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HYDROXAMATE INHIBITORS OF HUMAN GELATINASE B (92 kDa).

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Abstract: Gelatinase B is potently inhibited by peptide hydroxamates, including molecules that have a R_1' group which is larger than the side chains of the natural amino acids.

The matrix metalloproteinases (MMPs) are a family of homologous enzymes whose substrates are molecules of the extracellular matrix.[1] The MMPs are postulated to have a major role in normal and pathological matrix turnover, and are targets of therapeutic inhibitor design.[2] Gelatinases A (72 kDa) and B (92 kDa) are a subgroup of the MMP family whose macromolecular substrates include proteins such as gelatin, elastin, and collagens type IV and V. The specifity of the gelatinases for peptide substrates is similar to that of the interstitial collagenases and stromelysin.[3] As secreted from cells, latent gelatinase is complexed to a molecule of TIMP: gelatinase A with TIMP-2 and gelatinase B with TIMP-1. Upon activation, the gelatinases can be inhibited by binding a second molecule of TIMP. A TIMP-free form of gelatinase B is found in the specific granules of polymorphonuclear leukocytes (PMNs), and this was the source of gelatinase B in this study.[4]

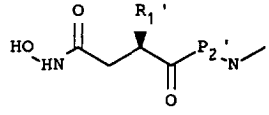
The gelatinases are expressed by a number of transformed cells, and their role in tumor invasion and metastasis is an area of intense interest.[5,6] Recently, gelatinase B was shown to be expressed at high levels in human osteoclasts, which suggests a role in bone remodelling.[7]

In this report, we show that hydroxamate inhibitors that are known to be potent inhibitors of other MMPs are also potent inhibitors of gelatinase B.

Human gelatinase B was purified by a modification of an earlier method.[4] Crude buffy coats from four units of blood were washed by centrifugation in phosphate-buffered saline until the supernatant was clear (three times). The polymorphonuclear leukocytes (PMNs) of the washed buffy coats were degranulated by phorbol myristate acetate as described[4], and the cells were removed by centrifugation. The supernatant (200 ml) was supplemented with 1 mM phenylmethyl-sulfonylfluoride, 0.3 M NaCl, 0.02% sodium azide, and 0.05% Brij-35, and passed over a 10 ml column of gelatin-agarose (G-5384, Sigma). The gelatin-agarose column was washed with 0.05 M tricine, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, 0.02% sodium azide (buffer), and then the bound material was eluted with buffer containing 5% dimethyl sulfoxide. The eluted peak was concentrated with a Centicon-30 (Millipore) and yielded approximately 0.7 A_{280} units from about 3 x 10^9 PMNs. The eluted fraction consisted of a single major band of about 90 kDa after analysis by sodium dodecyl sulfate-

dolyacrylamide gel electrophoresis (SDS-PAGE). Gelatin zymography of the eluted material gave the characteristic triple band pattern of gelatinase B from PMNs.[4] Zymographic analysis also showed that absorption of gelatinase B by the gelatin-agarose column was complete. Neutrophil collagenase was assayed by using SDS-PAGE to detect the formation of the characteristic TC^A/TC^B fragments after incubation with rat tail tendon collagen in the presence of 1 mM *p*-acetoxymercuri-aniline at 22°C. Collagenolytic activity was present in the degranulated PMN supernatant and the gelatin-agarose column flowthrough, but was not detectable in the eluted gelatinase B peak. An extinction coefficient for progelatinase B, 112 mM^{-1} cm^{-1}, was calculated[8] from the deduced content of 13 Trp and 27 Tyr residues.[9] Using this estimate of concentration, gelatinase B was concentrated to approximately 2 μM, and was activated by 1 mM *p*-acetoxymercuri-aniline for 4 h at 37°C. The mercurial was removed by gel filtration, and the activated material was stored at -20°C. This treatment did not

Table 1. Inhibition of MMPs by peptide hydroxamates.



#	R_1'	P_2'	gelatinase B K_i, nM (sd◊)	HNC K_i, nM (sd)	HFC K_i, nM (sd)	HFS K_i, nM (sd)
1	$-CH_3$	Trp	500 (20)	4200	8500	40000
2	$-CH_2CH_3$	Trp	20 (.5)	100	180 (10)	2700
3	$-CH_2CH(CH_3)_2$	Trp	0.69 (.03)	<1	2 (0.5)	46
4	$-CH_2CH(CH_3)_2$	Phe	1.4 (.3)	<1	6 (1)	89 (17)
5	$-(CH_2)_4CH_3$	Val+	330 (9)	190 (8)	300 (10)	1700 (300)
6	$-(CH_2)_4OPh$	Phe	1.2 (.12)	<2	8 (1)	28 (2)
7	$-(CH_2)_5OPh$	Phe	0.31 (.06)	<2	26 (1)	14 (1)
8	$-(CH_2)_4OCH_2Ph$	*t*-butylGly	11 (1.3)	4.7 (2)	1400 (300)	39
9	$-(CH_2)_4OCH_2Ph$	Phe	3.3 (0.1)	<2	1500 (540)	15 (4)
10	$-(CH_2)_4OCH_2Ph$	Gly	5600 (1100)	170 (70)	>100 μM	3500 (700)
11	$-(CH_2)_6Ph$	Phe	1.3 (.2)	<2	8400 (4000)	11 (1)
12	$-(CH_2)_4OPh$-4-$(CH_2)_4CH_3$	*t*-butylGly	3.8 (0.1)	not done	1900 (500)	14 (7)

