Effect of Species Differences on Stromelysin-1 (MMP-3) Inhibitor Potency

AN EXPLANATION OF INHIBITOR SELECTIVITY USING HOMOLOGY MODELING AND CHIMERIC PROTEINS

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For an animal model to predict a compound's potential for treating human disease, inhibitor interactions with the cognate enzymes of separate species must be comparable. Rabbit and human isoforms of stromelysin-1 are highly homologous, yet there are clear and significant compound-specific differences in inhibitor potencies between these two enzymes. Using crystal structures of discordant inhibitors complexed with the human enzyme, we generated a rabbit enzyme homology model that was used to identify two unmatched residues near the active site that could explain the observed disparities. To test these observations, we designed and synthesized three chimeric mutants of the human enzyme containing the single (H224N and L226F) and double (H224N/L226F) mutations. A comparison of inhibitor potencies among the mutant and wild-type enzymes shows that the mutation of a single amino acid in the human enzyme, histidine 224 to asparagine, is sufficient to change the selectivity profile of the mutant to that of the rabbit isofrom. These studies emphasize the importance of considering species differences, which can result from even minor protein sequence variations, for the critical enzymes in an animal disease model. Homology modeling provides a tool to identify key differences in isoforms that can significantly affect native enzyme activity.

Cell-matrix interactions provide cells with an assessment of their immediate environment and afford signaling events that result in proliferation, differentiation, migration, or programmed cell death (1). These matrix interactions can be altered through the activities of a family of extracellular zinc- and calcium-dependent endopeptidases, the matrix metalloproteinases (MMPs). Collectively, these enzymes are responsible for the proteolytic degradation of extracellular matrix macromolecules with the subsequent release of stromal contacts (2).

MMP activity is essential for tissue remodeling or cellular migration and is required for physiological processes such as fetal development, wound healing, angiogenesis, or inflammatory cell trafficking. However, it is critical to these processes that the matrix degradation be locally confined and temporally limited. Consequently, MMPs are highly regulated enzymes and, in general, are induced only in response to specific stimuli such as cytokines and growth factors. When induced, they are transiently expressed as latent enzymes that require complex interactions for activation (3). Once activated they are subject to rapid autodigestion. In addition, enzyme activity is modulated by potent and specific natural inhibitors, the TIMPs that are expressed under many of the same conditions that induce MMP activity (4). If the balance between induction, activation, and inhibition processes becomes even slightly dysregulated, the result can be chronic and debilitating diseases such as cancer, arthritis, inappropriate angiogenesis, autoimmune disease, aneurysm, atherosclerosis, or heart failure (5).

Because MMP expression in normal tissue is very low, enzyme activity can be associated with specific disorders through the correlation of disease severity with either mRNA or active protein expression. Stromelysin-1 (MMP-3) has been directly implicated in the pathologies of osteo- and rheumatoid arthritis and many types of cancers through these kinds of correlations (6–8). In addition, this enzyme provides the initial step in the activation cascades for many members of the MMP family and as such may play a less direct role in other disease states as well (9). As a result, MMP-3 inhibition has been an attractive target for pharmaceutical drug discovery.

The MMPs are a homologous family of enzymes with a conserved multidomain structure that serves functionally to enable the proteolytic degradation of complex matrix macromolecules. The enzyme structure comprises a prodomain, which maintains enzyme latency, the catalytic domain that contains the active site, and a C-terminal domain, which is responsible for matrix interactions and substrate and TIMP recognition (10). The catalytic domain can be expressed independently and functions as a competent protease with substrate and inhibitor specificities typical of the full-length parent enzyme (11–14). Several structures of MMP catalytic domains (15–32) and one full-length enzyme (33) have been reported and describe a common protein fold for the MMP active-site domain. This consists of an open-face α-β sandwich made up of three α-helices packed against a 5-stranded β-sheet. The overall structure is ellipsoidal with a flat, surface-exposed substrate-binding cleft on one face of the surface. The zinc-binding active site is contained in the second helix and adjacent loop, followed by a characteristic 1–4 β-turn (Met-turn) which positions an invariant methionine near the catalytic zinc to support the active site.
substructure. After a series of turns that define the $S_1'$ pocket, the structure terminates in the third helix.

