cyclization Method	Notes
cWGF1-6	Fmoc-Asp-OAll, Rink	Aapptec Automated Synthesis	On-Resin, PyBOP	24 hr
cWGF7-9	Fmoc-Asp(OAll)-OH, Rink	CEM Microwave Synthesis	On-Resin, PyBOP	24 hr
cWGF10,11	Fmoc-Asp-OAll, Rink	CEM Microwave Synthesis	On-Resin, PyBOP	24 hr
cWGF12	Fmoc-Asp-OAll, Rink	Aapptec Automated Synthesis	On-Resin, PyCLOCK	24 hr, with 13-19
cWGF13	Fmoc-βAla-OH, Wang	Aapptec Automated Synthesis	Solution-Phase, PyCLOCK	8 hr with 12, 14-19
cWGF14-19	Fmoc-Asp(OAll)-OH, Rink	Aapptec Automated Synthesis	On-Resin, PyCLOCK	24 hr with 12, 13
cWGF20	Fmoc-β-homoalanine-OH, Wang	Aapptec Automated Synthesis	Solution Phase, PyCLOCK	8 hr, with 21-23
cWGF21-23	Fmoc-Asp(OAll)-OH, Rink	Aapptec Automated Synthesis	On-Resin, PyCLOCK	24 hr, with 20
cWGF24	Fmoc-β-homothreonine-OH, Wang	CEM Microwave Synthesis	Solution-Phase, PyCLOCK	8 hr
cWGF26	Fmoc-Asp(OAll)-OH, Wang	CEM Microwave Synthesis	On-Resin, PyCLOCK	24 hr
bcWGF1-8	Fmoc-Asp(ODmab)-OH, Rink	Aapptec Automated Synthesis	On-Resin, PyCLOCK	24/48 hr
bcWGF9	Fmoc-Asp(ODmab)-OH, Wang	CEM Microwave Synthesis	On-Resin, PyCLOCK	24/48 hr

Table 2: Summary of synthetic MMP inhibitors. Peptides have been synthesized via a number of methods, referred to in the text. For cyclic peptides, cyclization time is in "notes" along with other peptides synthesized in the same parallel automated synthesis . Cyclization and bicyclization times for cyclic peptides are separated by slashes.

In Vitro MMP Assays:

MMP activity was measured using a FRET assay. An MMP-2 cleavable substrate from the Anaspec Sensolyte® 520 MMP-2 Assay kit was used. This substrate is cleavable by recombinant MMP-2, and has 5-FAM (fluorophore) and QXL520™ (quencher) groups on either end. The degree of fluorescence is relative to the activity of MMP-2. This is incubated with a synthetic MMP inhibitor peptide along with activated MMP-2.

MMP-2 is activated by incubating 50ug/mL enzyme with 0.5mM 4-Aminophenyl Mercuric Acetate (APMA). APMA is prepared by adding 0.5 µL of 100 mM APMA in DMSO into 49.5 µL of 0.1 M NaOH, then diluted 1:10 in the assay buffer (50 mM Tris (pH 7.6), 150 mM NaC1, 5 mM $CaC1_2$, 1 µM $ZnC1_2$ 0.15% Brij 35).[62] A 5 ug/mL stock (activation solution diluted 1:10) is added to the assay. Equal volumes of this final dilution of APMA and MMP-2 were combined and incubated at 37^0C for one hour. Inhibitors are prepared by diluting from 10 mM pure DMSO stocks to 0.5 mM 5% DMSO stocks, a 1:20 dilution, in assay buffer.



Scheme 3: Sensolyte™ MMP-2 assay. Cleavage of linker by MMP-2 releases quenched fluoresceine, resulting in fluorescence. Inhibition of MMP-2 results in little cleavage, so less fluorescence. Therefore, activity of MMP is proportional to fluorescence. Assays for MMP-1, MMP-14, and MMP-19 all follow a similar schematic, but with different substrates.

A number of inhibitors were tested at high concentrations against a fixed concentration of MMP-2. First, an inhibitor (200 µM in assay) and activated MMP-2 (0.2 µg/mL) are added to the assay, with the volume adjusted to 20µL total. These members are pre-incubated for fifteen minutes before addition of the substrate. The stock solution of substrate is diluted 1:100 in assay buffer, and 20uL is added to the assay to make the final concentration of inhibitor and MMP-2 100 µM and 0.1 µg/mL respectively. Positive and negative controls include the disulfide-bridged peptide CTT and the linear analogue STT. Measurements of 100% MMP activity are measured with no inhibitor, which requires 12µL of buffer and 8µL of activated MMP-2 before addition of the substrate. Measurements of 0% MMP activity are wells with the substrate alone.

This assay is repeated three days in a row, and measurements are scaled according to the 0% and 100% data points taken on each individual day to yield relative MMP activity data. These data are

then represented on a bar graph. Measurements were taken using a Tecan® Plate Reader and an excitation wavelength of 490 nm and emission wavelength of 525 nm. The level of fluorescence is directly proportional to the relative activity of MMP-2. Results form each reaction are normalized to 100% and 0% MMP-2 activity, based on the controls.

Activity Assays of other MMPs:

Preparation of MMP-9 and MMP-1 included dilution of 1 µg of enzyme into 100µL of assay buffer (same buffer as above). These enzymes are catalytic domains alone, so there is no need to activate them with APMA. MMP-14 obtained from Anaspec is activated with APMA for one hour using the same procedure as above for MMP-2. In a 384-well plate, 8µL of 500 µM inhibitor (in 5% DMSO Assay buffer solution) is added in duplicate. 8µL of 1:10 diluted enzyme is also added, along with 4µL of assay buffer. After 15 minutes of pre-incubation of enzyme and inhibitor, 20µL of substrate (diluted 1:100 in assay buffer) is added to each well. The substrate is specific to each enzyme being tested. Measurements are scaled to the 100% and 0% MMP activity to obtain a normalized relative activity measurement.

Titration Assay:

A separate assay is run to determine the IC_{50} of synthesized inhibitors. In this assay, a single inhibitor is tested at a number of concentrations. First, a set of three wells are set up with double the normal volumes as in a high-concentration assay (so a total of 40µL, with 16µL of enzyme and 16µL of inhibitor). Nine adjacent wells are set up with 8µL of diluted MMP and 12µL of assay buffer. Then, the concentrated inhibitor is serially diluted 1:2 in each adjacent well to create an assay ranging from 100 µM to 9.8 nM. This array is preincubated for 15 minutes at 37^0C. Then, 20µL of diluted substrate is added to each well under low direct light. The fluorescence intensity is measured after one and two hours of incubation at 37^0C using the same methods as described above. Measurements are scaled to the 100% and 0% MMP activity to obtain a normalized relative activity measurement.

Serum Stability Assays:

In this assay, the stability of peptides in human serum was tested. The peptides test were normalized to a final concentration of 0.587mg/mL, and 6.25 µL was added to 243.75 µL 10% sterile PBS in human serum (pH 7.2). The final concentration of DMSO in solution was 2.5%. Each reaction was performed in triplicate. After addition of peptide to the serum, and initiation of proteolysis, 50 µL of solution is removed and quenched with 200 µL of ethanol. This is spun down using a tabletop centrifuge at 13,200 RPM for ten minutes. The remaining serum was placed in the 37^0C incubator. Time points were taken at 1, 5, 29, and 50 hours. Degradation of the peptide was monitored using Analytical RP-HPLC.[63] 100 µL peptide was injected onto the HPLC for each reaction run.

Data Analysis:

Microsoft Excel is used to process and graph all data. Kaleidagraph™ is used to calculate IC_{50}curves. For molecular modeling, Molecular Operating Environment (MOE) was used, and the PDB file 2OVZ was used. The MMP inhibitors synthesized were tethered into MMP-9 in a similar place where the aromatic groups from the phosphinate inhibitor are docked. Chemical structures were drawn using Chemsketch to first draw all cyclic peptides, then the structures were imported and edited in ChemDraw. Figures were formatted using Microsoft Powerpoint.

Results and Discussion

Series 1: Testing Macrocycle Length and Stereochemistry

Eleven cyclic peptides were synthesized using the methods described above, and made up the first cWGF series of CTT mimics. They are listed in Figure 6. The aim of this series of peptides was to modify the overall length and stereochemistry of the macrocycle in order to achieve an active conformation of the cyclic peptide. This was done by modifying the stereochemistry at the alanine in position 2. D-Alanine, L-Alanine, and Glycine (elimination of stereochemistry) were used to attempt to preserve the position of the N-terminus in CTT. The macrocycle size was altered by substituting Glycine, β-Alanine (β), or γ-amminobutyric acid (γ) in the first position.

Peptide	n	*Asn*	Stereochemistry
cWGF1	1	*n-*	L
cWGF2	2	*n-*	L
cWGF3	3	*n-*	L
cWGF4	1	*n-*	D
cWGF5	2	*n-*	D
cWGF6	3	*n-*	D
cWGF7	2	*i-*	L
cWGF8	3	*i-*	L
cWGF9	2	*i-*	Gly
cWGF10	N/A	*n-*	L-Pro-Gly
cWGF11	N/A	*n-*	D-Pro-Gly

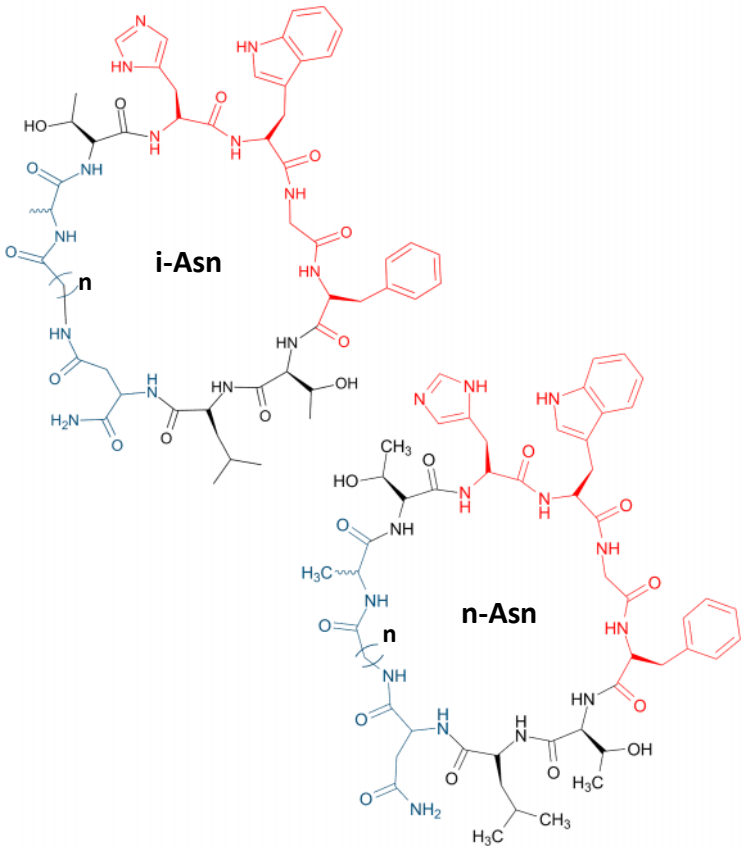


Figure 6: Plan for the iterative design of cWGF1 through cWGF11. Focus is on the linker region (blue), where the stereochemistry of the linker as well as the overall macrocycle length were modified using different amino acids. Also incorporated were sidechain and C-terminus cyclization methods, resulting in either a normal Asn sidechain or a carboxamide group.

