STRUCTURE-BASED DEVELOPMENT OF A NOVEL COLLAGEN INHIBITOR FOR MMP-1: RE-DESIGNING THE FUNCTIONS OF A MATRIX PROTEIN

James M Chen [1] and Li-An Yeh [2]

[1] Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, [2] Harvard Center for Neurodegeneration and Repair, 65 Landsdowne Street, Cambridge, MA 02139

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1. ABSTRACT

Collagenases are a highly specific class of enzymes (1). In their native states, collagenases cleave only native triple helical collagen molecules at a single peptide bond between Gly775-Leu776 for Type I collagen and Gly775-Ile776 for Type II collagen (1, 2, 3). The linear sequence of collagen is about 1050 amino acids in length, where three linear peptide sequences are required to form a triple helical collagen molecule. At present, there exist no crystallographic structures of collagenase bound to native triple helical collagen; nor has it been shown that collagenase recognizes the triple helical conformation of collagen. In our study, we have used an inhibitor design structure-activity based approach to show that collagenase recognizes and cleaves triple helical collagen conformations in preference to non-triple helical collagen conformations (4).

2. INTRODUCTION

Structural information is critical in the process of inhibitor design towards pharmaceutical leads. Whether X-ray (5), NMR (6), or modeling (7) based information is provided, the creative utilization of this information can result in the efficient discovery of interesting and biologically active molecules. During the start of this study, the x-ray structures of collagenases had not been solved, nor that of triple helical collagens. In this study, carried out in 1994, published structural information pertaining to collagen was utilized to model (8-13) and design novel inhibitor probes (4) for studying the interactions of the collagenase, matrix metalloprotein (MMP-1) with its native triple helical collagen substrate.

Under normal biological conditions and functions, maintaining the balance of matrix remodeling is crucial (1, 2, 3). The matrix metalloproteinases are involved in the regulation of the extracellular matrix components of which collagens are the major proteins. MMP expression and activity are highly controlled and, because of the degradative nature of these enzymes, if there is a loss in their regulation, pathological destruction of connective tissue always ensues. Thus, the MMPs are a highly active set of targets for the design of therapeutic agents within the disease areas of arthritis and oncology (1, 14). At present, there have been many MMPs identified of which collagenase, MMP-1, is highly specific for Type-I triple helical collagen proteins. Collagenase cleaves collagen at one specific sequence, Gly775-Leu776, and is thought to be the initial upstream trigger of collagen degradation. Although there is no direct evidence, it has been suggested that the native-collagen conformation must undergo a conformational change at Gly775-Leu776 in order to expose the peptide bond for cleavage by MMP-1. Hypothetically, the native collagen conformation at Gly775-Leu776 may unravel into a coil-like conformation prior to MMP-1 recognition.

However, we show that MMP-1 prefers a defined collagen conformation for substrate recognition. In this study, scaffold-ligand conjugates were synthesized (4). Each scaffold was made of three cross-linked polypeptide chains, wherein the three polypeptide chains each contain repeat sequences of Gly-Pro-Hyp (where Hyp represents hydroxyproline) aligned to form a triple helix coil. The tripeptide scaffold-ligand molecule, in which each peptide sequence contains four repeats of Gly-Pro-Hyp, is the minimum length that has been shown, experimentally, to form the native structure of collagen (15-19). A set of inhibitors based on collagen's native structure, have been synthesized and the results showed that biological recognition
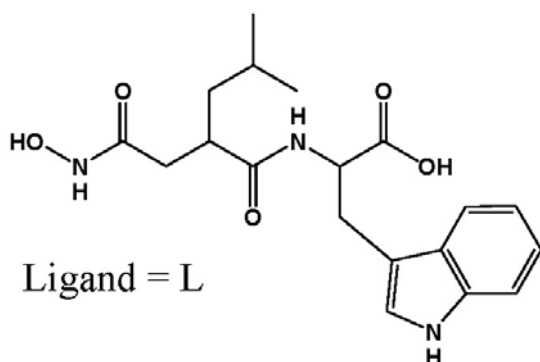
Ligand = L

Figure 1. Structural formula of the peptido-mimetic inhibitor molecule (4). Chemical formula is: N-[2-isobutyl-3-(N'-hydroxycarbonylamido)propanoyl]-L-Tryptophan.

of the collagenase active site for collagen, is conformation-dependent.

