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EVALUATION OF THE INHIBITION OF OTHER METALLOPROTEINASES BY MATRIX METALLOPROTEINASE INHIBITORS

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Two series of compounds synthesized as specific matrix metalloproteinase (MMP) inhibitors have been evaluated for their inhibition of non-MMPs. In a series of substituted succinyl hydroxamic acids, some were found to be significant ($IC_{50} < 1\ \mu M$) inhibitors of leucine (microsomal) aminopeptidase, neprilysin (3.4.24.11), and thermolysin. Macrocyclic compounds in which the alpha carbon of the succinyl hydroxamate is linked to the side chain of the P2′ amino acid were found to be good inhibitors of aminopeptidase, but not of neprilysin or thermolysin. Compounds of neither series were found to be significant inhibitors of angiotensin converting enzyme or carboxypeptidase A.

Keywords: Matrix metalloproteinases; MMPs; Hydroxamic acid inhibitors

Abbreviations: MMP(s), matrix metalloproteinase(s); MMP-1, fibroblast collagenase; MMP-2, gelatinase A; MMP-3, stromelysin; MMP-8, neutrophil collagenase; MMP-9, gelatinase B; MMP-13, collagenase 3.

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INTRODUCTION

The inhibition of the matrix metalloproteinases (MMPs) has been the subject of intense research interest in the pharmaceutical industry in recent years.[1–3] Among the possible uses of such inhibitors are new drugs for the treatment of arthritis[4] and cancer.[5,6] A major objective has been the discovery of agents which inhibit the MMPs believed to be major contributors to the disease process, sparing enzymatic activity required for normal matrix remodeling. As the specificity profile depends on the therapeutic target, some inhibitors, proposed as cancer therapeutics, have relative specificity for the gelatinases (MMP-2, MMP-9).[7] Other compounds, designed as anti-arthritis agents, are relatively specific inhibitors of neutrophil collagenase (MMP-8)[8,9] whereas compounds with relative inhibitory specificity for the collagenases (MMP-1, MMP-8, MMP-13)[10] or stromelysin (MMP-3)[11] have also been described.

However, compounds devised as MMP inhibitors may not necessarily inhibit only this class of enzymes. Other proteolytic enzymes also contain zinc at the active site and might be inhibited by such compounds, which most commonly contain a metal chelating group attached to a peptide or peptide mimetic.[2,3] In an effort to examine more fully the specificity of selected inhibitors, we have studied the potency of two series of compounds as inhibitors of five metalloproteinases which are not members of the MMP family of enzymes. The enzymes tested are porcine leucine (microsomal) aminopeptidase, rat neprilysin (3.4.24.11), the bacterial metalloendoproteinase thermolysin, bovine carboxypeptidase A, and rabbit angiotensin converting enzyme.

MATERIALS AND METHODS

Leucine aminopeptidase (porcine kidney microsomes), thermolysin (*Bacillus thermoproteolyticus rokko*), carboxypeptidase A (bovine pancreas), and angiotensin converting enzyme (rabbit lung) were purchased from Sigma. The commercially available substrates and standard inhibitors described below were also purchased from Sigma. Neprilysin (neutral metalloendopeptidase 24.11) was partially purified from rat kidney microsomes (through the concanavalin A column) using a published method.[12]

All assays were carried out in a 96-well microtiter plate format (0.15 mL/well) using either an absorbance or fluorescence plate reader. Leucine aminopeptidase was assayed by monitoring the increase at 405 nm upon hydrolysis

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of leucine-*p*-nitroanilide (0.5 mM).[13] Carboxypeptidase A was assayed by monitoring the decrease at 328 nm upon its reaction with *N*-(3-[2-furyl] acryloyl)Phe-Phe (0.2 mM),[14] and similarly angiotensin converting enzyme was assayed using *N*-(3-[2-furyl]acryloyl)Phe-Gly-Gly (0.2 mM).[15] Neprilysin and thermolysin were found to cleave a fluorescent substrate of the sequence Ac-Gly-Glu(EDANS)-Gly-Pro-Leu-Gly-Leu-Tyr-Ala-Lys(DABCYL)-Gly (0.01 mM). A 30-fold enhancement of the fluorescence (ex: 335 nm, em: 485 nm) of the EDANS group is observed upon hydrolysis of the peptide.