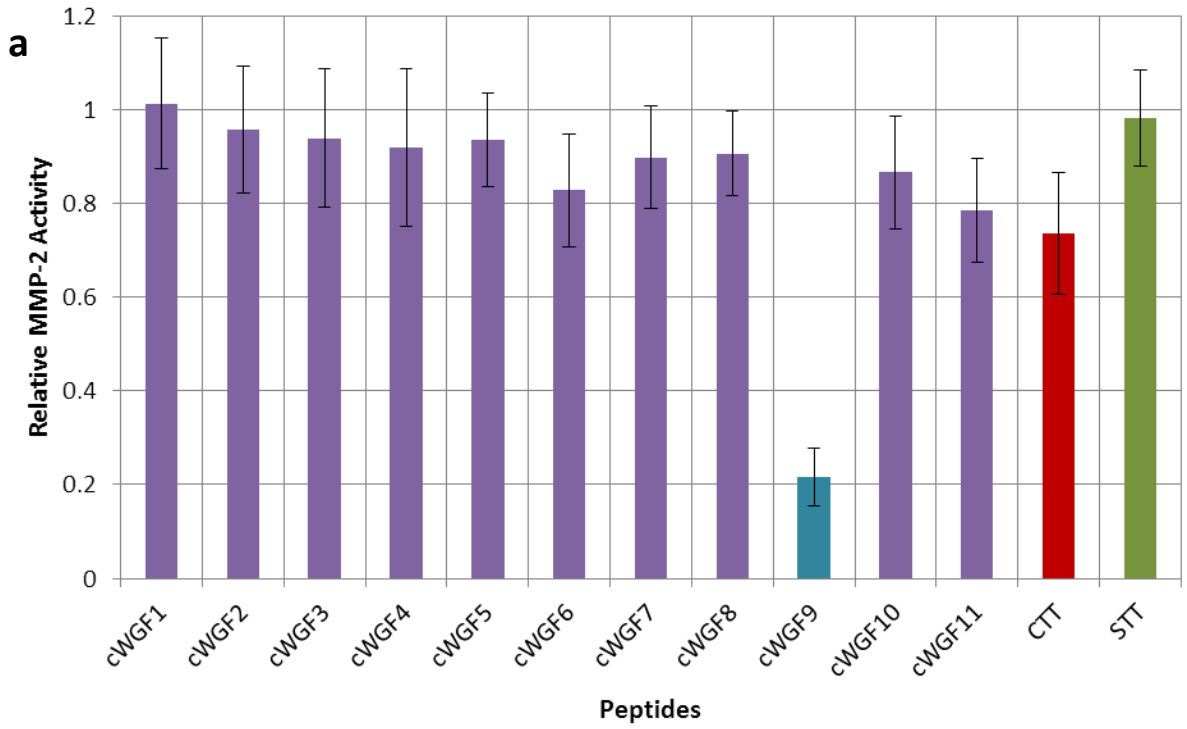
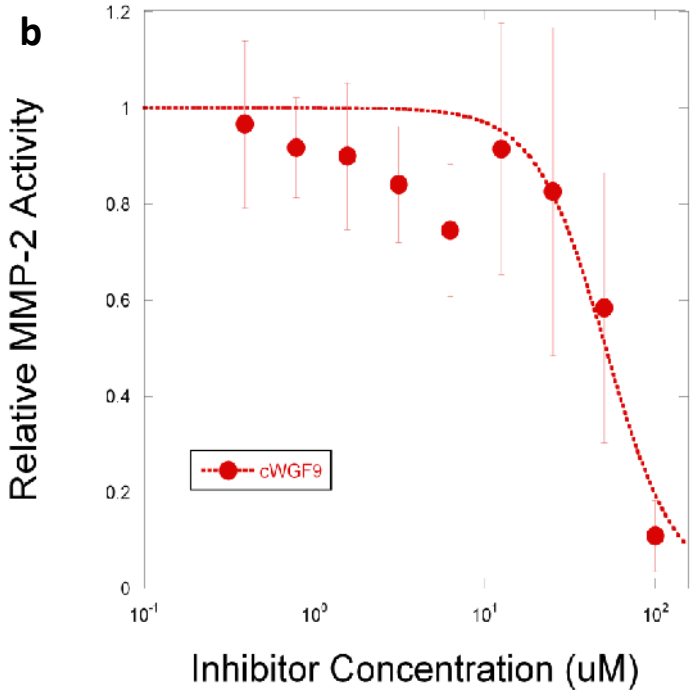


Figure 7: (**a)** Relative inhibitory activity of first-generation CTT analogues. cWGF9 showed significant improvement over original peptides, and will be the lead compound in this study. (**b**) Titrations of cWGF9 calculate a roughly 50 µM IC_{50} against MMP-2. CTT was too ineffective against MMP-2 in this context to calculate IC_{50} curves, but cWGF9 appears four times more effective than its parent compound.

cWGF7-9 were synthesized with a sidechain to head cyclization rather than head to tail. This increases the size of the macrocycle by one methylene unit. Also, this different method of cyclization results in a sidechain with one fewer methylene unit. cWGF10 and cWGF11 are peptides with Proline-Glycine motifs, which have been shown to promote β-turns. These peptides



were synthesized to rigidify the linker and sample a different conformational space.

The results showed that cWGF9 is a cyclic peptide inhibitor of Gelatinases (Figure 7). This peptide had an IC_{50} of 51 µM against MMP-2. It shows a roughly fourfold improvement over the measured IC_{50} of CTT. No other peptide in the initial series was able to inhibit MMP-2 to a degree greater than CTT.

This suggests that the recognition motif, HWGF, is highly sensitive to small changes in macrocycle size and stereochemistry of the linker. There were several distinct structural features of cWGF9. First, elimination of stereochemistry at the Alanine position suggests that the linker must be flexible. Substitution of either D or L Alanine at that position restricts the Ramaschandran angles such that the correct conformation cannot be achieved. The iso-Asparagine moiety incorporated both a longer macrocycle size and a carboxamide group. Further series should test the importance of the iso-

Peptide	Modification	Testing
cWGF21	G6→A	Sterics around catalytic domain
cWGF22	G6→a	Sterics around catalytic domain
cWGF23	H4→A	Necessity of His

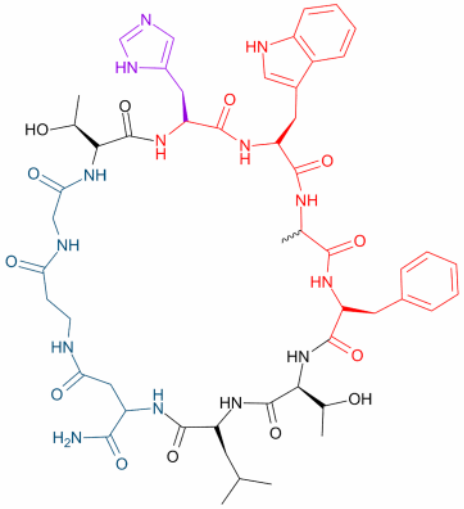


Figure 8: Synthetic plan for Series 2. Here, the attributes of the HWGF motif are tested. The recognition motif Glycine is substituted for L and D Alanine (grey), and His is substituted to Ala (purple).

Asparagine group and attempt to determine its function in the cyclic peptide.

Series 2: Probing Active-Site Structure-Activity Relationships

The first SAR series attempted to confirm elements of the SAR performed with the original CTT peptide in the context of cWGF9. cWGF21 and 22 replaced the active-site Glycine with L-Alanine and D-Alanine, and cWGF22 replaced the His with Alanine (Figure 8). The results of this series showed that the recognition motif cannot tolerate L-Alanine, but D-Alanine is mildly tolerated, and cWGF9's activity is recovered. Substitution of Histidine to Alanine results in a peptide whose activity is similar to that of CTT (See Figure 10).

The fact that Histidine is important in this context is interesting, and suggests that this residue may be involved in possibly chelating the Zinc, or forming a separate hydrogen bond intramolecularly or

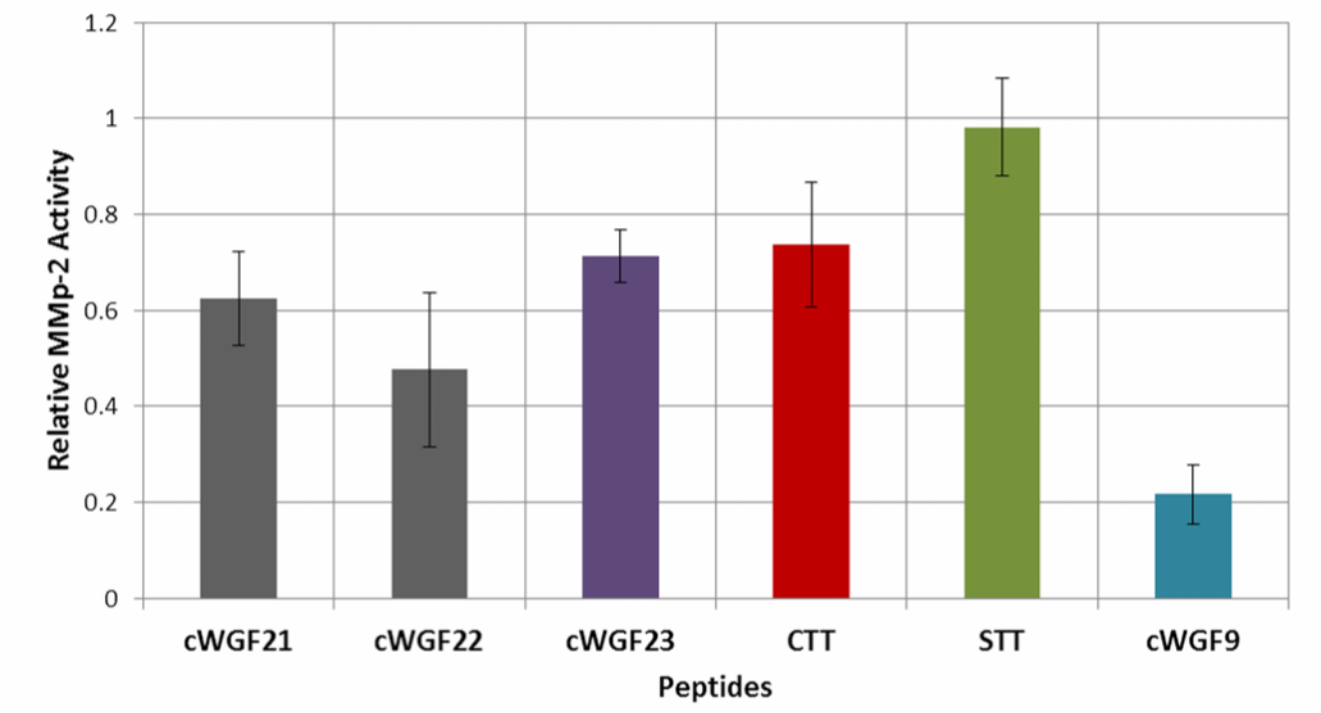


Figure 9: The second series attempted to examine elements of the conserved HWGF sequence. cWGF22 is mildly active against MMP-2, though not to the level of cWGF9 (grey). cWGF23 is minimally active, roughly to the level of CTT (purple).

to another site on the protein. In the original SAR study for CTT, the Histidine was unimportant to function. The importance of Histidine in this context suggests that cWGF9 may inhibit MMPs in an entirely different manner than CTT.

The substitution of L- and D- Alanine into the active site was to test to see how addition of steric bulk into the recognition motif affects its ability to achieve the correct conformation. Substitution with D-Alanine did not impair inhibitory activity as much as with L-Alanine, which suggests one of two things. First, the recognition motif may be able to still bind to Gelatinases with the additional bulk from a D-Alanine. Alternatively, the conformation of the methylene unit in the recognition-motif Glycine is such that it can accommodate the Ramaschandran angles of a D- substation, but not an L- substitution. This again shows that the conformation of the overall peptide is highly sensitive to change.

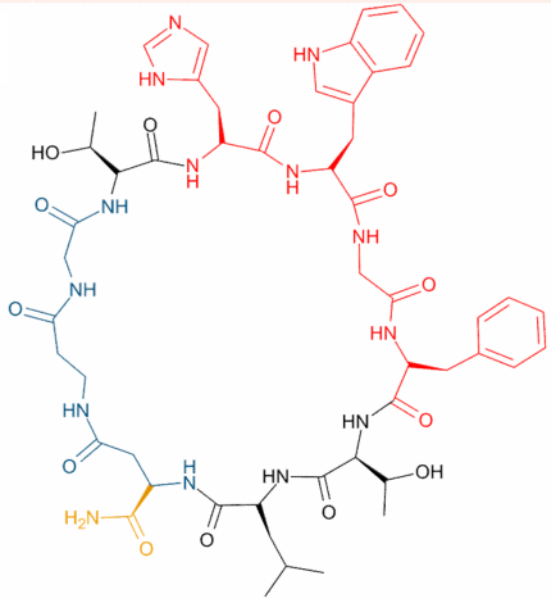
Series 3: Testing the Importance of the carboxamide group on the peptide

The second SAR series is centered on the role of the iso-Asparagine in cWGF9's function (Figure 10). The peptide cWGF12 uses a normal Asparagine sidechain. This is to test an Asparagine peptide with an elimination of stereochemistry at the 2 position. cWGF13 uses β-Alanine to eliminate the sidechain entirely and test overall macrocycle length, and cWGF20 added a methyl group. This peptide was designed to test the necessity of the carboxamide group while keeping the Ramaschandran angles the same. In addition, three peptides were synthesized to measure the function of the carboxamide group. cWGF24 replaces the carboxamide with a threonine sidechain, to test for the importance of hydrogen bond donation, cWGF25 replaces it with an isopropyl group to test steric bulk, and cWGF26 replaces it with a carboxylate to test to see if the importance is as a hydrogen bond acceptor or as a polar group.

a

Peptide	Modification	iAsn Sidechain	Testing
cWGF12	iN→N	Asparagine (-1 macrocycle)	Importance of Carboxamide, Macrocycle Size
cWGF13	iN→β	No sidechain	Importance of macrocycle size
cWGF20	iN→β³A	Methyl	Sterics of iAsn position
cWGF24*	iN→β³T	1-hydroxyethyl	Hydrogen bond donor
cWGF25*	iN→β³V	isopropyl	Sterics
cWGF26	iN→iD	Carboxylate	Hydrogen Bond Acceptor

b



c

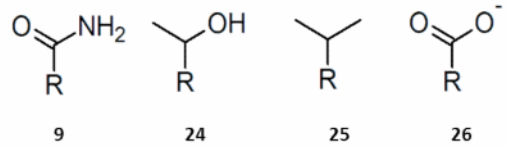


Figure 10: Peptides synthesized for series 3, to investigate the purpose of the iso-asparagine in cWGF9 function (**a** and **b**). Peptides synthesized were designed to simulate hydrogen bonding, sterics, and electronics (**c**).