3. MATERIALS AND METHODS

Molecular modeling methods utilized the Sybyl software from Tripos (20). The computational models of Type I & II collagen systems are described elsewhere (10-13). The development and study of these molecular models lead to the results described within this report. The structure of MMP-1 used was taken from the Protein Data Bank labeled 1FBL (21). All experimental procedures for synthesis and biological evaluations of this data set are described in the corresponding US patent (4).

4. RESULTS AND DISCUSSION

4.1. Collagenase recognizes the collagen triple helix

We have used the hydroxamate collagenase inhibitor called "L" in Figure 1 as a prototypical peptido-mimetic inhibitor molecule. We have attached the carboxyl terminus of this inhibitor to the amino terminus, forming a peptide bond, of a series of different collagen-like sequences as shown in Figure 2A. Our hypothesis is that collagenase recognizes triple-helical collagen-like polypeptides and attachment of the hydroxamate inhibitor to triple-helical collagen-like polypeptides should therefore result in enhanced binding of the inhibitor to collagenase-1. The collagen-like polypeptides therefore act as scaffolds for the hydroxymate inhibitor.

To test the efficacy of this approach, we attached the inhibitor to each of the peptide scaffolds shown in Figure 2A. Each scaffold polypeptide set consists of a repeating tripeptide sequence of (Gly-Pro-Hyp), and at least one biologically active ligand (labeled 'L' in Figure 1 & Figure 2A) attached to the scaffold via covalent peptide bonding with each collagen chain. For each chain, at the carboxyl terminal end of the collagen-prototype Gly-Pro-Hyp sequences, an interface sequence (4, 15-17), -(Gly-Pro-Pro-Gly-Ser-Ser)- is added that links the collagen-like repeat sequences to the covalent cross-bridge-forming amino acid, epsilon-amino-caproic acid sequence, Acp in Figure. 2B. The interface sequence probably serves to separate the actual collagen sequence from the tri-cross-

linker motif; thus, enabling the collagen peptides to fold properly without potential interference by the cross-linker group. As shown in Figures 2A & B, in one of the three scaffold chains, the tri-cross-linker group, labeled TCL, is added and enables the covalent attachment of three identical polypeptide chains. The carboxyl terminus of the polypeptide chain is covalently attached to the free amino group of Acp through formation of a peptide bond.

Shown in Figure 2A, in the final synthesis, the hydroxamate inhibitor was linked to an un-tri-cross-linked chain consisting of four Gly-Pro-Hyp repeats (LTL in Figure 2A); and to a series of fully tri-cross-linked scaffolds, containing one Gly-Pro-Hyp unit (NHT1-L), two units (NHT2-L), three units (NHT3-L) and four units (THT-L). Each of these scaffold-inhibitor molecules was assayed for its ability to inhibit collagenase-1.

Figure 3 summarizes the results for the four inhibitor series. As can be seen in this figure, addition of the Gly-Pro-Pro tripeptide units, up to three units, results in no increase in inhibition. Addition of the fourth unit results in at least a twenty-fold decrease in the IC_{50} value, or enhanced inhibition. Based on both a computational approach (10) and on several systematic experimental studies (15-19), it has been found that a minimum of four

repeating (Gly-Pro-Hyp) units, in each tri-crosslinked chain, is required for collagen triple helix formation in solution. This result strongly supports our hypothesis that the nucleation of the collagen helix enhances the inhibition potency of the small molecule hydroxamate inhibitor.

4.2. The triple helix is required for catalysis

The biological ligand (Labeled 'L' in Figure 4) utilized in this study is a peptido-mimetic inhibitor of a collagen (Gly-X-Y) tripeptide; where the Gly position is replaced with a hydroxymate, making it stable towards MMP-1 cleavage (Figure 4). Once covalently linked to each $(Gly-Pro-Hyp)_n$-(Gly-Pro-Pro)-(Gly-Ser-Ser)-COO^- sequence, Figure 2A, the triple helical scaffold (THT-L) where n = 4 should induce and constrain each biological ligand into collagen's conformation. Figure 5 is a theoretical diagram depicting the folding properties as shown and published for similar sets of synthetic collagen polypeptides (15 –18). In Figure 5, where n = 1-3, the collagen helix will probably not form, and the ligands would not mimic collagen's conformation. Based on the resulting enzyme potency, Figure 3, it is clear that the IC_{50}'s increase (i.e., decreasing potency) somewhat as the polypeptide chain lengthens from n=1-3. This observation is likely due to increasing conformational flexibility as the unfolded peptide chain gets longer. It has also been shown, using circular dichroism techniques, that there is no characteristic collagen helical conformations for collagen scaffolds where n = 1-3 (15-17). Based on these studies, it has been confirmed experimentally, that the conformation of native collagen is formed only when n=4 (15-17). In fact, only the THT-L inhibitor, for which n=4, nucleates the stable collagen triple helix and consequently