For each enzyme a control inhibitor was employed to verify the assay, and the inhibition constants observed were consistent with reported values. The standard inhibitors were bestatin for leucine aminopeptidase;[16] phosphoramidon for neprilysin[17] and thermolysin;[8] DL-benzylsuccinic acid for carboxypeptidase A;[19] and captopril for angiotensin converting enzyme.[20] The inhibitory potencies were calculated by plotting the logit function of the percentage inhibition against the logarithm of the concentration of the inhibitor, and then calculating the IC_{50} by a linear regression analysis.

The general methodology for synthesis of these inhibitors has been described.[21–23] The determinations of fibroblast collagenase (MMP-1) and gelatinase A (MMP-2) inhibition are listed for comparison. The IC_{50}s listed for previously reported compounds are values determined in our laboratory, and are generally in agreement with published data. The methods employed and some of the assay data have previously been reported.[21–23]

RESULTS

The compounds in this study are of two classes. Ten compounds are substituted acyclic succinyl hydroxamates, illustrated in Table I, all of which contain an *iso*-butyl group at R^2. Several of these compounds have been reported by others: for example, compound **1** is Ro 31-9790,[24] compound **2** is marimastat (BB-2516),[25] and compound **8** is GI 129471.[26] They are potent, broad spectrum inhibitors of MMPs, but they are not exclusive in their inhibition, as many of the compounds have significant inhibitory potency against some of the other metalloproteinases (Table II). Neprilysin inhibition is not greatly affected by a hydroxyl group at R^1, but hydrophobic substituents at this position lead to compounds **7–10** with much reduced activity. Conversely, the compounds with hydrophobic R^1 substituents have enhanced potency as aminopeptidase inhibitors. A benzyl substituent at R^3 seems to be somewhat preferred to *tert*-butyl in inhibitors of both neprilysin and aminopeptidase M. The most potent inhibitors of

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TABLE I Structures of acyclic succinyl hydroxamate MMP inhibitors

	R^1	R^2	R^3	R^4
1	H	*iso*-butyl	*tert*-butyl	$NHCH_3$
2	OH	*iso*-butyl	*tert*-butyl	$NHCH_3$
3	OH	*iso*-butyl	*tert*-butyl	NH–phenyl
4	OH	*iso*-butyl	*tert*-butyl	NH–2-pyridyl
5	H	*iso*-butyl	CH_2–phenyl	$NHCH_3$
6	OH	*iso*-butyl	CH_2–phenyl	$NHCH_3$
7	CH_3	*iso*-butyl	CH_2–phenyl	$NHCH_3$
8	CH_2-S–phenyl	*iso*-butyl	CH_2–phenyl	$NHCH_3$
9	$(CH_2)_3$–CH=CH$_2$	*iso*-butyl	CH_2–phenyl	$NHCH_3$
10	$(CH_2)_3$–CH=CH$_2$	*iso*-butyl	CH_2–3-indolyl	$NHCH_3$

thermolysin are unsubstituted at the alpha carbon (compounds **1** and **5**), but significant inhibition by compounds with either a hydroxyl or hydrophobic group at R^1 is also observed. Although the compounds are more potent inhibitors of the representative MMPs (MMP-1 and MMP-2) than these other enzymes, in some cases the relative selectivity is only about 10-fold.

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TABLE II Inhibition of metalloproteinases by succinyl hydroxamate MMP inhibitors (IC_{50} [Molar])

