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PROTEOGLYCAN DEGRADATION BY A CHONDROCYTE METALLOPROTEASE

EFFECTS OF SYNTHETIC PROTEASE INHIBITORS

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Abstract—Synthetic inhibitors of a chondrocyte metalloprotease (CMP) were assessed for potency. Proteoglycan core protein was used as substrate. The IC_{50} values were between 2×10^{-6} and 7×10^{-6} M for two types of inhibitors, thiol tripeptides and *N*-carboxyalkyl peptides. Hydroxamic acid peptides were more potent, with IC_{50} values of 3.2×10^{-8} to 6.0×10^{-8} M. These results confirm inhibitory concentrations reported using a proteoglycan-polyacrylamide bead assay. The slopes of the dose–response curves for the thiol compounds were steeper than the slopes for the other two types of compounds. All of the culture media tested inhibited CMP to some extent. Some media also interfered with inhibitor activity. In Ham's F10 nutrient medium, minimum CMP inhibition occurred, and all four hydroxamic acid peptides retained their activity for 1–2 days at 37°. One thiol peptide compound assayed lost activity in 1 hr in thiocyanate-treated serum. All four hydroxamic acid peptides assayed retained activity in thiocyanate-treated serum after 3 days at 37°. The hydroxamic acid peptides may provide a way to block endogenous CMP activity *in vivo* and to assess the role of CMP in normal and experimentally altered cartilage. They are more potent than other known CMP inhibitors. They retain activity in culture media and serum conditions used for *in vivo* and *in vitro* tests of CMP activity and toxicity.

Metalloproteases that degrade proteoglycan and collagen are produced by chondrocytes *in vitro* [1]. Upon stimulation with interleukin-1, chondrocytes isolated from rabbit articular cartilage release these enymes in latent form. APMA† activates both collagenase and PG-degrading activities in the culture medium. These enzymes separate on Ultrogel AcA 54. They display molecular weights between 21,000 and 36,000 daltons for collagenase and between 30,000 and 40,000 daltons for the PG-degrading protease [2]. Both enzymes are calcium dependent. They are inhibited by metalloprotease inhibitors but not by inhibitors of other types of proteases [1, 2]. These metalloproteases resemble enzymes isolated from bovine chondrocytes, rabbit cartilage explant cultures, and extracted human cartilage described by other laboratories [3–5].

Di- and tripeptides with a thiol, carboxylic acid, or hydroxamic acid functional group are known inhibitors of metalloproteases, including angiotensin-converting enzyme [6–8] and thermolysin [9, 10], and of enkephalin-degrading metallopeptidases [11]. The functional group is thought to chelate the metal at the active site of the enzyme. Recently, we synthesized several novel peptide compounds with these functional groups that are potent inhibitors of rabbit CMP, assayed by measuring carbohydrate released over 6–20 hr from PG entrapped in beads [1]. The carboxyalkyl peptides do not inhibit collagenase, while the other synthetic inhibitors are less potent as collagenase inhibitors than as CMP inhibitors [12].

Roles for cartilage metalloproteases have been proposed in osteoarthritis [13, 14], animal models of arthritis [15], and *in vitro* cartilage autolysis [16–18]. Phenanthroline and EDTA have been administered *in vivo* to probe the role of metalloproteases in several experimental models of osteoarthritis [19, 20] and in cartilage autolysis *in vitro* [16–18]. In some systems, decreased cartilage matrix loss occurs upon adding inhibitors. A toxic effect of the compounds on chondrocytes, rather than enzyme inhibition, cannot be ruled out as a mechanism of action of these inhibitors [17].

The present studies were designed to study the effects of the synthetic inhibitors of CMP on degradation of core protein during the first hour of degradation. Degradation of the core protein portion of PG was measured in an assay that uses PG radiolabeled in the core protein. Degradation products were separated from substrate on an ion exchange column. This assay also provides a way to measure inhibitor activity in serum. We evaluated the stability and potency of these inhibitors in culture medium and serum.