L = N-[2-isobutyl-3-(Hydroxymate)-propanoyl)]-L-tryptophan

LT-L (Linear Non-Triple-Helical Inhibitor)
NH2-(Gly-Pro-Hyp)$_4$-(Gly-Pro-Pro-Gly-Ser-Ser)-COO-

TCL (Tri-Linker)
-(Acp)--|
-(Acp-Lys-Lys-Gly)-COO-
-(Acp)-------|

NHT1-L (Non-Helical Inhibitor 1)
(NH2)-(Gly-Pro-Hyp)$_1$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)--|
(NH2)-(Gly-Pro-Hyp)$_1$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp-Lys-Lys-Gly)-COO-
(NH2)-(Gly-Pro-Hyp)$_1$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)-------|

NHT2-L (Non-Helical Inhibitor 2)
(NH2)-(Gly-Pro-Hyp)$_2$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)--|
(NH2)-(Gly-Pro-Hyp)$_2$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp-Lys-Lys-Gly)-COO-
(NH2)-(Gly-Pro-Hyp)$_2$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)-------|

NHT3-L (Non-Helical Inhibitor 3)
(NH2)-(Gly-Pro-Hyp)$_3$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)--|
(NH2)-(Gly-Pro-Hyp)$_3$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp-Lys-Lys-Gly)-COO-
(NH2)-(Gly-Pro-Hyp)$_3$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)-------|

THT-L (Helical Inhibitor)
(NH2)-(Gly-Pro-Hyp)$_4$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)--|
(NH2)-(Gly-Pro-Hyp)$_4$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp-Lys-Lys-Gly)-COO-
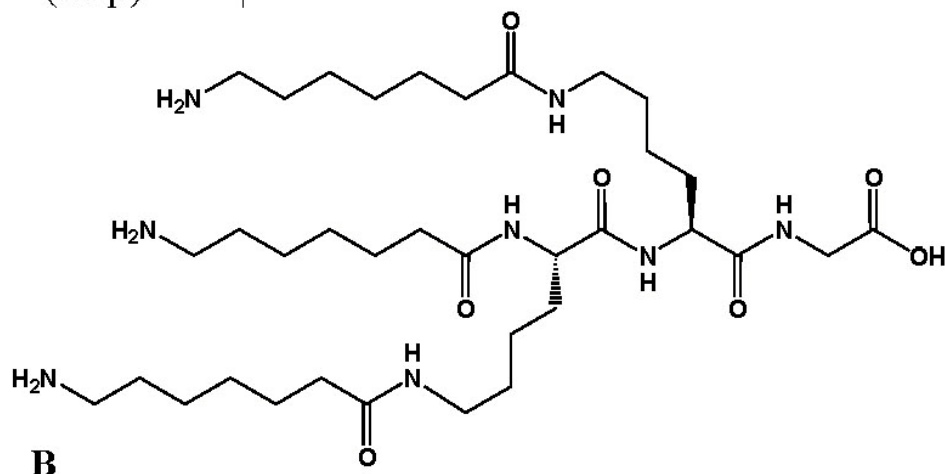(NH2)-(Gly-Pro-Hyp)$_4$-(Gly-Pro-Pro-Gly-Ser-Ser)- (Acp)-------|