Compound number	*MMP-1*	*MMP-2*	*Aminopeptidase M*	*Neprilysin*	*Thermolysin*	*Carboxypeptidase A*	*Angiotensin converting enzyme*
1	1.3×10^{-9}	7.5×10^{-9}	5.4×10^{-6}	6.2×10^{-7}	8.9×10^{-8}	$> 10^{-4}$	$> 10^{-4}$
2	4.7×10^{-10}	3.8×10^{-10}	5.0×10^{-6}	1.6×10^{-6}	1.7×10^{-6}	$> 10^{-4}$	$> 10^{-4}$
3	3.2×10^{-9}	2.0×10^{-9}	4.2×10^{-6}	4.2×10^{-7}	3.7×10^{-7}	$> 10^{-4}$	$> 10^{-4}$
4	3.4×10^{-9}	1.0×10^{-9}	5.6×10^{-6}	5.2×10^{-7}	3.7×10^{-7}	$> 10^{-4}$	$> 10^{-4}$
5	2.5×10^{-9}	7.0×10^{-10}	5.7×10^{-7}	8.1×10^{-8}	2.6×10^{-8}	$> 10^{-4}$	$> 10^{-4}$
6	3.3×10^{-9}	8.0×10^{-10}	1.5×10^{-6}	7.2×10^{-8}	1.3×10^{-7}	$> 10^{-4}$	$> 10^{-4}$
7	1.3×10^{-9}	1.3×10^{-9}	8.2×10^{-7}	3.6×10^{-5}	8.6×10^{-7}	$> 10^{-4}$	$> 10^{-4}$
8	1.4×10^{-9}	1.0×10^{-9}	1.2×10^{-7}	$> 10^{-4}$	4.1×10^{-7}	$> 10^{-4}$	$> 10^{-4}$
9	1.6×10^{-9}	1.4×10^{-9}	6.3×10^{-8}	5.0×10^{-6}	3.8×10^{-7}	5.7×10^{-5}	$> 10^{-4}$
10	6.0×10^{-10}	3.9×10^{-9}	4.3×10^{-8}	6.1×10^{-6}	1.5×10^{-6}	$> 10^{-4}$	$> 10^{-4}$

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The second set of inhibitors consists of 14 compounds in which the alpha substituent of the succinate backbone is connected to the side chain of the amino acid producing a macrocyclic bridge, as illustrated in Tables III and IV. We have recently reported[22] the inhibition of MMPs by compounds of this type, and similar inhibitors have been reported by others.[27] In Table V, we show that nearly all of these compounds are more potent inhibitors of aminopeptidase M than the acyclic compounds. As with examples previously reported,[22] compounds with large groups at R^2 (Table IV) exhibit some selectivity in their inhibition of MMP-2, and this is also consistent with the reported specificity of acyclic MMP inhibitors.[28] However, the nature of the R^2 substituent does not greatly influence the inhibition of aminopeptidase M. None of the macrocyclic compounds significantly inhibits neprilysin or thermolysin, which may be the result of some steric conflict between the active site clet of these enzymes and the macrocyclic ring. These findings are in accord with hydrophobic substituents at R^1 in the acyclic

TABLE III Structures of macrocyclic MMP inhibitors with *iso*-butyl substituents at R^2

	R^2	R^4
11	*iso*-butyl	$NHCH_3$
12	*iso*-butyl	$NH\text{-}CH_2\text{-}CH_2\text{-}C_6H_4\text{-}SO_2NH_2$
13	*iso*-butyl	NH–(2-pyridyl)
14	*iso*-butyl	$NH\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_3$
15	*iso*-butyl	$NH\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)_2$
16	*iso*-hexyl	$NHCH_3$

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TABLE IV Structures of macrocyclic MMP inhibitors with hydrophobic substituents at R^2

	R^2	R^4
17	$(CH_2)_3-C_6H_4-CH_3$	$NH-CH_2-CH_2-N(CH_3)_2$
18	$(CH_2)_3-C_6H_4-CH_3$	N (piperidine ring)
19	$(CH_2)_3-C_6H_4-CH_3$	HN– (thiazole ring, N, S)
20	$(CH_2)_3-C_6H_4-CH_3$	$NH-CH_2-CH_2-NH-SO_2-N$ (morpholine ring, O)
21	$(CH_2)_3-C_6H_4-CH_2-CH_2-CH_3$	$NHCH_3$
22	$(CH_2)_3-C_6H_4-Cl$	$NHCH_3$
23	$(CH_2)_3-C_6H_4-O-CH_2-CH_3$	$NHCH_3$
24	$(CH_2)_3$–(naphthyl)	$NHCH_3$

compounds having enhanced aminopeptidase inhibitory activity whereas the neprilysin inhibitory activity is decreased.