The results of this series show that the carboxamide is not only important to the inhibition of Gelatinases, but it may have a specific structural function (See Figure 11). Substitution of Asparagine rather than iso-Asparagine decreased activity, which shows that the iso-Asparagine is an important element of cWGF9's inhibitory activity. Elimination of the sidechain in cWGF13 as well as substitution of the carboxamide functionality with a methyl resulted in little inhibitory activity, which suggests that the carboxamide group contributes to the function of cWGF9. cWGF24 also did not show inhibitory activity. This substitution of the carboxamide with beta-homothreonine resulted in loss of activity suggests that the carboxamide group may not act as a hydrogen-bond donor. cWGF26 was nearly as effective an inhibitor of MMP-2 as cWGF9 at 58 µM. Carboxylate substitution resulted in increased activity. This

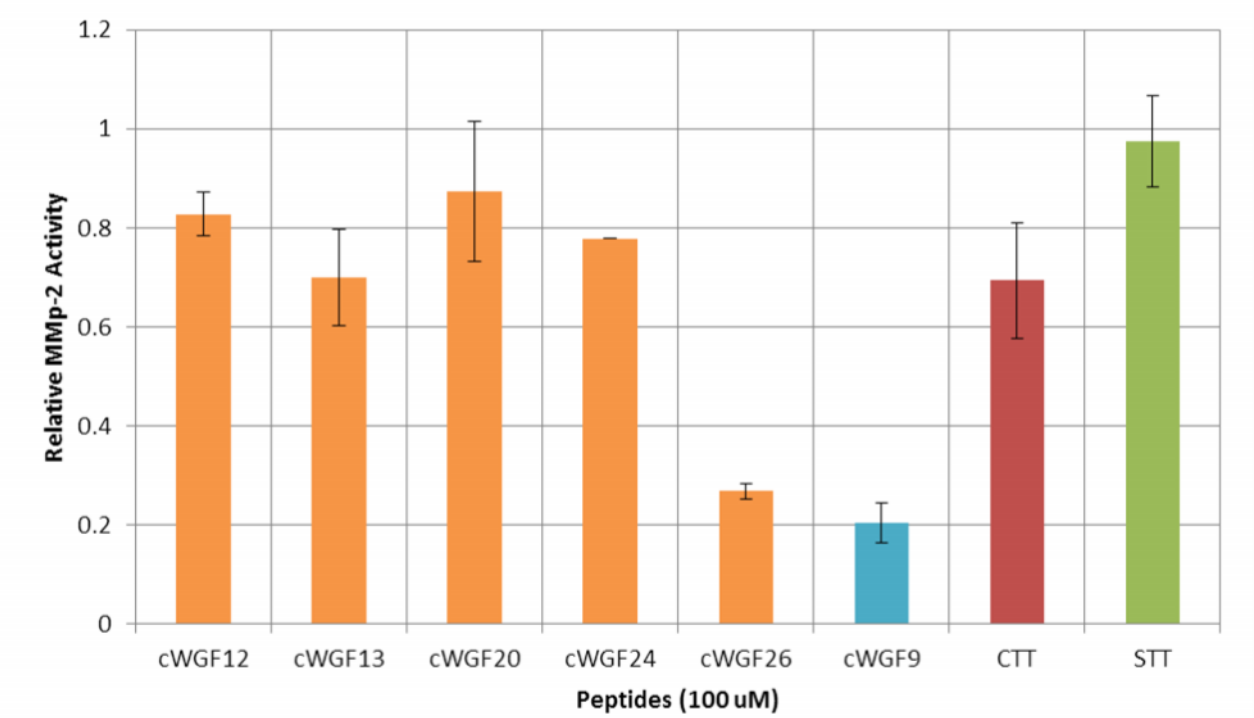


Figure 11: Results for series 3. cWGF26 was synthesized to a poor yield and is at a concentration of 58 µM. Substitution of the carboxamide group with a number of functionalities had a number of consequences on cWGF9's affinity for Gelatinases. Results from cWGF24 suggest that hydrogen bonding may not be the primary purpose of the carboxamide, but as evidenced by cWGF26, the substitution of a carboxylic acid may increase the efficacy of cWGF9.

suggests that the carboxamide is necessary as either a hydrogen bond acceptor or as a polar group. Substitution to a carboxylate increased its ability to perform both of these functions.

Series 4: Lysine Scan to Determine Bicyclization Sites

Bicyclization of peptides is an effective way to gain selectivity and affinity. The previous results have showed that the conformation of cWGF9 inhibition is highly specific: small changes in amino acid composition or macrocycle size result in drastically decreased affinity. By rigidifying the peptide using cross-links, we can lock the peptide in an active conformation. This would increase affinity, because the entropic cost of binding to MMPs would be much less: in bicyclization, we are paying the entropic penalty to inhibition up front. Also, rigidifying the linker could increase the selectivity of the bicyclic to Gelatinases, but disallowing it to relax into a conformation where it could be promiscuous to other

Peptide	Modification	Linker Region
cWGF14	T3→K	βG
cWGF15	T3→k	βG
cWGF16	T8→K	βG
cWGF17	T8→k	βG
cWGF18	L9→K	βA
cWGF19	L9→k	βG

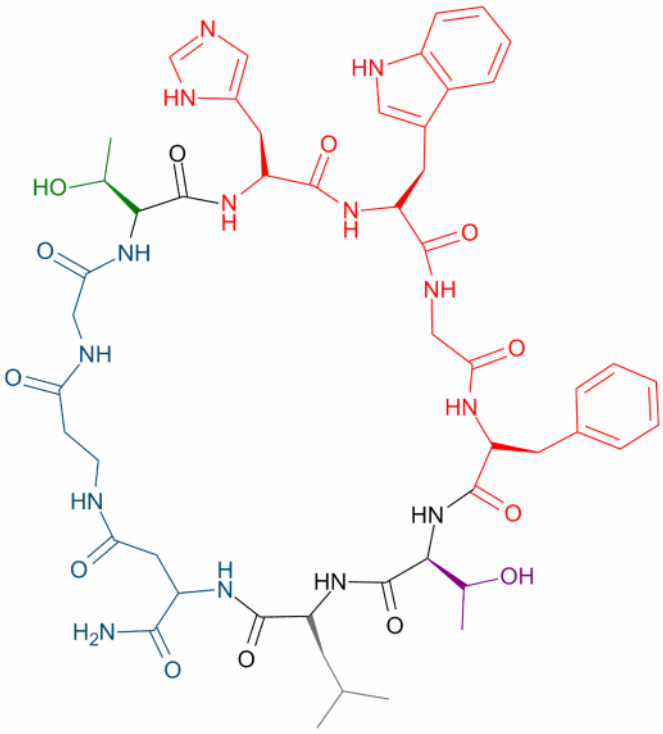
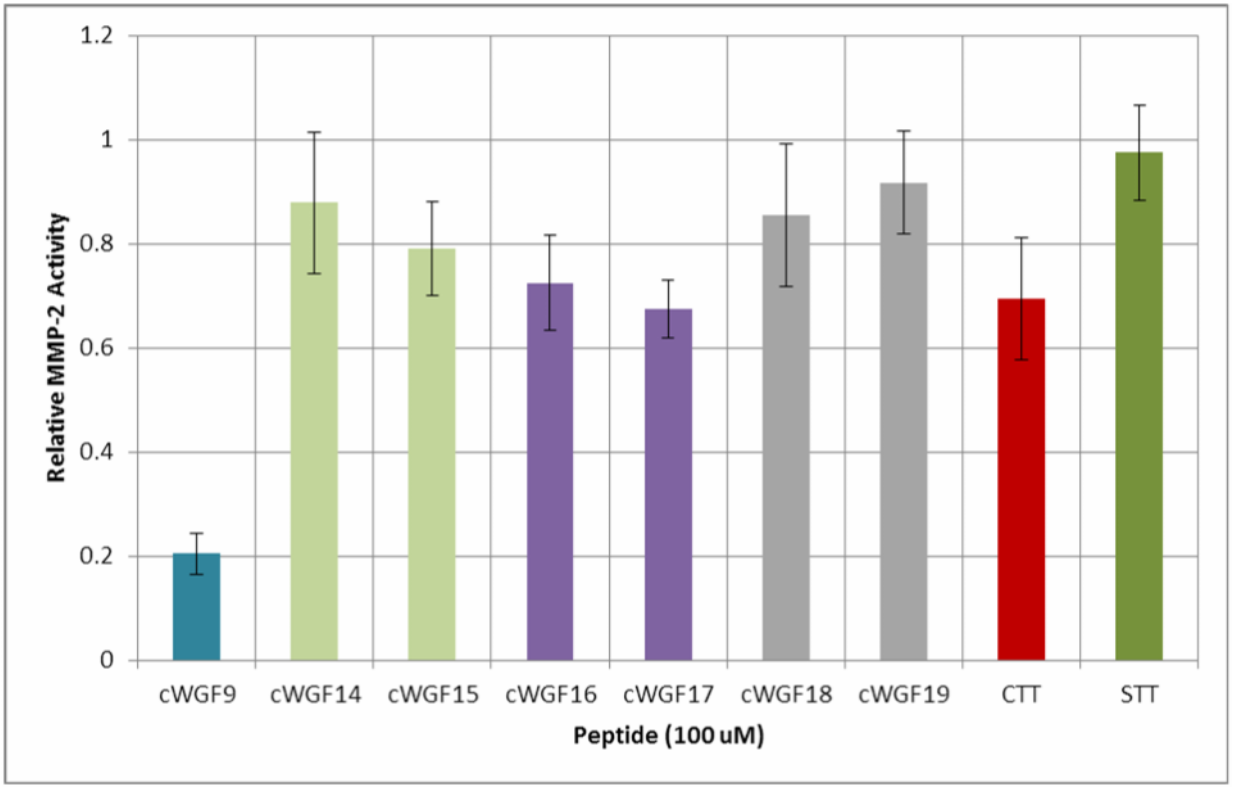


Figure 12: (above) To discover potential bicyclizatoin sites on cWGF9, nonessential residues were substituted with L-Lysine or D-Lysine. The residues substituted were Threonine-3 (green), Threonine-8 (purple), and Leucine 9 (grey). (Below) Substitution of T3 with either D or L Lysine, and T8 with D-Lysine resulted in cyclic peptides which had activities at or above the level of CTT.



MMPs. Finally, bicyclization would theoretically improve serum stability of the tested peptides, which would increase their lifetime *in vivo*.

There are few residues on cWGF9 that have not already been shown explicitly to be absolutely essential for inhibitory activity. The residues which appear to not be as crucial to function will be the sites to produce bicyclic peptides. These residues were substituted with Lysine in order to incorporate a long, bulky polar group that would mimic the

A scan of nonessential positions on cWGF9 had to be undertaken in order to synthesize bicyclic peptides. Residues were substituted with Lysine, because a position that could tolerate the addition of a long polar group would be a potential site to bicyclize. In this series, cWGF14-19 were different substitutions of the two Threonines and Leucine to both L- and D- Lysine (Figure 12). Macrocycle length and all other residues on the peptide were unchanged.

Substitution of the Threonine in the third position to D-Lysine and substitution of Threonine in the eighth position with both L-Lysine and D-Lysine yielded peptides that had roughly the same inhibitory activity against MMP-2 as CTT, though were significantly worse than cWGF9 (Figure 13.) Because substitutions at these positions were the least detrimental to function, they were the sites where the peptides were bicyclized.

This study may have several confounding factors. The use of Lysine as a mimic for a bicyclization linker may be flawed. The positive charge on lysine could result in peptides which would be different than the desired pre-bicyclic probes. In the future, acylated lysine should be used to mimic the lactam better.