A

Tri-Cross-Linker Formula

-(Acp)--|
-(Acp-Lys-Lys-Gly)-COO-
-(Acp)-------|

B

Figure 2. (A) Chemical formulas representing all the synthetic molecules studied (4). (B) Chemical structure of the Tri-cross-linker molecule utilized within this study. The exposed amino groups are the attachment points for the synthetic collagen polypeptides. The carboxyl group of the tri-cross-linker is the attachment point for the solid phase synthesis column.
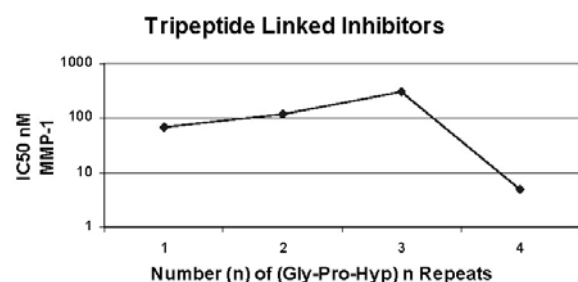
Figure 3. This figure compares the potency of the four tri-crosslinked inhibitors. When n = 1-3, as the sequence length increases, the IC50's also increase (or decreasing potencies). The correlation can be attributed to increasing conformational flexibility as the peptide length is increased; the greater the length = disfavored conformational entropy upon binding = higher IC50 values. When n = 4, the peptide length is greatest, but the potency is greatest. THT-L corresponds to a design sequence that folds into the three dimensional structure of collagen. Upon folding, the binding entropy becomes favorable compared to THT1, THT2 and THT3.
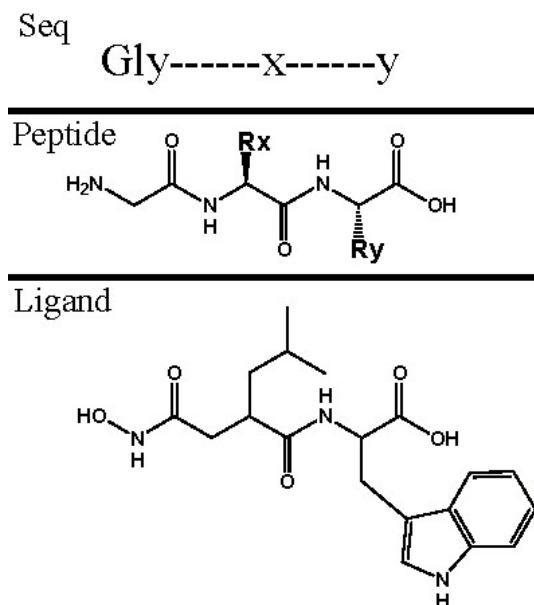

Figure 4. Seq. represents the repeat sequence known for triple helical collagens. Glycine is always the first position followed by variations at the X and Y positions. Peptide represents the corresponding and aligned amino acid sequence. Ligand represents the peptido-mimetic inhibitor of the above Peptide and Seq. formulas.

has a much lower IC_{50}. The peptide length and better binding potency of THT-L contradicts the conformational flexibility defined by inhibitors where n=1-3 (Figure 2A). Thus, THT-L likely forms the collagen structure, resulting in greater inhibition.

4.3. The three helical strands must be cross-linked for short helical sequences
Finally, LT-L defines an inhibitor where the scaffold sequence contains the full $(Gly-Pro-Hyp)_4$ tetra-tri-

peptide repeat, but without the crosslinker; in this case, the inhibitor does not form the collagen triple helix because the three chains now only associate weakly (15-17). As shown in Figure 6, this results in an over100-fold increase in the IC_{50} for LT-L.

Figure 7 depicts the three dimensional model for inhibitor THT-L. It is clear that the inhibitor ligands are able to form most of the interactions required for maintaining the native conformation of collagen. Figure 8 shows the inhibitor THT-L docked into the catalytic zinc containing active site of MMP-1. Figure 9 depicts that the hydroxymate inhibitor (L), constrained into collagen's conformation, is able to fit into the known binding site of MMP-1. The hydroxymate chelates the catalytic zinc atom and the P1' isobutyl group fits into the S1' hydrophobic pocket, while maintaining the original triple helical collagen conformation.

4.4. Overview of the implications of the modeling studies
In summary, our modeling studies predicted that the conformation of collagen at the cleavage site, Gly775-Leu776, should be that of the native conformation. Since collagen triple helical molecules are also packed tightly into fibrils (13), un-coiling a part of the collagen peptide chain for interactions with MMP-1 would likely be energetically unfavorable. Herein, we have designed a set of collagen peptido-mimetic inhibitors of which only one design type (THT-L) would form the three dimensional structure of collagen (15). It was predicted that the folded form of the inhibitor would be the most active.