Although angiotensin converting enzyme and carboxypeptidase A also act on peptide substrates and contain zinc at the active site, inhibition of these enzymes was observed with some compounds only at high concentrations (30–100 μM), and in most cases the inhibition did not reach 50% at 100 μM. These concentrations are greater than 1000-fold higher than those used for inhibition of the MMPs. As many of the reported inhibitors of

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TABLE V Inhibition of metalloproteinases by macrocyclic MMP inhibitors (IC_{50} [Molar])

Compound number	*MMP-1*	*MMP-2*	*Aminopeptidase M*	*Neprilysin*	*Thermolysin*	*Carboxypeptidase A*	*Angiotensin converting enzyme*
11	1.8×10^{-9}	5.5×10^{-9}	2.8×10^{-8}	6.9×10^{-5}	7.2×10^{-5}	$> 10^{-4}$	$> 10^{-4}$
12	3.0×10^{-9}	1.9×10^{-9}	2.2×10^{-9}	$> 10^{-4}$	9.5×10^{-5}	$> 10^{-4}$	$> 10^{-4}$
13	4.6×10^{-9}	2.1×10^{-9}	8.8×10^{-9}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
14	2.7×10^{-9}	3.8×10^{-9}	1.5×10^{-8}	$> 10^{-4}$	3.9×10^{-5}	$> 10^{-4}$	$> 10^{-4}$
15	6.6×10^{-9}	6.5×10^{-9}	1.7×10^{-7}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
16	5.8×10^{-9}	1.0×10^{-9}	2.2×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
17	3.1×10^{-8}	2.7×10^{-10}	5.4×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
18	1.9×10^{-7}	1.4×10^{-10}	3.6×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	*ca.* 10^{-4}
19	9.3×10^{-8}	8.1×10^{-10}	6.4×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	4.1×10^{-4}	$> 10^{-4}$
20	1.2×10^{-8}	2.6×10^{-9}	1.9×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
21	5.4×10^{-8}	1.1×10^{-10}	3.6×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
22	2.3×10^{-9}	2.6×10^{-10}	9.8×10^{-9}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
23	6.8×10^{-9}	7.9×10^{-11}	4.3×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
24	2.8×10^{-8}	2.3×10^{-10}	6.6×10^{-8}	$> 10^{-4}$	$> 10^{-4}$	*ca.* 10^{-4}	$> 10^{-4}$

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angiotensin converting enzyme and carboxypeptidase A contain a carboxylate group to mimic those of the products of these enzymatic reactions,[19,29] the absence of inhibition of these enzymes may not be surprising, since these MMP inhibitors do not incorporate such a moiety. However, none of the 24 compounds tested contain a free amino group which could bind to the natural site of the aminopeptidase substrate, yet many potent aminopeptidase inhibitors have been found among the compounds synthesized as MMP inhibitors.

DISCUSSION

Bestatin (ubenimex), an aminopeptidase inhibitor discovered as a natural product,[16] has been used in clinical trials as a biological response modifier in adjuvant cancer therapy;[30] and it has been shown to augment natural killer cell activity and the tumor killing activity of peripheral blood lymphocytes and spleen cells via macrophage activation.[31] Inhibitors of neprilysin have been developed for the treatment of cardiovascular diseases,[32] as this enzyme has been shown to degrade atrial natriuretic factor and other vasoactive peptides both in animal models[12] and in humans.[33,34] Compounds which inhibit both neprilysin and angiotensin converting enzyme, termed dual metalloproteinase inhibitors, have been devised,[35] and a clinical study of such an agent has been reported.[36] Therefore the degree of inhibition of mammalian aminopeptidase and neprilysin by compounds being developed as MMP inhibitors must be taken into consideration.

Although the enzymes used for these comparisons are from animal sources, human and rat neprilysin are 94% identical in sequence,[37] and the porcine and human leucine (microsomal) aminopeptidases are 80% identical.[38] It has also been shown that the human myeloid plasma membrane glycoprotein CD13 has the same sequence as microsomal aminopeptidase.[39] Thus the inhibition of the rat and porcine enzymes is likely to be predictive of inhibition of the corresponding human proteinases. The inhibition of thermolysin by acyclic MMP inhibitors should not be of clinical relevance and it is not surprising, as peptide hydroxamates have been previously shown to inhibit this bacterial metalloproteinase.[40]

This work demonstrates that inhibitors of one class of metalloproteinases can be inhibitors of other such enzymes, thus *a priori* predictions of specificity must be tested experimentally. Even though the macrocyclic compounds were devised through modeling the interaction of acyclic compounds in an MMP active site,[22] they are coincidentally also potent aminopeptidase

inhibitors. These findings indicate that, although the enzymes differ in substrate specificity, the active site clefts of the MMPs and microsomal aminopeptidase must be similar in structure. Other series of compounds should be similarly evaluated with non-MMPs in the development of inhibitors which have specific action against target MMPs.

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