Peptide	m	n	3 -D or L	Orientation
bcWGF1	1	2	D	A
bcWGF2	1	2	D	B
bcWGF3	2	2	D	A
bcWGF4	2	2	D	B
bcWGF5	1	2	L	A
bcWGF6	1	2	L	B
bcWGF7	2	2	L	A
bcWGF8	2	2	L	B

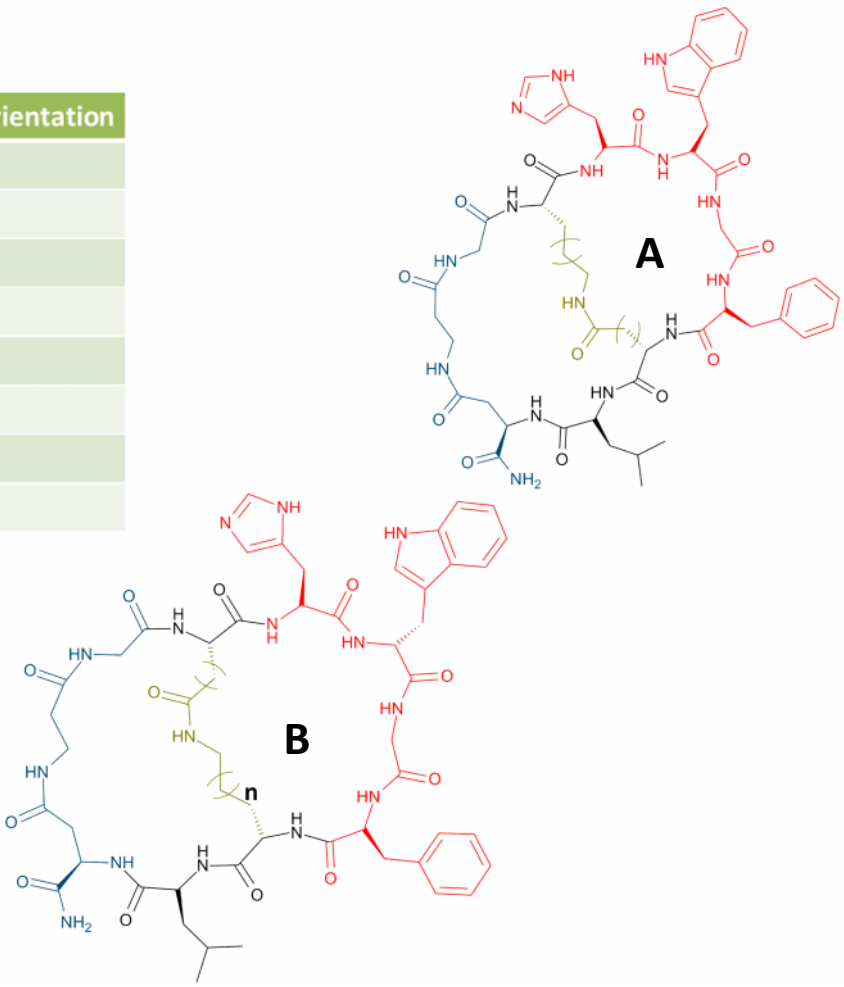


Figure 13: Design for a first series of bicyclic inhibitors of MMPs. Different linker lengths and stereochemistries (green) were scanned in order to find the optimal conformation for inhibition. Design is based on Lysine scan results, where position 3 can be either L or D, and position 8 is always D stereochemistry.

Series 5: Bicyclic Analogues of cWGF9

The first series of bicyclic peptide analogues of cWGF9 use the data from the fourth cyclic series (Figure 13). Aspartate, Glutamate, and Lysine in both D and L stereochemistries were used to synthesize the peptides in this series. The goal of the series was to test a series of peptides where the cross-linker length as well as the stereochemistry was sampled. In addition, the positions of the acid and amide were switched to account for both conformational differences of switching as well as the possibility of the lactam participating in inhibition of Gelatinases. These compounds are described in Figure 14.

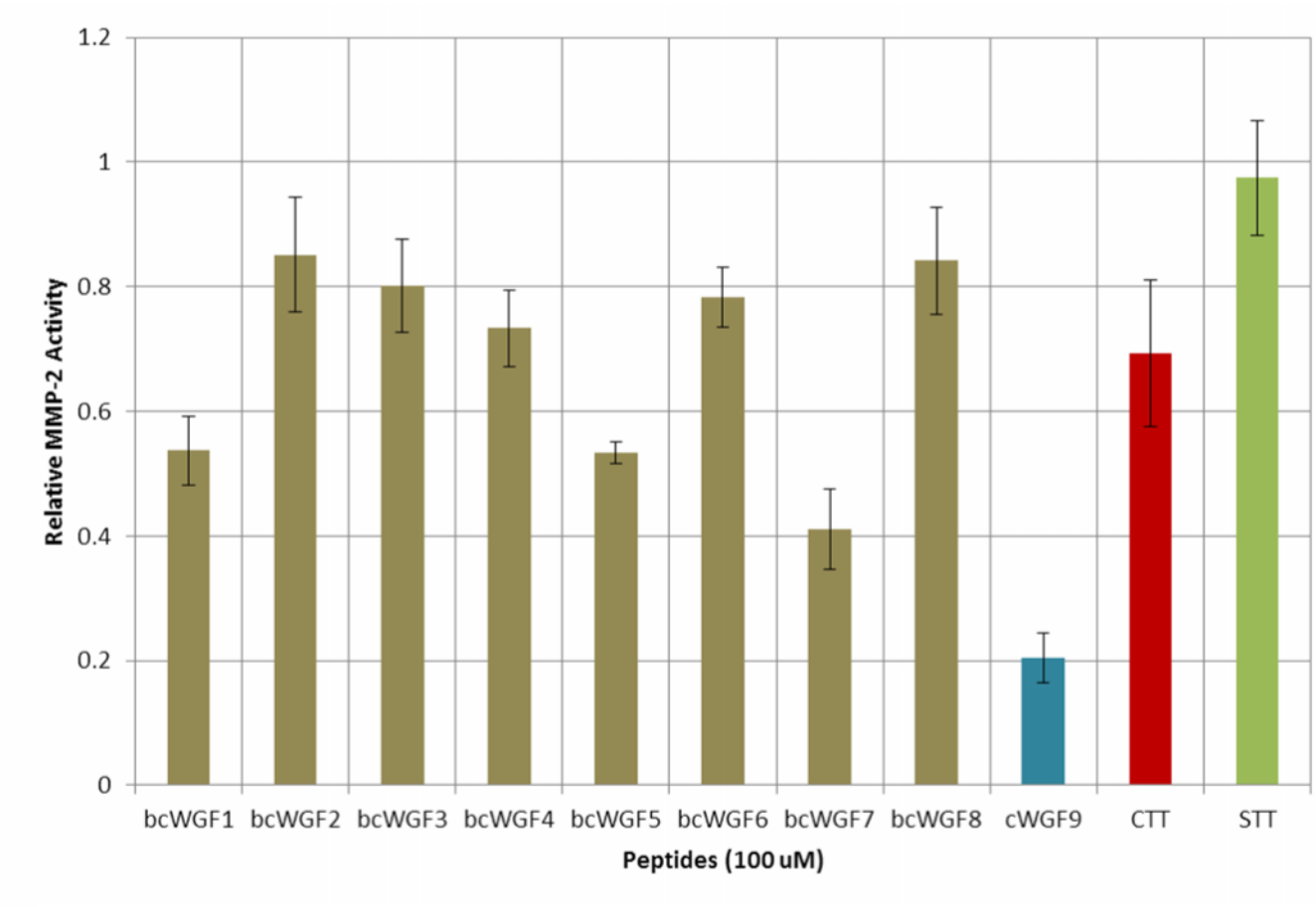


Figure 14: Results for bicyclized analogues of cWGF9. Here, bcWGF, bcWGF5, and bcWGF7 all show increased affinity for MMP-2 compared to CTT. bcWGF7 is the most improved peptide at 100 µM, and will be the basis for improvement in bicyclic peptides.

bcWGF7 was the bicyclic analogue which had the greatest affinity for MMP-2. This bicycle is linked via a D-Glutamate and L-Lysine, is the most effective inhibitor of MMP-2 tested (Figure 14). This compound was able to lower MMP-2 activity to 35%, and has an IC_{50} value of 67.91 µM against MMP-9 (Figure 15). Other effective inhibitors included bcWGF1 and bcWGF5.

The bicyclized analogues could only tolerate a very specific cross-linker length, stereochemistry, and lactam position. This could imply specific contacts with the peptide or the protein by the lactam cross-linker. Further investigations of cross-linker structure could potentially lead to a more stabilized inhibitor of Gelatinases. This observation makes it necessary to investigate some of the structure-activity relationships uncovered in the context of cWGF9. Unlike the cyclic inhibitor, substitution to the

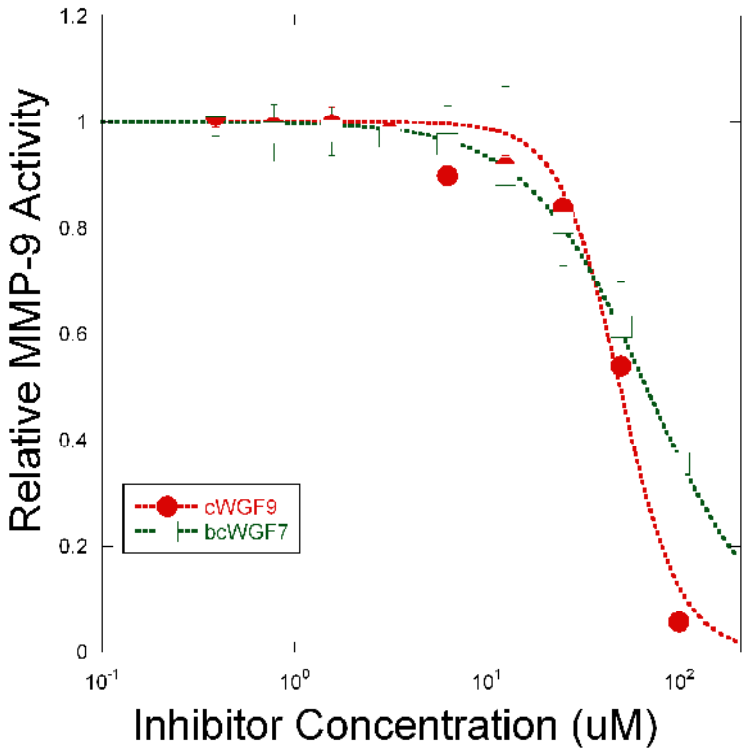
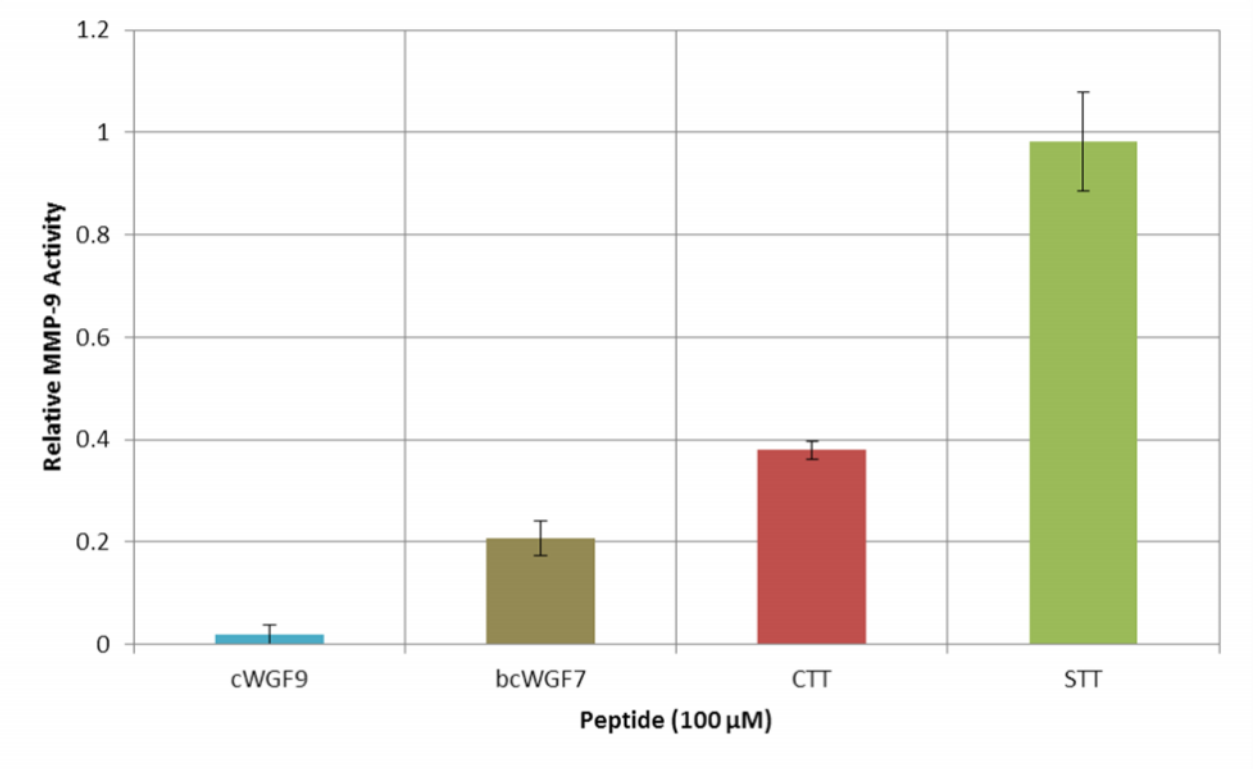


Figure 15: IC_{50} curves for bcWGF7 and cWGF9 against MMP-9. The $IC_{50}$of cWGF9 was 49.67uM and that of bcWGF7 was 67.91 µM. The IC_{50} of bcWGF7 against MMP-9 could not be calculated because its affinity was too low.