The substrate-binding cleft is quite shallow, and most of the binding energy of synthetic inhibitors comes from two functional groups, a substituent that chelates the catalytic zinc and a hydrophobic portion that binds in the $S_1'$ pocket (34). The most variable region among MMP structures is this deep, hydrophobic pocket (35). Sequence differences within the $S_1'$ region define the shape and character of the pocket and appear to be responsible for much of the observed substrate specificity of a given MMP (32). These differences have been exploited to develop inhibitors with increased selectivity for (ideally) a single MMP, or (more realistically) a limited subset of family members. Targeting an MMP inhibitor to the specific enzyme(s) associated with a disease should limit the potential side effects of therapeutic use and allow safe, long-term drug treatment for chronic disorders.

With the development of MMP inhibitors with improved enzyme selectivity, it has become increasingly important not only to identify the enzyme associated with a given disease, but also to characterize animal models to assure that they are dependent on that particular MMP. However, even with an established correlation for the disease model, cognate MMPs from different species may differ in the composition of the $S_1'$ pocket, and inhibitor activity in an animal model may not adequately predict efficacy for the human disease.

The rabbit and human isoforms of stromelysin-1 are highly homologous enzymes, and, in general, inhibitors demonstrate comparable potencies when tested against the purified enzymes. However, we have found that certain very selective human stromelysin inhibitors possess reduced potency against the rabbit enzyme. Using crystal structures of human stromelysin catalytic-domain-inhibitor complexes as guides, we constructed a rabbit stromelysin catalytic domain homology model, which was used to identify specific amino acid interactions within the $S_1'$ pocket that could explain the observed disparity in inhibitor potencies. We then synthesized chimeric mutants of human stromelysin-1, which allowed us to characterize the inhibitor interactions predicted from homology modeling and to identify the specific region of the $S_1'$ pocket that was responsible for the observed differences in inhibitor selectivity of the wild-type isoforms.

**EXPERIMENTAL PROCEDURES**

**Gene Constructs and Reagents**—The synthetic gene for human MMP-3 CD was constructed using previously reported methodology (36). Codons were optimized for Escherichia coli expression, and silent mutations were introduced to maximize the number and distribution of unique restriction sites throughout the gene. The amino acid sequence of the expressed protein was identical to that previously reported for MMP-3 CD from this laboratory (37). The natural gene for rabbit MMP-3 CD was a gift from D. Kelner and S. Hunt (Parke-Davis Pharmaceutical Research, Ann Arbor, MI) and was designed to express a protein corresponding to the human catalytic domain. All constructs were expressed from the pGEMEX-1 plasmid (Promega, Madison, WI) and was designed to express a mammalian vector for the unique sites introduced with the oligo cassettes. Mutant gene sequences were confirmed by dideoxy sequencing of the complete coding region.

**Protein Expression and Purification**—MMP-3 CD plasmids were transformed into BL21(DE3)pLysS competent cells. Two liters of the freshly transformed expressing strains were grown overnight in shaking flasks at 37 °C in Superbroth with phosphates supplemented with ampicillin (50 μg/ml) and chloramphenicol (50 μg/ml) and induced with 1 mM isopropyl-thio-β-D-galactopyranoside for 2 h before harvesting by centrifugation. Mutant and wild-type MMP-3 CD were overexpressed in the presence of 1 mM Mlu and AurII and lyophilized (calf intestine alkaline phosphatase). Plasmids were transformed into BL21(DE3)pLysS competent cells and screened by restriction mapping for the unique sites introduced with the oligo cassettes. Mutant gene sequences were confirmed by dideoxy sequencing of the complete coding sequence.