Biological evaluation of the set of Inhibitors in the MMP-1 assay resulted in the IC_{50} values (in nM) for L, NHT1-L, NHT2-L, NHT3-L, LT-L, THT and THT-L, that are 500, 67, 116, 288, 1400, more than 50,000 and 7, respectively. THT-L showed an inhibitory potency better than NHT1-L, NHT2-L, NHT3-L, L, and LT-L (See Figure 3). In Figure 3, it is clear that a triple helical conformation is required for better potency against MMP-1. If one compares NHT1-L, NHT2-L, NHT3-L, the increasing peptide chain length corresponds with higher conformational entropy, thereby decreasing enzyme potency. But also shown in Figure 3 is THT-L, where the collagen chain length is longest and where one would expect the least potency. But THT-L is the most potent inhibitor against MMP-1; this can be attributed to favorable conformational entropy resulting from the folding of the cross-linked peptide chains into the collagen triple helix. Figure 6 compares the triple-helical Inhibitor (THT-L, Figure 2A) with the linear non-cross-linked Inhibitor version (LT-L, Figure 2A). Although LT-L contains the identical sequence as THT-L, LT-L is unable to form the collagen helix and is considerably less potent than THT-L against MMP-1.

5. CONCLUSION

5.1. Clinical implications of the modeling studies
Collagen is an abundant protein whose main functions are to provide an extracellular scaffolding or matrix.
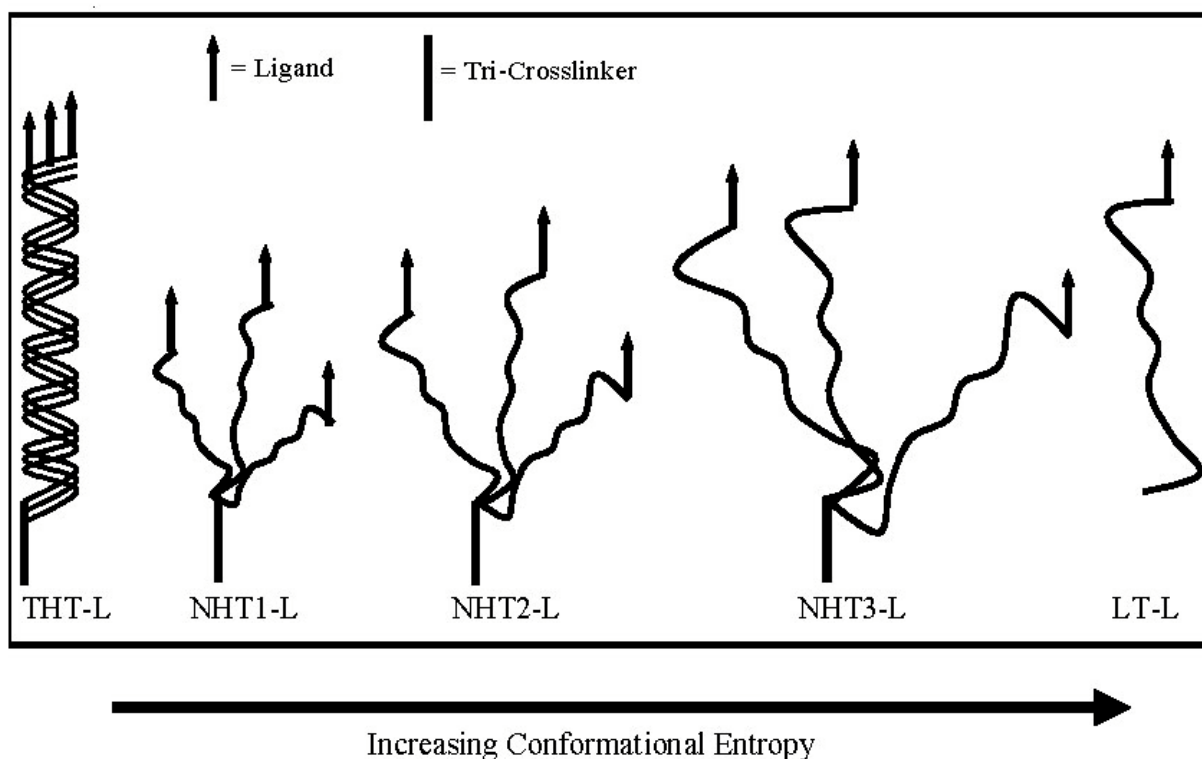
Figure 5. This scheme depicts the ability of each synthesized inhibitor to fold into the three dimensional structure of collagen. Only THT-L is able to fold into collagen; whereas, the squiggle lines (longer lines represents longer sequence length) for the rest of the inhibitors represent coiled structures. The small arrows represent the inhibitor ligand, and the thick line at the bottom of each inhibitor represents the tri-crosslinker motif.
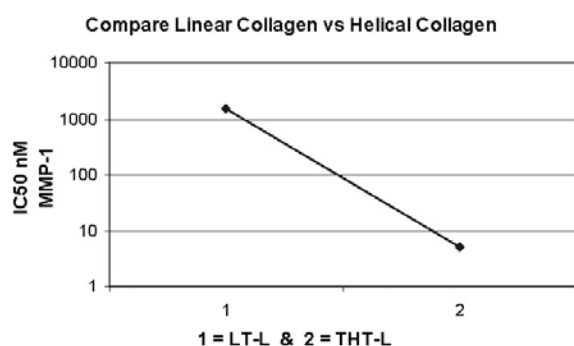