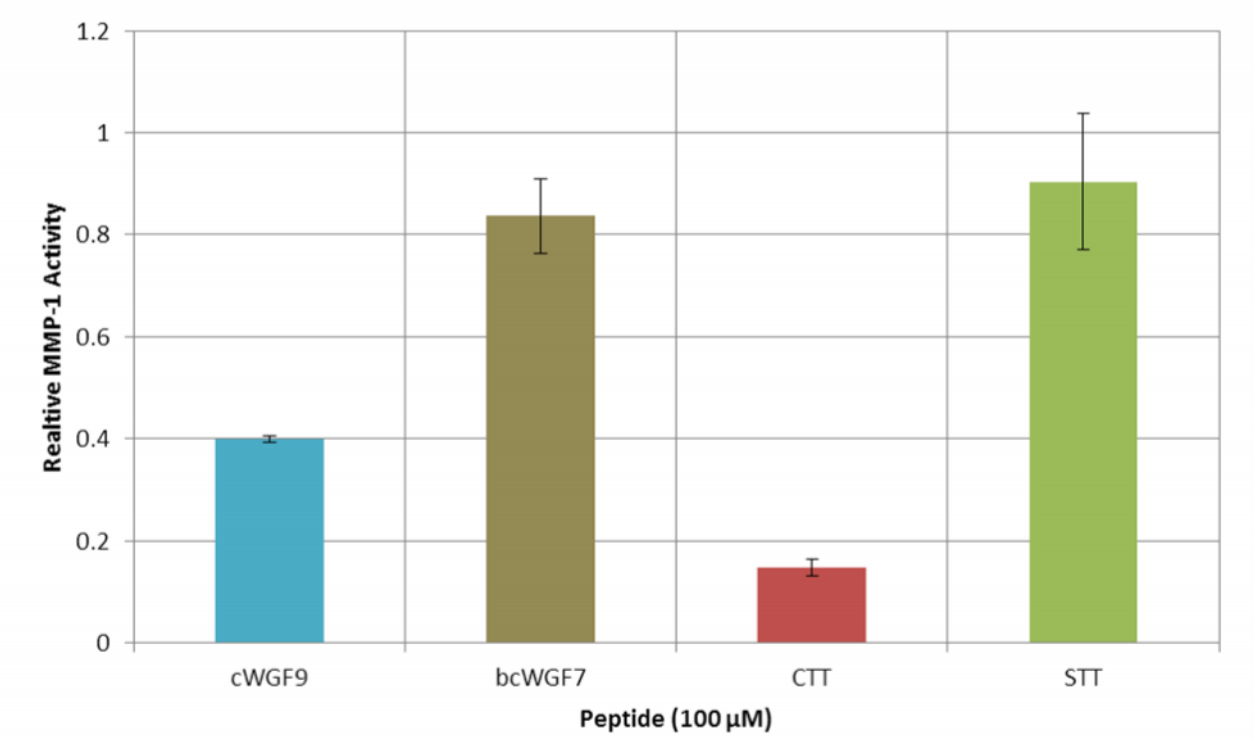
carboxylate had a very negative effect on inhibition of bcWGF7. This could be because of the cross-linker changing the conformation, or potentially detrimental interactions between the linker region and the cross-linker of bcWGF7.

Selectivity Studies:

Selectivity of CTT analogue peptides is an important aspect of their function. CTT was originally reported as a peptide that was selective for Gelatinases, and it is important that next-generation MMP therapeutics are highly selective. Using quenching assays for different MMPs, the selectivity of cWGF9 and bcWGF7 were measured. The selectivity of our inhibitors against MMP-9, MMP1, and MMP-14 were measured. Gelatinase B, or MMP-9, was the first reported target of CTT, and is the



Figures 16 and 17: (Above) Selectivity studies for synthesized Gelatinase inhibitors against MMP-9 (Gelatinase B). cWGF9 and bcWGF7 appear to be improved inhibitors of MMP-9 relative to CTT. In inhibition studies of Collagenase (MMP-1), bcWGF7 had decreased activity, while CTT and cWGF9 were mild inhibitors at 100 µM.



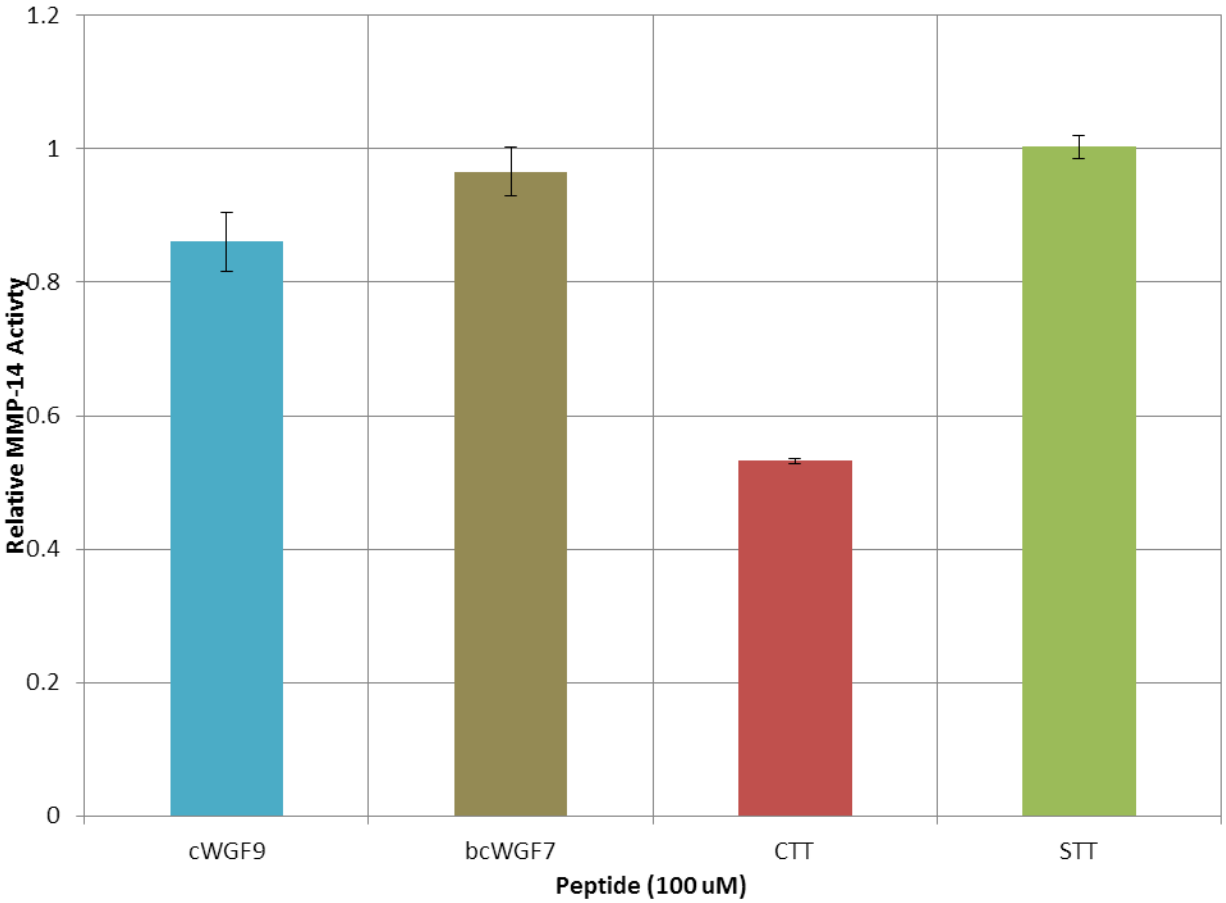


Figure 18: Selectivity studies of MMP-14 with effective Gelatinase inhibitors. A similar scene to inhibition of MMP-1 is seen, where CTT is a minor inhibitor, and bcWGF7 is a very poor inhibitor of MMP-14. These results suggest that bcWGF7 is a very selective inhibitor of Gelatinases.

enzyme which was reported to its target. The IC_{50} and maximal inhibition values for cWGF9 and bcWGF7 were lower against MMP-9 than they were against MMP-2 (See Figure 16). cWGF9 was able to completely inhibit MMP-9 activity at 100 µM,, and bcWGF7 was able to lower activity to less than 20%. The IC_{50} of cWGF9 was 49.9 µM, and of bcWGF7 was 67.91

Peptide (at 100 µM)	Activity of MMP-2	Activity of MMP-9	Activity of MMP-1	Activity of MMP-14
cWGF9	20.5±41%	1.9±1.8%	40.0±0.8%	86.0±4.4%
bcWGF7	41.0±6.5%	20.7±3.3%	83.7±7.3%	96.5±3.6%
CTT	67.6±13%	38.0±1.8%	14.8±1.7%	53.2±0.4%
STT	98.4±9.3%	98.2±9.8%	90.4±13%	100±1.7%

Table 3: Summary of selectivity data for CTT analogues. bcWGF7 is highly selective for MMP-2 and 9, while CTT and cWGF9 less so.

µM (Figure 15 and 16). The IC_{50} of CTT for MMP-9 could not be effectively measured because a curve could not be fitted to its titration.

The enzymes were also tested against MMP-1 (Interstitial collagenase), and it was found that bcWG7, the bicyclic analogue, had no inhibitory potency towards MMP-1, while the cyclic analogue and CTT were able to minimally inhibit it at 100 µM (Figure 17). Similar results were seen with MMP-14, where cWGF9 was a mild inhibitor, whereas bcWGF7 showed reduced affinity non-Gelatinase MMPs (Figure 18). CTT was shown to inhibit all the MMPs tested. These results are summarized in Table 3.

Series 6: Additional Modifications of bcWGF7

Previous data in the context of cWGF9 showed that substitution of a carboxylate group for the carboxamide may increase the affinity of the CTT analogue to MMP-2. bcWGF9 was synthesized using

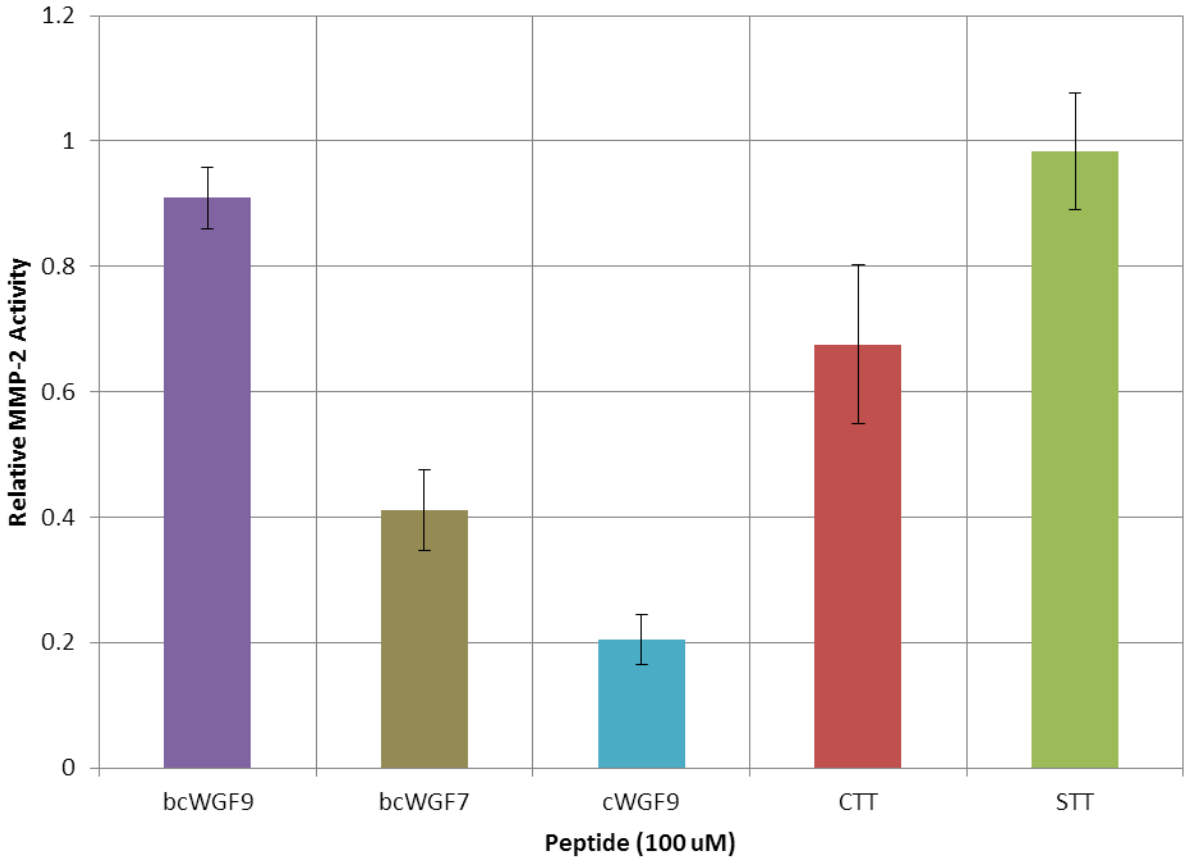


Figure 19: Substitution of the carboxamide with a carboxylic acid results in a bicyclic peptide with a reduced inhibitory potency for MMP-2.