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**Enzyme Assays and Steady State Kinetic Determinations**—Initial rates for hydrolysis of the thiopeptolide substrate coupled to a reaction with 5,5′-dithiobis(2-nitrobenzoic acid) were used to assess the catalytic activity of mutant and wild-type enzymes. The change of absorbance at 405 nm was monitored continuously at room temperature using a SPECTRAMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). A typical 100-μl reaction contained 50 mM MES, pH 6.0, or 50 mM HEPES, pH 7.0, with 10 mM CaCl₂, 100 μM substrate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 5 mM human or 10 mM rabbit enzyme. For $K_i$ determinations, inhibitors were included in the assay at appro
Amino acid modifications were reasonably well represented among all three small molecule subsets. Modifications were, as a class, much less active against the rabbit enzyme. We had previously shown that the tertiary structure of human stromelysin-1/inhibitor interactions at acidic pH (13). Deletion of other MMP family members, with a general shift to neutral-pH probably reflects diverging substrate preference resulting from the pH dependence of Km. Nevertheless, the observed catalytic efficiencies of human and rabbit stromelysin-1 at pH 6 are reasonably similar, and most synthetic inhibitors show comparable inhibition profiles when tested against these two species isoforms. However, some very selective human MMP-3 inhibitors were, as a class, much less active against the rabbit enzyme. Fig. 2 shows the correlation of Ki values determined for other MMP inhibitors. The test group included the four general classes of synthetic, substrate-competitive inhibitors described in Fig. 3: (a) dibenzofuran, (b) biphenyl, (c) diphenylpiperidine, and (d) reference peptidic compounds. The functional groups interacting with the active site zinc were varied to provide each set with compounds that demonstrated a fairly wide range of Ki values and included both strong and weak zinc-chelating groups.

As a class, diphenylpiperidines segregate from the other inhibitors demonstrating significantly less activity against the rabbit enzyme. We had previously shown that the tertiary phenyl ring in this series was crucial to activity against the human enzyme (50) and expected that the differences between rabbit and human enzyme potencies were due to interactions deep within the S1’ binding pocket. Analysis of the primary sequence of the S1’ region identified 5 residues (Fig. 1) that differed between the two isoforms, yet these differences represented conservative mutations which, on the surface, did not explain the observed disparity of inhibitor potencies. Using the crystal structures of inhibitor-complexed human MMP-3 CD, we constructed a homology model of the rabbit enzyme and used it to identify a 3-residue sequence that could affect inhibitor binding within the S1’ pocket (residues 224 through 226 in Fig. 1). Because the side chain of alanine 225 (serine in the human sequence) was solvent exposed and positioned outside the pocket, it was not expected to interact significantly with the diphenylpiperidine inhibitors and was not included in the mutagenesis studies. In contrast, asparagine 224 and phenylalanine 226 both had side chains that extended into the S1’ pocket (Fig. 4).

The rabbit homology model was constructed to represent an apoenzyme and is shown here with the “collapsed” S1’ subsite characteristic of MMP-3 CD structures where the P1’ side chain does not penetrate deeply into the specificity pocket (26). Although asparagine 224 in this model is stabilized by a hy-
Species Differences for MMP Inhibition

**Fig. 2.** Comparison of inhibitor potencies between human and rabbit stromelysin-1 catalytic domains. IC_{50} values were determined for substrate competitive, synthetic MMP inhibitors at pH 6.0 in the presence of 100 μM substrate and 10 mM CaCl_2 and converted to K_i using the relationship K_i = IC_{50}/k_{cat} + [S]/K_m. K_i for the rabbit enzyme = 929 μM. K_{m} for human MMP-3 CD = 598 μM. Closed circles represent diphenylpiperidines, a class of highly selective human MMP-3 inhibitors; and ×, a selection of other MMP inhibitors.

**Fig. 3.** Representative classes of synthetic MMP inhibitors. Four sets of inhibitors were used to characterize the effect of species differences on inhibitor potencies for stromelysin-1. Examples within each set were chosen so that the portion of the molecule that interacted with the active site zinc would vary to describe a wide range of inhibitor activities, including both strong and weak zinc chelators.

The recent burst of structures for matrix metalloproteinases complexed with small molecule inhibitors has shown that the S_1' pocket is critical in defining inhibitor selectivity. Minor sequence differences within this region can change the shape and hydrophobic character of the pocket resulting in wide variations in inhibitor potencies for a particular compound among members of this family of homologous enzymes (32). These binding interactions have been exploited to develop increas-
ingly potent and selective inhibitors through structure-based drug design processes. Improving selectivity for the human target, however, may result in altered activity for the cognate enzyme in a given animal model of disease. Since it is impractical to test all inhibitors against the purified enzymes from all species used for animal models, it may be possible to predict the species-specific activity of synthetic inhibitors using computer-generated homology models of the animal enzymes.