Figure 6. This figure compares two inhibitors containing identical peptide sequences. THT-L is tri-crosslinked and LT-L is not linked. This comparison also confirms that folding is critical towards MMP-1 potency since LT-L is unable to fold without the crosslinker.

These proteins are the major components of skin, cartilage, tendons, blood vessels, cornea and bone. Under normal conditions, collagen matrix remodeling requires a delicate balance in the production and degradation of the matrix components, and the MMPs are involved in the degradative process. Thus, MMP expression and activities are highly controlled due to the degradative nature of this class of enzymes, where the apparent loss in this regulation results in the pathological destruction of connective tissue and the ensuing disease state. For these reasons, the MMPs are a highly active set of targets selected for the design of therapeutic agents in the disease areas of arthritis and oncology (1, 4, 6, 14). The MMPs are generally categorized based on their substrate specificity, where the collagenase subfamily of MMP-1, MMP-8, and MMP-13 selectively cleaves native interstitial collagens (types I, II, & III) (14). Collagenases, such as MMP-1 in Type I collagen systems, are thought to be the initial trigger of collagen degradation where a single bond in a linear collagen sequence containing more than 1040 bonds is specifically cleaved. It is, thus, important to understand the structural requirements of substrate recognition for MMP-1.

5.2. The structure-function relationship for collagenase activity

A structure-based approach to designing inhibitor probes was taken in this study. A set of inhibitors have been synthesized and tested against MMP-1 (4). We have shown that only one inhibitor, THT-L (see Figure 2A), that was able to obtain the three dimensional structure of collagen, was the most potent, IC50 = 7 nM. This potency was not due to the collagen scaffold, THT more than 50,000 nM, nor was it due to the small molecule inhibitor, L = 500nM. Furthermore, LT-L was a version of THT-L where the tri-cross-linker was not present, and this inhibitor also lacks collagen's conformation; thus, its potency was also weak, IC50 = 1400 nM. These results suggest that MMP-1 binds to the native conformation of the collagen triple helix. It is unlikely that the Gly775-Leu776 substrate site uncoils out of its native
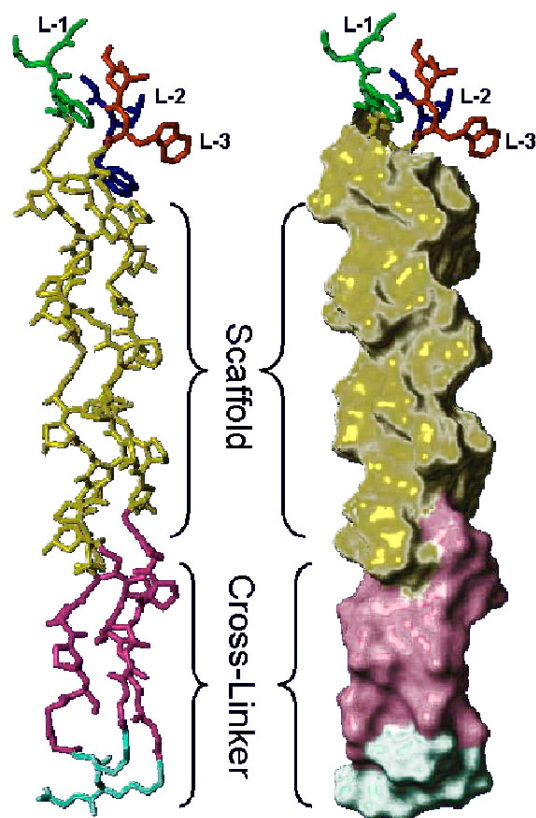
Figure 7. This figure depicts the energy minimized structure THT-L. Each inhibitor design region is labeled. L1, L2 and L3 represent the chemically linked ligand on each chain. Since each small molecule ligand mimics the original tripeptide, the original collagen interactions are maintained and the ligands can adopt the triple-helical collagen conformation.
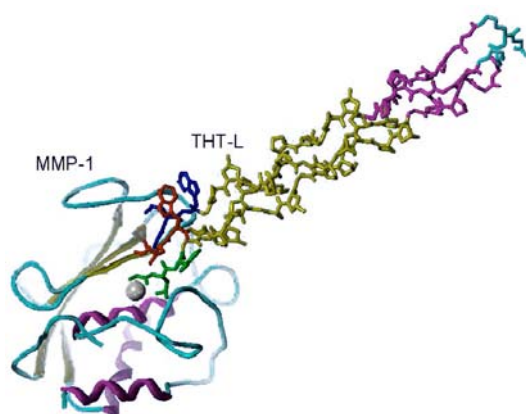