Wang-loaded Aspartate to achieve the carboxylic acid functional group (See table 2). This peptide is designed to confirm the observation that a carboxylic acid increases the inhibitory activity of the CTT analogues tested.

The results suggest that, in the context of bicyclized inhibitors, substitution of a carboxylate group does not improve affinity (Figure 19). This result could be because of the cross-linker changing the conformation of the recognition motif such that the substitution of a carboxylic acid did not have the same positive effect on inhibitory potency. Alternatively, there could be potentially detrimental interactions between the linker region and the cross-linker of bcWGF7 with a carboxylic acid substitution.

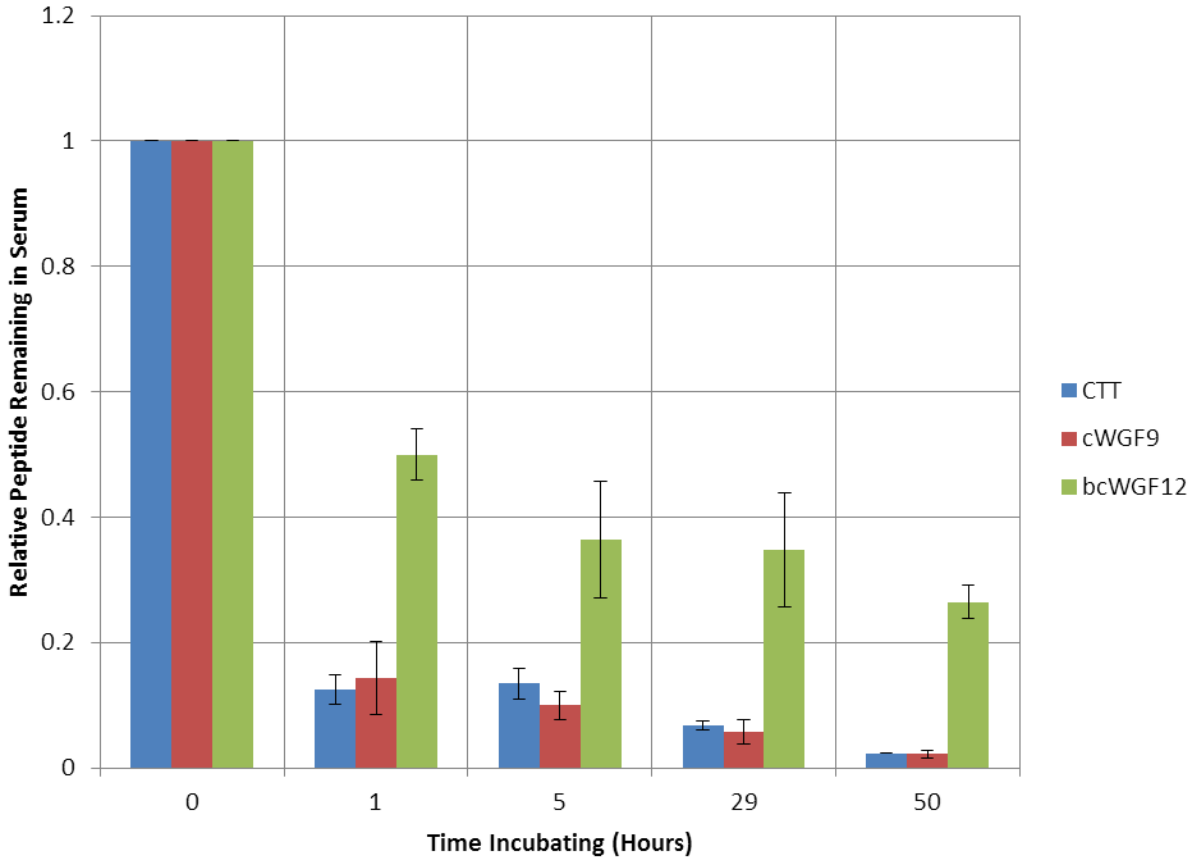


Figure 20: Serum Stability experiments show that bicyclic peptides are significantly more stable in serum.

Serum Stability of CTT Analogues

The stability of CTT analogues in human serum was also tested via incubation in human serum, then analytical RP-HPLC analysis. Results show that disulfide bridged peptides are degraded quickly in human serum. Cyclic analogues survive slightly better in serum, and bicyclic analogues have roughly 20% peptide remaining after 50 hours in serum (Figure 20). These results suggest that the bicyclized peptides are more serum stable than their cyclic or disulfide-bridged analogues.

An important factor of the efficacy of protease inhibitors is their serum stability: an inhibitor with a much longer lifetime will be much more effective. The result that bicyclic peptides are significantly more stable than their monocyclic or disulfide-bridged counterparts suggests that bcWGF7, while having a lower affinity to Gelatinases than cWGF9, could be an effective starting point for inhibiting cancer progression *in vivo*. Its longer lifetime in serum would result in lower doses having a greater inhibitory effect over time.

Future Directions:

These results suggest that *in vivo*, bcWGF7 could be effective at limiting tumor growth due to MMP expression. However, the lower efficacy of bcWGF7 compared with cWGF9 necessitates the further creation of a number of new bicyclic peptides, utilizing a number of new strategies and crosslinking chemistries. In addition, the creation of different bicyclic analogues may also shed light on the function of certain structural elements of bcWGF7.

All the collected data suggests that the inhibition of MMPs by CTT and CTT analogues is highly dependent on conformation. Small changes in the macrocycle length and conformation of the linker have large effects on inhibitory potency. The smaller affinity of bcWGF7 is likely due to the cross-link locking the peptide into a suboptimal configuration. In order to synthesize an effective bicyclic, several strategies should be taken. The lactam linker can be modified and tested further. It is the author's opinion that substitution with longer versions of lysine or glutamic acid will increase flexibility

of the linker, which may enable the bicycle to better access the conformational space occupied by cWGF9, but retain the same selectivity profile and serum stability of bcWGF7.

Previous studies of CTT's activity against MMP-9 have shown that substitution of 5-fluorotryptophan on CTT led to increased inhibitory potency. This is a simple modification to include in future studies, and could also improve cWGF9 as well as bcWGF7's inhibitory potency. Synthesis of this peptide would also lend evidence to this mode of inhibition being similar or different than the mode by which CTT inhibits Gelatinases. The data collected, especially the result that the Histidine is important for function in cWGF9, suggests that our cyclic analogues may have a different mode of binding.

Other methods of cyclization offer interesting alternatives to bcWGF7, and will be included in further rounds of iterative design. These altered bicyclic peptides would be able to access different conformational and electronic space in the cross-linker region, which could positively affect the conformation of the recognition motif. Currently olefin metathesis is being employed to synthesize olefin-containing bicyclic peptides.[64,65,66] The addition of an alkene group to the peptide would increase its hydrophobicity. Olefin metathesis has been used to both conformationally restrict as well as increase cell permeability of alpha-helix mimetics.[67,68] These bicyclic peptides would therefore be liposome soluble, like CTT. These peptides could also then be displayed on Liposomes to home to Gelatinase-producing tumor cells.

In addition, olefin-containing bicyclic peptides could test the necessity for a polar lactam linker, providing interesting structure-activity relationship data. To test this, a peptide with an olefin linker of the same size, and with the position of the olefin equivalent to the lactam position on the original bicyclic peptide should be synthesized. This peptide will hypothetically have the same macrocycle length and hybridization of bcWGF7. This peptide would test to see if bcWGF7's potency as an inhibitor has any relationship to the lactam group.

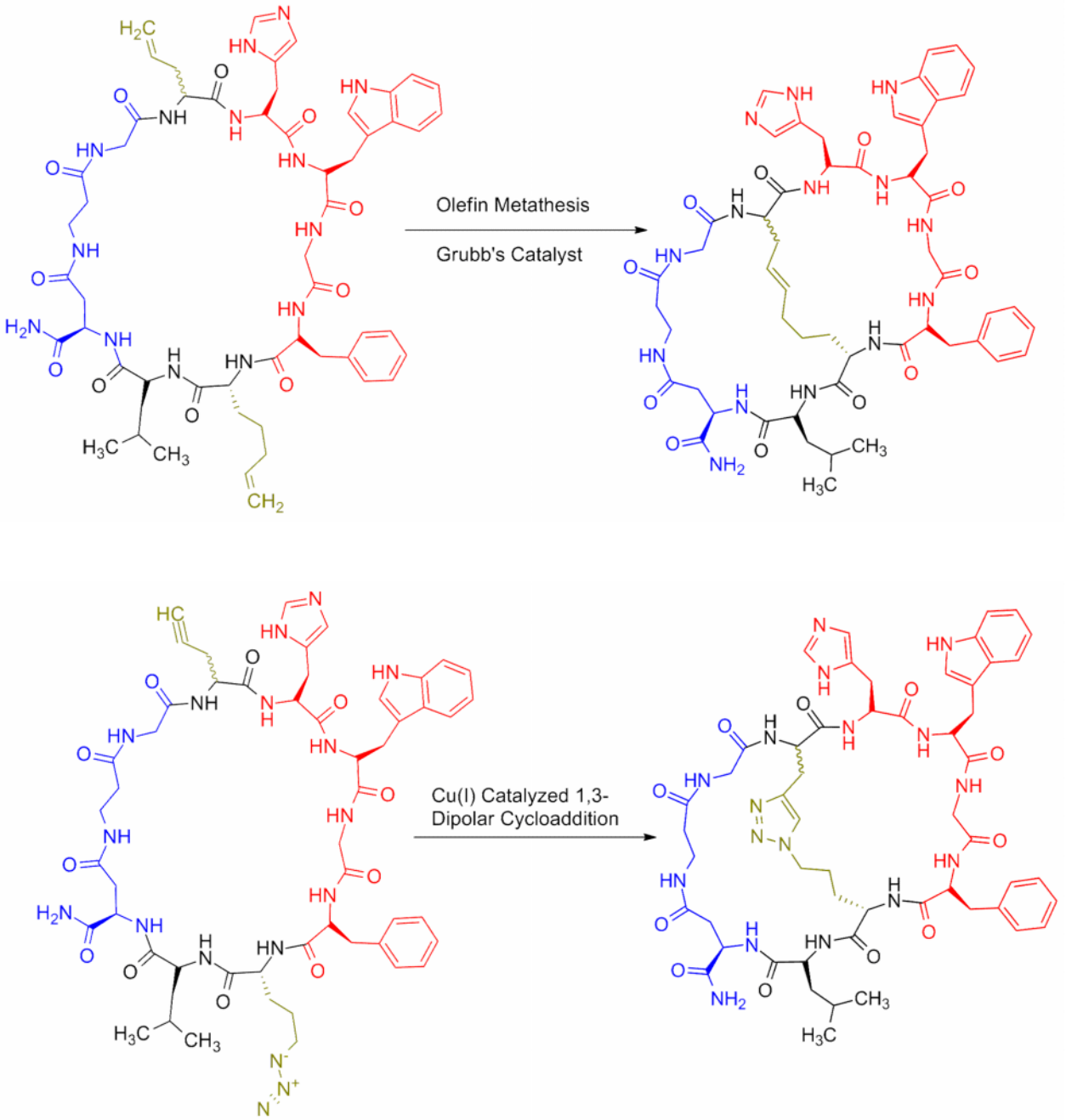


Figure 21: Alternate cyclization strategies will be used to access different conformational and chemical space in the cross-link (brown). Olefin metathesis and "Click" – Cu(1) catalyzed 1,3-dipolar cycloaddition chemistry have been proposed.

Click chemistry is a popular area of synthetic chemistry, and offers an interesting alternative to lactam-bridged cyclization. On-resin peptidic copper-catalyzed 1,3-dipolarcycloaddition has been used to synthesize cyclic and bicyclic peptides has been performed recently, and is a robust process.[69,70,71,72] The amino acids needed to perform this chemistry are also commercially available. This method could

be used to restrict the overall conformation of the cross-linker. Use of Ruthenium or Copper catalysts for this reaction can result in either 1,3 or 1,5 substitution, which offers versatility in producing a *cis* or *trans* peptide bond mimic in the highly conformationally sensitive crosslinking.

Determination of cWGF9's mode of binding

Determination of the mode of binding by CTT analogues is a problem that will likely require multiple different techniques to solve. 2D-NMR studies of effective bicyclic peptides can better elucidate their lowest-energy structures and better facilitate computational docking of these inhibitors into MMPs. Determination of the structure of these bicyclic decapeptides will also provide a glimpse into the role the backbone stabilization plays on conformation. In addition, NMR studies of the protein and the protein complexed with MMP-9 may provide interesting data on the exact location and effect that bcWGF7 is having on MMP-2 or MMP-9. NMR peaks of the protein that are shifted upon binding of the inhibitor suggest close contact with the bicyclic peptide.