+ P_3' = 2-hydroxymethylpyrrolidino instead of methylamide. ◊Standard deviation (sd).

result in full activation, as monitored by SDS-PAGE, but was a compromise between activation of the latent species and degradation of the active species. Recombinant catalytic domains of human neutrophil collagenase (HNC), fibroblast collagenase (HFC), and fibroblast stromelysin (HFS) were prepared as described[10]. Inhibition was determined using 300 μM benzoyl-Pro-Leu-Ala-Leu-Tyr(O-methyl)-NH-$(CH_2)_4NHMe_2$ as a substrate.[10] K_i values were calculated from IC_{50} values assuming competitive inhibition using the following K_m values in μM: gelatinase B: 40, HNC: 96, HFC: 220, HFS: 760. Compound **5**, actinonin, was purchased from Sigma (A-6671). The general methods of synthesis of all compounds and the specific syntheses of **6**, **7**, **9**, and **12** are described.[10]

Table 1 shows the K_i values for inhibition of gelatinase B by a group of hydroxamate inhibitors, along with a comparison to three other MMPs. Prototypical MMP inhibitors such as **3** and **4**, which contain the analog of Leu at P_1' and an aromatic amino acid at the P_2' position, are nanomolar inhibitors of gelatinase B. Potent inhibition requires a hydrophobic group with more than two methylene units at the P_1' postition. Thus, there is a 1380 fold loss of activity when R_1' = methyl and a 29 fold loss of activity when R_1' = ethyl compared to R_1' = *i*-butyl (**1** versus **3** and **2** versus **3**, respectively). For compounds **1-4**, there is a trend of decreasing potency of inhibition across the panel of the MMPs as they are listed in Table 1: A typical compound is most potent versus gelatinase B, somewhat less potent versus HNC, somewhat less potent again versus HFC, and least potent versus HFS. This trend corresponds to the trend of K_m values seen for the substrate used in this study. The correlation of K_i and K_m may suggest that gelatinase B has greater ground state binding, versus transition state binding, of peptide substrates and peptide hydroxamate inhibitors than other MMPs.[11] Compounds **3** and **4** (R_1' = *i*-butyl) gives nearly optimal potency. As the R_1' group increases in size, **6-9** and **11-12**, the potency is maintained at the nanomolar level. The most potent inhibition of gelatinase B, 0.31 nM, is achieved with compound **7** (R_1' = phenoxypentyl-). Given the uncertainty of the concentration of active gelatinase B in our assays, this value is an upper limit, due to potential depletion of the inhibitor by titration with the enzyme. Actinonin, which has R_1' = *n*-pentyl, cannot be properly compared in this series because of differences in the P_2'-P_3' region, but is included as a reference compound.

At P_2', a Gly for Phe substitution results in a 1700-fold loss of gelatinase B potency (**10** versus **9**). A Trp for Phe substitution gives a two fold increase in potency (**3** versus **4**) and a *t*-butylGly for Phe substitution gives a three fold loss in potency (**8** versus **9**). Thus, a hydrophobic R_2' group is required for potent inhibition.

In the structure of the complex of HFC[12] with **4**, the S_1' specificity pocket of HFC is delimited to approximately the size of an isobutyl group by the side chain of Arg-214. However, the structure of the complex of HNC with the same inhibitor showed that HNC has a much larger S_1' specificity pocket.[13] In HNC, residue 214 is Leu, not Arg, and the *i*-butyl side chain does not close off the S_1' pocket. We have shown that inhibitors with large P_1' groups are potent inhibitors of both HNC and HFS, which also has Leu-214.[10, 15] Among the MMPs, only HFC has Arg at residue 214; HFS, HNC, and gelatinase B have Leu at this position.[14] Given the

high sequence identity among MMPs (>50%), it is likely that they have very similar folds and specificity pockets. Indeed, the HFC and HNC structures are extremely similar.[13] It is likely that the presence of Leu-214, and the absence of Arg-214, is a general indicator of the presence of a large S_1' pocket. The nanomolar potency of **6-9** and **11-12** versus gelatinase B argue that this is the case for this MMP. Modelling studies with the HNC structure or a homology model of HFS suggest that the R_1' group of **12** can traverse the complete length of the S_1' pocket, or tunnel, and that the terminal methyl of the *n*-pentyl group of **12** reaches the solvent on the opposite side of the protein from the active site.[10] Again, the nanomolar potency of **12** versus gelatinase B argues for the presence of an analogously large S_1' pocket as is found in HNC (and HFS).

We are unaware of any previous reports on the inhibition of gelatinase B by synthetic inhibitors. The largest R_1' group previously reported in any MMP inhibitor is $-(CH_2)_4Ph$, which was present in a gelatinase A (72 kDa) inhibitor,[16] although $-(CH_2)_3Ph$ was the optimally sized R_1' group in that study.

The physiological significance of the ability of MMPs to accommodate very large P_1' groups is unknown at this time. Indeed, the properties of substrates with R_1' groups larger than those of the natural amino acids have not yet been examined.

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