Figure 8. This figure depicts the THT-L molecule docked into the inhibitor binding site of MMP-1 (21). This computer models shows that the catalytic site of MMP-1 is able to position itself to bind to the collagen-based inhibitor, L-1. L-2 and L-3, as seen, are not hindering this interaction. It is likely that collagenase depends on its carboxyl domain for recognition interactions (see 1FBL (21), which then allows for the positioning of the catalytic domain over the substrate cleavage site.

conformation in order to expose the cleavage site. Computer

models of Type I and II collagen microfibrils depict a highly stable and tightly packed cluster of collagen triple helices (13). In addition, the uncoiling of a substrate region would require the breaking of many strong hydrogen bonds known for triple helical collagens.

It is known that the collagenases are multi-domain proteins containing a common catalytic domain and a larger carboxyl domain (see structure 1FBL in PDB (21)). It is also known that collagen does not exist, in-vivo, as monomeric, triple helical molecules. Collagens pack against each other to form microfibrils (3, 13). The microfibril structures pack together to form thicker fibril structures (3, 13). Our molecular models of the type I and type II collagen microfibrils depict that collagenases are actually exposed to a larger collagen surface resulting from the axially packed collagen triple helical molecules (Figure 10, ref.13). This surface, surrounding the actual cleavage site, is highly specific (13). And it is likely that the carboxyl terminus domain of collagenase interact specifically at the microfibril surface, while positioning the smaller, zinc-containing catalytic domain, for peptide bond cleavage. Figure 10 shows the x-ray structure of MMP-1 (1FBL (21)), positioned with respect to a collagen microfibril structure (3, 13). It is evident MMP-1's recognition or interaction with collagen may occur at the carboxyl-terminus domain. The catalytic domain and carboxyl domains are connected by a flexible coiled 'hinge' sequence, allowing for the zinc contain catalytic site to 'swing' into position for peptide bond cleavage (at the Gly775-Leu776 sequence). Thus, the catalytic site is not required to bind or fit to the complete triple helical structure; only the linear peptide region to be cleaved is required for interaction and catalysis.

5.3. Our studies suggest a new drug scaffold
Our work pertaining to this collagen-based inhibitor system has resulted in an interesting molecule (the in-vivo activities of THT-L) in addition to a drug design concept based on the structure function relationships of the collagen triple helical scaffold (4). The computationally designed inhibitor, THT-L, satisfies both potency and specificity. Potency is due to pre-organization of binding, and specificity is due to the inhibitor's shape being complementary for the native collagen structure. Since the overall shape of THT-L is that of collagen, it is assumed that this inhibitor would have intrinsic delivery properties for the matrix environment. This pre-designed delivery property may solve the potential toxicity problems due to non-specific interactions known for pharmaceutical drugs. We envision that optimized versions of THT-L would efficiently delivery the drug or 'itself' to the site of disease action.