In addition, x-ray crystallography is an option for determination of the binding of these peptides. Crystal formation and calculation of an x-ray crystal structure for Matrix Metalloproteinases is a well-established process. Collaboration with an experienced crystallography lab once a higher-potency inhibitor is synthesized could offer an accurate snapshot of the binding mode of these inhibitors.

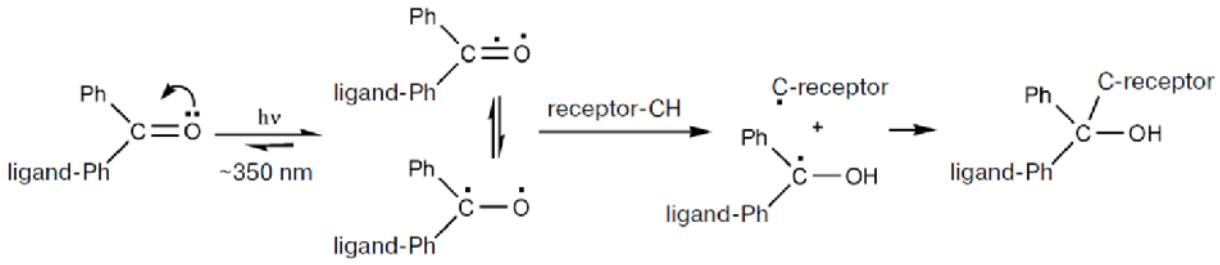


Figure 22: Mechanism of photocrosslinking to a protein (receptor) by benzophenone (ligand). Taken from reference 73.

Photocrosslinking is another area of potential study, where a photolabile group such as a benzophenone is irradiated in the presence of the enzyme. Exposure of a photocrossliking group to light results in formation of a radical. This radical can then form a C-C bond to the protein (See figure 22).[73] Trypsin digest and subsequent LC-MS/MS analysis can determine which amino acid the benzophenone binds to and determine the potential binding sites of bcWGF7. This can also help determine the binding mode of CTT analogue peptides.

Determination of an NMR structure as well as photocrosslinking studies can inform the lowest-energy conformation of the bicyclic peptide. This model can then be docked into an existing x-ray crystal structure of MMP-9 using docking or molecular modeling software. This can then provide an *in silico* model of the bicyclic binding to an MMP. .

Hypothesis of the binding mode:

Most small molecule Gelatinase inhibitors bind by chelating the zinc. Specificity to Gelatinases is given by inclusion of a large hydrophobic group to bind to the S2 specificity pocket of MMP-2 and MMP-9. This pocket in other MMPs is relatively shallow, so the large aromatic group prevents chelation of the zinc by steric clash of the aromatic group. A preliminary computer model was made of cWGF1 bound to

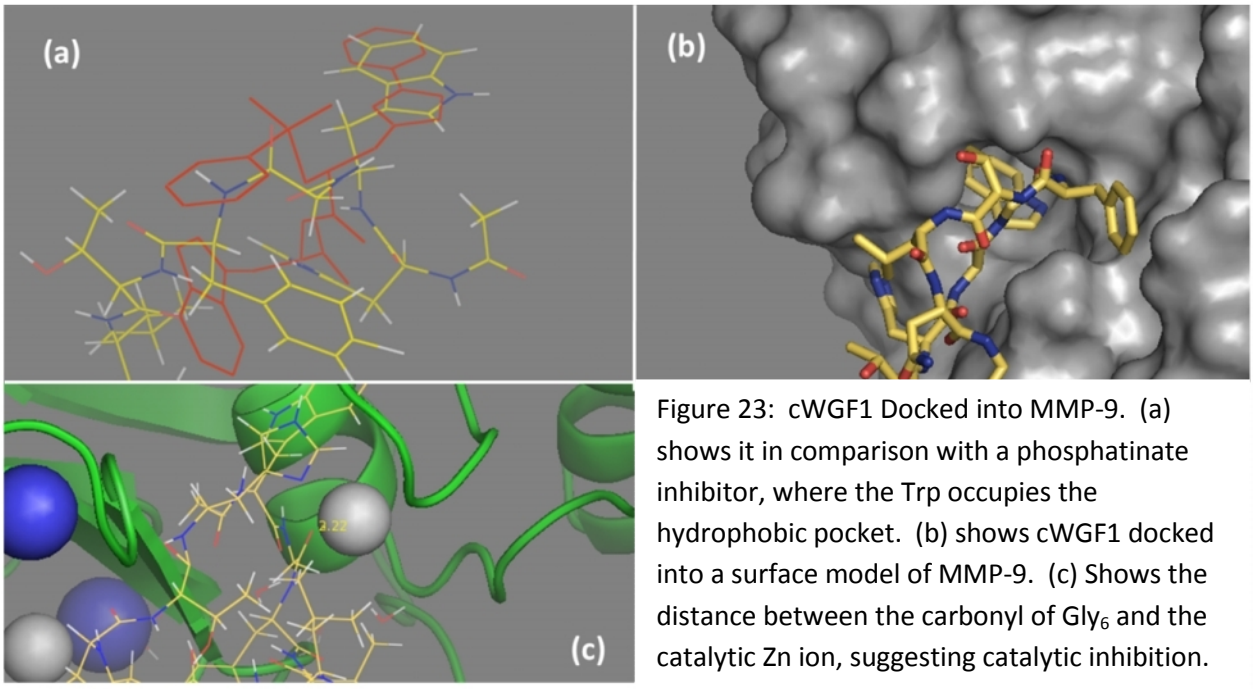


Figure 23: cWGF1 Docked into MMP-9. (a) shows it in comparison with a phosphatinate inhibitor, where the Trp occupies the hydrophobic pocket. (b) shows cWGF1 docked into a surface model of MMP-9. (c) Shows the distance between the carbonyl of Gly_6 and the catalytic Zn ion, suggesting catalytic inhibition.

MMP-9. This is a speculative model that offers only a hypothesis for binding.

An x-ray crystal structure of a peptidomimetic phosphinate inhibitor (resembling Tryptophan) was used as a template for an energy-minimized structure of cWGF1 peptide binding to MMP-9.[74] In this model (Figure 23), the Tryptophan of cWGF1 was tethered into the S2 pocket and the cyclic peptide was energy minimized in the context of MMP-9. It appeared that the phenylalanine from the recognition motif was making contacts in the S1' specificity pocket. The carbonyl group on the active-site Glycine is 2.22 Å from the catalytic Zinc. This is well within hydrogen bonding radius, so it is possible that in this model, the glycine carbonyl could coordinate the Zinc. This model predicts that cWGF-1 could inhibit MMP-9 by binding in their specificity pockets with large aromatic groups as well as coordinating the zinc ion.

While this model is promising, it is speculative. Further experiments described above can yield a more accurate model of either a bicyclic peptide inhibitor that can be used in more computationally rigorous studies of inhibition. Data gleaned from crystallography, photocrosslinking, or protein NMR will yield information about where on the protein this inhibitor binds. Then, an *in silico* model will be more trustworthy.

Conclusion

Cyclic and Bicyclic Peptides can be effective inhibitors of Matrix Metalloproteinases. In creating analogues of CTT, a highly specific stabilized bicyclic inhibitor, bcWGF7 was found. bcWGF7 is an inhibitor of MMP-9 and MMP-2, and is highly specific for Gelatinases. In addition, this peptide features much greater serum stability, which would improve its *in vivo* effectiveness over time. Further SAR suggests that the carboxamide group on these inhibitors is essential to function, though the function of this group may differ between cyclic and bicyclic analogues. Further studies will attempt to improve affinity of the bicyclic analogues to Gelatinases, attempt to characterize the *in vivo* efficacy of peptidic inhibitors, and perform a number of structural studies to determine the mode of binding to Gelatinases.

The lack of a strong Zinc chelating group on these peptides suggests that their mode of MMP inhibition may be a novel one. Further study of the binding mode of these peptides is therefore essential in understanding their function as potentially next-generation MMP therapeutic agents.

---

[1]J. Gross, C.M. Lapiere. "Collagenolytic activity in amphibian tissues: a tissue culture assay." *Proc. Natl. Acad. Sci.* 48 (1962), pp. 1014–1022 .

[2]A. Gutierrez-Fernandez, M. Inada, M. Balbin, A. Fueyo, A.S. Pitiot, A. Astudillo, K. Hirose, M. Hirata, S.D. Shapiro, A. Noel et al. "Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8)." *FASEB J.*, 21 (2007), pp. 2580–2591

[3]W.C. Parks, C.L. Wilson, Y.S. Lopez-Boado. "Matrix metalloproteinases as modulators of inflammation and innate immunity." *Nat. Rev. Immunol.*, 4 (2004), pp. 617–629

[4]Hu, Jialiang, Philippe E. Van den Steen, Qing-Xiang A. Sang and Ghislain Opdenakker. "Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases." *Nature Reviews Drug Discovery* 6, 480-498 (June 2007)

[5]B.C. Patterson, Q.A. Sang. "Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and Gelatinase B/type IV collagenase (MMP-9)." *J. Biol. Chem.*, 272 (1997), pp. 28823–28825

[6] Polette M, Birembaut P. "Membrane-type metalloproteinases in tumor invasion." *Int J Biochem Cell Biol*. 1998 Nov;30(11):1195–1202.

[7]Coussens et al. "Matrix Metalloproteinase Inhibitors and Cancer—Trials and Tribulations." *Science* 29 March 2002

[8] Jed F. Fisher and Shahriar Mobashery. "Recent advances in MMP inhibitor design." *Cancer Metastasis Rev* (2006) 25: 115–136.

[9]Keith Brew, Deendayal Dinakarpandianb, Hideaki Nagase. "Tissue inhibitors of metalloproteinases: evolution, structure and function." *Biochimica et Biophysica Acta*. Volume 1477, Issues 1–2, 7 March 2000, pp 267–283.

[10] Irina Massova, Lakshmi P. Kotraa, Rafael Fridmanb and Shahriar Mobasherya. "Matrix metalloproteinases: structures, evolution, and diversification." *The FASEB Journal.* 1998;12:1075–1095 (1998)

[11] M.D. Sternlicht, Z. Werb. "How matrix metalloproteinases regulate cell behavior." *Annu. Rev. Cell Dev. Biol.*, 17 (2001), pp. 463–516

[12] Stryer, L. "Proteases." *Biochemistry.* N.Y.: Freeman and, 1997. Print.

[13]Kai Kessenbrock, Vicki Plaks, Zena Werb. "Matrix Metalloproteinases: Regulators of the Tumor Microenvironment." *Cell*. Volume 141, Issue 1, 2 April 2010, Pages 52–67

[14]K. Wolf, Y.I. Wu, Y. Liu, J. Geiger, E. Tam, C. Overall, M.S. Stack, P. Friedl "Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion." *Nat. Cell Biol*., 9 (2007), pp. 893–904

[15] P. Friedl, K. Wolf "Tube travel: the role of proteases in individual and collective cancer cell invasion." *Cancer Res*., 68 (2008), pp. 7247–7249

[16]F. Sabeh, I. Ota, K. Holmbeck, H. Birkedal-Hansen, P. Soloway, M. Balbin, C. Lopez-Otin, S. Shapiro, M. Inada, S. Krane et al. "Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP" *J. Cell Biol*., 167 (2004), pp. 769–781

[17] J. Wyckoff, W. Wang, E.Y. Lin, Y. Wang, F. Pixley, E.R. Stanley, T. Graf, J.W. Pollard, J. Segall, J. Condeelis. "A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors." *Cancer Res*., 64 (2004), pp. 7022–7029

[18] Agrawal, P. Anderson, M. Durbeej, N. van Rooijen, F. Ivars, G. Opdenakker, L.M. Sorokin. "Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis." *J. Exp. Med*., 203 (2006), pp. 1007–1019

[19]H. Nagase, R. Visse, G. Murphy. "Structure and function of matrix metalloproteinases and TIMPs" *Cardiovasc. Res*., 69 (2006), pp. 562–573

[20] P.A. Rupp, R.P. Visconti, A. Czirok, D.A. Cheresh, C.D. Little "Matrix metalloproteinase 2-integrin alpha(v)beta3 binding is required for mesenchymal cell invasive activity but not epithelial locomotion: a computational time-lapse study." *Mol. Biol. Cell*, 19 (2008), pp. 5529–5540

[21] J. Massague. "TGFbeta in Cancer." *Cell*, 134 (2008), pp. 215–230

[22] Q. Yu, I. Stamenkovic. "Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis." *Genes Dev*., 14 (2000), pp. 163–176

[23] S.L. Dallas, J.L. Rosser, G.R. Mundy, L.F. Bonewald. "Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts. A cellular mechanism for release of TGF-beta from bone matrix." *J. Biol. Chem*., 277 (2002), pp. 21352–21360

[24] O. Tatti, P. Vehvilainen, K. Lehti, J. Keski-Oja. "MT1-MMP releases latent TGF-beta1 from endothelial cell extracellular matrix via proteolytic processing of LTBP-1." *Exp. Cell Res*., 314 (2008), pp. 2501–2514