The rabbit and human isoforms of stromelysin-1 are highly homologous enzymes, demonstrating over 90% identity for the primary amino acid sequence of the catalytic domains. Catalytic efficiencies for synthetic substrates are similar, and, in general, inhibitors show comparable potencies when tested against the purified enzymes. Unexpectedly, one class of very selective inhibitors for human stromelysin-1 was much less potent against the rabbit enzyme. Using crystal structures of these inhibitors complexed with the human catalytic domain, we generated a rabbit homology model that was used to identify specific amino acids within the S1 pocket that might be responsible for the disparity in inhibitor potencies. The model showed that of the several amino acid differences between the two enzymes, only asparagine 224 and phenylalanine 226 of the rabbit enzyme were positioned within the S1 pocket, and both could potentially impact the binding efficiency of the diphenylpiperidine class of inhibitors. Using human to rabbit chimeric mutants, we showed that the mutation of a single amino acid, histidine 224 to asparagine, changed the inhibitor selectivity of the human enzyme to that of the rabbit isoform.

Species Differences for MMP Inhibition

As a family the MMPs are neutral endopeptidases, demonstrating broad bell-shaped curves for the dependence of catalysis on pH with generally uniform and robust activity from pH 5–9. Human stromelysin is unique among the MMP family members in that it has a sharp catalytic optimum between pH 5.5 and 6.0 (46, 47). The fall-off of catalytic efficiency at neutral pH is dramatic with proteolytic activity at pH 7.0 about 32% of the activity at pH 6.0 (Table I). This appears to be an intrinsic property of stromelysin-1 since this effect is observed for both peptide and natural substrates, using either the catalytic domain or full-length enzyme (37, 46–49). Latent stromelysin-1 activates spontaneously at pH 5.5 (51) and is subject to much more rapid autodegradation (37). In addition, TIMP interactions with active MMP-3 are weakened at acidic pH (52), indicating that the exceptional pH dependence of stromelysin activity may serve as an additional layer of post-translational regulation in vivo.

Holman and co-workers (46) constructed the H224Q mutant of human MMP-3 CD and showed that this single mutation released the enzyme from its acidic pH optimum resulting in strong catalytic activity over a broad pH range. In this case, the effect of the mutation was believed to be the stabilization of a pH-dependent S1 pocket that is required in the wild-type enzyme for efficient substrate binding and catalysis. His-224 has been shown to form a hydrogen bond across the S1 pocket with the alanine 217 carbonyl, an interaction that tethers the flexible outer loop of the S1 pocket and, in effect, serves to stabilize the S1 pocket (26, 31). The unprotonated form of His-224 cannot form this bond, and, above its pK_a (predicted to be near pH 6), this interaction would be lost in the wild-type but not the mutant enzyme. In our characterization of the rabbit isofrom (asparagine at position 224), we have also observed a broad pH dependence for catalytic activity that is, not surprisingly, very similar to the H224Q mutant activity. Considering these results, it is possible that human stromelysin-1
has somewhat different physiologic regulation and substrate activity than the corresponding rabbit and rodent enzymes, and disease models in these species may not accurately describe the underlying mechanism of human disease if indeed stromelysin-1 plays a key role in the pathology.

The studies described here emphasize the importance of understanding inhibitor and substrate interactions with species isoforms of the enzymes believed to be active in a given model of human disease. This goes beyond a simple accounting of primary amino acid sequence similarity or identity, since even a single amino acid difference can result in significant differences in potency between the human and animal enzymes. Using a homology model of rabbit stromelysin-1 based on the crystal structure of the human enzyme, we predicted specific amino acid interactions within the $S_1'$ pocket that could be responsible for the differences in inhibitor potency between the rabbit and human enzymes. The biochemical analyses of chimeric mutants confirmed and clarified the model predictions. These data support the use of homology modeling coupled with site-directed mutagenesis to assess inhibitor interactions that may be due to minor sequence differences between cognate enzymes from separate species.

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