In fact, it would appear that THT (Figure 2A) can be applied as a universal drug design template. Although drugs developed using this template are likely to be larger than the usually pharmaceutical products, < 1,000 Dalton, these molecules are still much smaller compared to blockbuster drugs like Epogen™ and Enbrel™. Furthermore, drugs developed using our template concept can be synthesized utilizing solid phase
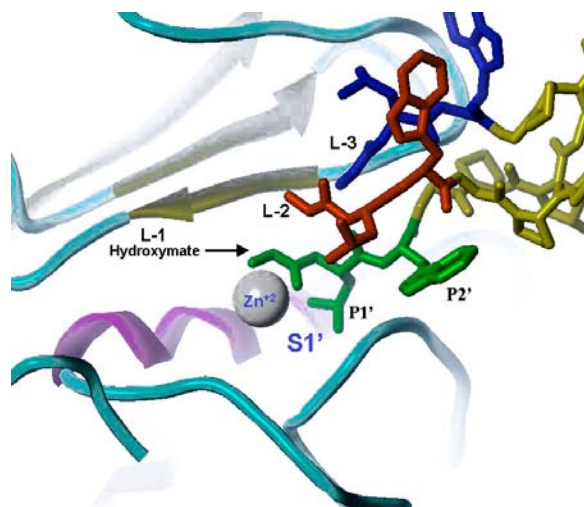
Figure 9. This figure is focused on the catalytic site of MMP-1. This shows that the known binding groups, hydroxymate, P1' isobutyl and P2' tryptophan interact with their corresponding binding sites.
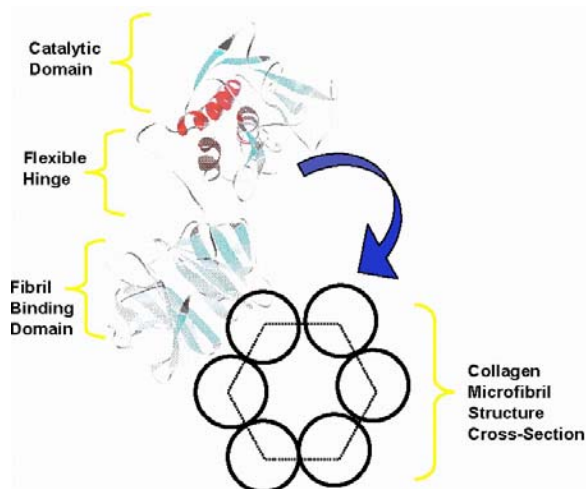

Figure 10. This figure describes the potential interactions of the full collagenase structure (1FBL (21)) to that of the collagen microfibril model. It is evident that MMP-1 interacts with a large fibril surface and not a smaller individual collagen triple helical surface. Our model depicts specific and separate functions for the carboxyl and catalytic domains of the collagenases.

chemistry; thus, this technology lends itself towards automation.

Drug design applications based on our template molecule(s) are highly favorable. First, the native structure of collagen is highly stable and resistant towards non-specific proteolysis. In the folded form, most of the peptide bonds are internalized and not exposed for cleavage by most proteases. Additional stability can be added by using D-amino acids or amino acid mimetics for synthesis. Second, the THT template allows for greater design diversity. Drug properties such as specificity, selectivity and physical properties can be easily incorporated due to the greater design surface area. For example, drug properties pertaining to the molecules polarity (i.e., solubility and cell permeability) can be optimized by adding more hydrophilic or hydrophobic amino acids, respectively. Third, due to the greater design potentials, our template concept allows for the ability to design drug molecules that mimic the favorable in-vivo properties of therapeutic protein targets. Compounds that have similar properties to their corresponding target proteins may lower the risk of drug related toxicities.

In summary, our drug design concept is based on the realization that the three dimensional structure of the collagen triple helix may be one of nature's methods for designing a universal scaffold. The collagen scaffold serves as a platform for constraining peptide sequences into productive binding conformations. Thus, this scaffold may be utilized as a form of Structure Activity Template for the design of biologically relevant therapeutics, which will be described elsewhere (22).

6. ACKNOWLEDGMENTS

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Send correspondence to: Dr James Chen, Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, Tel: 650-522-5269, Fax: 650-522-5899, E-mail: jchen@gilead.com