[25] N.E. Hynes, H.A. Lane. "ERBB receptors and cancer: the complexity of targeted inhibitors." *Nat. Rev. Cancer*, 5 (2005), pp. 341–354

[26] K.D. Cowden Dahl, J. Symowicz, Y. Ning, E. Gutierrez, D.A. Fishman, B.P. Adley, M.S. Stack, L.G. Hudson. "Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells." *Cancer Res*., 68 (2008), pp. 4606–4613

[27] G. Bergers, R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb et al. "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis." *Nat. Cell Biol*., 2 (2000), pp. 737–744

[28] G. Bergers, R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb et al. "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis." *Nat. Cell Biol*., 2 (2000), pp. 737–744

[29] L.E. Littlepage, M.D. Sternlicht, N. Rougier, J. Phillips, E. Gallo, Y. Yu, K. Williams, A. Brenot, J.I. Gordon, Z. Werb. "Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression." *Cancer Res*., 70 (2010), pp. 2224–2234

[30] T.P. Padera, A. Kadambi, E. di Tomaso, C.M. Carreira, E.B. Brown, Y. Boucher, N.C. Choi, D. Mathisen, J. Wain, E.J. Mark et al. "Lymphatic metastasis in the absence of functional intratumor lymphatics." *Science*, 296 (2002), pp. 1883–1886

[31] G.O. Ahn, J.M. Brown. "Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: Role of bone marrow-derived myelomonocytic cells." *Cancer Cell*, 13 (2008), pp. 193–205

[32] R.N. Kaplan, R.D. Riba, S. Zacharoulis, A.H. Bramley, L. Vincent, C. Costa, D.D. MacDonald, D.K. Jin, K. Shido, S.A. Kerns et al. "VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche." *Nature*, 438 (2005), pp. 820–827

[33] S. Hiratsuka, A. Watanabe, H. Aburatani, Y. Maru. "Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis." *Nat. Cell Biol*., 8 (2006), pp. 1369–1375

[34] S. Hiratsuka, A. Watanabe, Y. Sakurai, S. Akashi-Takamura, S. Ishibashi, K. Miyake, M. Shibuya, S. Akira, H. Aburatani, Y. Maru. "The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase." *Nat. Cell Biol*., 10 (2008), pp. 1349–1355

[35] M. Bond, R.P. Fabunmi, A.H. Baker, A.C. Newby. "Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B." *FEBS Lett*., 435 (1998), pp. 29–34

[36] B. Heissig, K. Hattori, S. Dias, M. Friedrich, B. Ferris, N.R. Hackett, R.G. Crystal, P. Besmer, D. Lyden, M.A. Moore et al. "Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand." *Cell*, 109 (2002), pp. 625–637

[37] Brinckerhoff , Constance E. and Lynn M. Matrisian. "Matrix metalloproteinases: a tail of a frog that became a prince." *Nature Reviews Molecular Cell Biology* 3, 207-214 (March 2002)

[38]Serra P, Bruczko M, Zapico JM, Puckowska A, Garcia MA, Martin-Santamaria S, Ramos A, de Pascual-Teresa B "MMP-2 Selectivity in Hydroxamate-Type Inhibitors." *Curr Med Chem*. 2012 Mar 1;19(7):1036-64.

[39] Turk, Boris. "Targeting Proteases: successes, failures, and future prospects." *Nature Reviews Drug Discovery*. Vol 5, Sept 2006 Pg 785-799

[40] Shelley Littin. "Drug Reduces Gum Disease, Risk of Osteoporosis, Heart Disease in Women." *University of Arizona News*. 09 March, 2011.

[41] R. Paul Beckett, Alan H. Davidson, Alan H. Drummond, Philip Huxley, Mark Whittaker. "Recent advances in matrix metalloproteinase inhibitor research." *Drug Discovery Today* Volume 1, Issue 1, January 1996, Pages 16–26

[42]Anna Tochowicz, Klaus Maskos, Robert Huber, Ruth Oltenfreiter, Vincent Dive, Athanasios Yiotakis, Matteo Zanda, Wolfram Bode, Peter Goettig. "Crystal Structures of MMP-9 Complexes with Five Inhibitors: Contribution of

the Flexible Arg424 Side-chain to Selectivity." *Journal of Molecular Biology*, Volume 371, Issue 4, 24 August 2007, Pages 989–1006.

[43] Owens, MW, Milligan SA, Grisham MB. "Effects of reactive metabolites of oxygen and nitrogen on gelatinase A activity." *Am J Physiol*. 273. 1997.

[44] Wang J, O'Sullivan S, Harmon S, Keaveny R, Radomski MW, Medina C, Gilmer JF. "Design of Barbiturate-Nitrate Hybrids that Inhibit MMP-9 Activity and Secretion." *J Med Chem*. 2012 Mar 8;55(5):2154-62. Epub 2012 Feb 23

[45]In Taek Lim, Stephen Brown, and Shahriar Mobashery. "A Convenient Synthesis of a Selective Gelatinase Inhibitor as an Antimetastatic Agent." *J. Org. Chem*. 2004, 69, 3572-3573

[46] Stephen Brown, M. Margarida Bernardo, Zhi-Hong Li, Lakshmi P. Kotra, Yasuhiro Tanaka, Rafael Fridman, and Shahriar Mobashery. "Potent and Selective Mechanism-Based Inhibition of Gelatinases." *J. Am. Chem. Soc*. 2000, 122, 6799-6800

[47]Erkki Koivunen, Wadih Arap, Heli Valtanen, Aija Rainisalo,Oula Penate Medina, Pia Heikkilä, Carmela Kantor, Carl G. Gahmberg, Tuula Salo, Yrjö T. Konttinen, Timo Sorsa, Erkki Ruoslahti, and Renata Pasqualini. "Tumor targeting with a selective Gelatinase inhibitor." *Nature Biotechnology*. Vol 17, August 1999. Pages 768-774

[48]Mikael Björklund, Heli Valtanen, Harri Savilahti and Erkki Koivunen. "Use of Intein-Directed Peptide Biosynthesis to Improve Serum Stability and Bioactivity of a Gelatinase Inhibitory Peptide." *Combinatorial Chemistry & High Throughput Screening*, 2003, 6, 29-35

[49]Justus Reunanen, Tanja-Maria Ranta, Oula Peñate-Medina, Juho Suojanen, Timo Sorsa, Tuula Salo e, Erkki Koivunen, Per E. J. Saris. "A chimera of green fluorescent protein with Gelatinase binding and tumor targeting peptide." *Protein Expression and Purification*. 72 (2010) 234–237

[50]Oula Penate Medina, Tim Soderlund, Liisa J. Laakkonen, Esa K. J. Tuominen, Erkki Koivunen, and Paavo K. J. Kinnunen. "Binding of Novel Peptide Inhibitors of Type IV Collagenases to Phospholipid Membranes and Use in Liposome Targeting to Tumor Cells in Vitro." *Cancer Research*, 61, May 15, 2001, 3978–3985

[51]Pia Heikkila, Juho Suojanen, Emma Pirila, Anu Vaananen, Erkki Koivunen, Timo Sorsa and Tuula Salo. "Human tongue carcinoma growth is inhibited by selective antigelatinolytic peptides." *Int. J. Cancer*: 118 (2006) , 2202–2209

[52]Tuula Salo. "Inhibition of Gelatinase activity by cyclic peptides—A support for oral cancer treatment?" Oral *Oncology* (2006) 42, 955– 956

[53]Wei Wang, Ruping Shao, Qingping Wu, Shi Ke, John McMurray, Frederick F. Lang Jr., Chusilp Charnsangavej, Juri G. Gelovani, Chun Li. "Targeting Gelatinases with a Near-Infrared Fluorescent Cyclic His-Try-Gly-Phe Peptide." *Mol Imaging Biol* (2009) 11:424-433

[54] Finely and Brent. "Two-hybrid analysis of genetic regulatory networks." *The yeast two-hybrid system* (ed. P.L. Bartel and S. Fields) Oxford University Press, Oxford, England

[55] Albertha J. M. Walhout and Simon J. Boulton, "The Yeast Two-Hybrid System." *Worm Book.* Online, 2006. Accessed January 2011. URL: <http://www.wormbook.org/chapters/www_biochemmolbio/biochemmolbio.html>

[56] J. Biol. Chem. 2003 Lee Oct;278(41):40224–40230

[57] Christopher M. Overall, Eric Tam, G. Angus McQuibban, Charlotte Morrison, U. Margaretha Wallon, Heather F. Bigg, Angela E. King and Clive R. Roberts. "Domain interactions in the gelatinase A center dot TIMP-2 center dot MT1-MMP activation complex - The ectodomain of the 44-kDa form of membrane type-I matrix metalloproteinase does not modulate gelatinase A activation" *Journal of Biological Chemistry*, 2000, 275, 39497-39506

[58] Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A.. "PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells." *Cell* 120: 303-313. (2005)

[59] Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K, Covic L, Kuliopulos A. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell* 137: 332-343. (2009)

[60] Markus Gude, Jeannine Ryf, and Peter D. White. "An accurate method for the quantitation of Fmoc-derivatized solid phase supports." *Letters in Peptide Science*, 9: 203-206, 2002.

[61] Trent Conroy , Katrina A. Jolliffe and Richard J. Payne. "Efficient use of the Dmab protecting group: applications for the solid-phase synthesis of N-linked glycopeptides." *Org. Biomol. Chem*., 2009, 7, 2255-2258

[62] Beekman, B., et al. 1996. FEBS Lett 390, 221

[63] Thomas A. Knappe, Florian Manzenrieder, Carlos Mas-Moruno, Uwe Linne, Florenz Sasse, Horst Kessler, Xiulan Xie, Mohamed A. Marahiel. "Introducing Lasso Peptides as Molecular Scaffolds for Drug Design: Engineering of an

Integrin Antagonist." *Angewandte Chemie International Edition*. Volume 50, Issue 37, pages 8714–8717, September 5, 2011

[64]Scott J. Miller, Robert H. Grubbs. "Synthesis of Conformationally Restricted Amino Acids and Peptides Employing Olefin Metathesis." *J. Am. Chem. Soc*., 1995, 117 (21), pp 5855–5856

[65] Blackwell, H. E.; Grubbs, R. H. "Highly Efficient Synthesis of Covalently Cross-Linked Peptide Helices by Ring-Closing Metathesis." *Angew. Chem. Int. Ed*. 1998, 37, 3281-3284.

[66]Young-Woo Kim, Tom N Grossmann, and Gregory L Verdine. "Synthesis of all-hydrocarbon stapled α-helical peptides by ring-closing olefin metathesis." *Nature Protocols* 6, 761–771 (2011)

[67] Kirshenbaum, K.; Arora, P. S. "Cross-dressing proteins by olefin metathesis" *Nature Chem. Biol*.2008, 4, 527-528

[68] Wang, D.; Liao, W.; Arora, P. S. "Enhanced Metabolic Stability and Protein-Binding Properties of Artificial Alpha-Helices Derived from a Hydrogen-Bond Surrogate: Application to Bcl-xL." *Angew. Chem. Int. Ed*. 2005, 44, 6525-6529.

[69]Raimo Franke, Christian Doll, Jutta Eichler. "Peptide ligation through click chemistry for the generation of assembled and scaffolded peptides." *Tetrahedron Letters*, Volume 46, Issue 26, 27 June 2005, Pages 4479–4482

[70]Marco Tischler, Daichi Nasu, Martin Empting, Stefan Schmelz, Dirk W. Heinz, Philipp Rottmann, Gerd Buntkowsky, Dr. Daniel Tietze, Olga Avrutina. "Braces for the Peptide Backbone: Insights into Structure-Activity Relationships of Protease Inhibitor Mimics with Locked Amide Conformations." *Angew. Chem. Int*. Ed. 2012, 51, 1 - 6

[71]Belhu B. Metaferiaa, Matthew Rittlerb, Jinesh S. Gheeyaa, Albert Leea, Heidi Hempela, Alberto Plazac, William G. Stetler-Stevensonb, Carole A. Bewleyc, Javed Khana. "Synthesis of novel cyclic NGR/RGD peptide analogs via on resin click chemistry." *Bioorganic & Medicinal Chemistry Letters*. Volume 20, Issue 24, 15 December 2010, Pages 7337–7340

[72]Rushia A. Turner, Allen G. Oliver, and R. Scott Lokey. "Click Chemistry as a Macrocyclization Tool in the Solid-Phase Synthesis of Small Cyclic Peptides." *Org. Lett*., 2007, 9 (24), pp 5011–5014

[73] E. L. Vodovozova . "Photoaffinity Labeling and Its Application in Structural Biology." *Biochemistry (Moscow)*, 2007, Vol. 72, No. 1, pp. 120

[74]Tochowicz, A., Maskos, K., Huber, R., Oltenfreiter, R., Dive, V., Yiotakis, A., Zanda, M., Bode, W., Goettig, P., "Crystal structures of MMP-9 complexes with five inhibitors: contribution of the flexible Arg424 side-chain to selectivity." *J.Mol.Biol*. 371: 989-1006. (2007)