MATERIALS AND METHODS

Materials. All culture media and media components were from GIBCO (Grand Island, NY). Fetal calf serum was from the Armour Pharmaceutical Co. (Kankakee, IL) and antibiotics were from the Schering Corp. (Kenilworth, NJ). Sodium

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† Abbreviations: APMA, amino-phenyl mercuric acetate; CMP, chondrocyte metalloprotease; CS, chondroitin sulfate; DEAE, diethylamino ethyl; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; IC_{50}, concentration that produces 50% inhibition of CMP activity; and PG, proteoglycan.

thiocyanate was from Fisher Scientific (Pittsburgh, PA). APMA and ortho-phenanthroline were from the Sigma Chemical Co. (St. Louis, MO). Interleukin-1 was purchased from the Genzyme Corp. (Boston, MA). PD-10 columns and DEAE Sephacel were from Pharmacia Fine Chemicals (Piscataway, NJ). All solvents were reagent grade.

The following compounds were synthesized:
U11790: α-(*S*)-acetylamino-*N*-[2-(*S*)-1-mercapto-4-methyl)pentyl]benzenepropanamide;
U11866: α-(*S*)-acetylamino-*N*-[2-(*S*)-(1-mercapto-4-methyl)-pentyl]-β-hydroxyethanamide;
U18598: α-(*R*,*S*)-acetylamino-*N*[2-(*S*)(1-mercapto-4-methyl)pentyl]-β-(3-indolyl)-propanamide;
U18857: α-(*S*)-[*N*-acetyl-D,L-phenylalanyl]-*N*-[2-(*S*)-(1-mercapto-4-methyl)pentyl]benzenepropanamide;
U19345: *N*-[4-methyl-2-(*R*,*S*)-mercaptomethyl-1-oxopentyl]-L-phenylalaninamide;
U19346: *N*-[4-methyl-2-(*R*,*S*)-mercaptomethyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide;
U22136: α-(*R*,*S*)-acetylamino-*N*-[2-(*S*)-(1-mercapto-3-methyl)pentyl]-β-(3-indolyl)-propanamide;
U22203: α-(*R*,*S*)-acetylamino-*N*-[2-(*S*)-(1-mercapto-3-methyl)pentyl]benzenepropanamide;
U22312: (*R*,*S*)-*N*-[(1-carboxy-3-methyl)butyl]-L-leucyl-L-leucyl-L-leucine methyl ester;
U22932: (*R*)-*N*-[(1-carboxy-3-methyl)butyl]-L-leucyl-L-leucyl-L-alaninamide;
U22989: (*R*)-*N*-[(1-carboxy-3-methyl)butyl]-L-leucyl-L-leucyl-L-phenylalaninamide;
U23161: (*R*)-*N*-[(1-carboxy-3-methyl)butyl]-L-leucyl-L-leucyl-L-leucyl-L-alaninamide;
U24278: (*R* or *S*)-*N*-[2-[2-(hydroxyamino)-2-oxoethyl]-1-oxoheptyl]-L-leucyl-L-phenylalaninamide;
U24279: (*R*,*S*)-*N*-[2-[2-(hydroxyamino)-2-oxoethyl]-1-oxoheptyl]-L-leucyl-L-alaninamide;
U24522: (*R*,*S*)-*N*-[2-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide; and
U24631: *N*-[2-[2-(hydroxyamino)-2-oxoethyl]-1-oxoheptyl]-L-valyl-L-alaninamide. (Abbreviated structures are provided in Table 1).

Source of rabbit chondrocyte metalloprotease (CMP). Enzyme was obtained from cultured chondrocytes as described previously [1]. Briefly, rabbit knee articular cartilage from the femur and tibia was removed under aseptic conditions, diced, and incubated sequentially with hyaluronidase, trypsin and collagenase to remove the extracellular matrix. The isolated chondrocytes were plated at a density of 3×10^4 cells per cm^2 in Ham's F12 nutrient mixture with 10% fetal calf serum and 25 μg/ml gentamicin. Upon reaching confluency, cultures were treated with 10–50 units of interleukin-1 per ml to stimulate CMP production. After 3 days, medium was removed from the cells and dialyzed exhaustively into 50 mM Tris buffer, pH 7.4, with 200 mM sodium chloride, 5 mM calcium chloride, and 0.02% azide. CMP was activated with 0.34 mM APMA for 6 hr at room temperature and then dialyzed into the Tris buffer to remove APMA. The average CMP activity for seventeen samples from different rabbit chondrocyte cultures was 12.4% of [^{35}S]methionine-labeled PG released per hr per 100 μl of sample (range, 5.9–29.6%) using the assay described below.

Assay for degradation of core protein. Radiolabeled PG monomer was prepared as described previously [21, 22]. Rat chondrosarcoma chondrocytes were isolated and cultured in the presence of radiolabeled [^{35}S]methionine and [^{3}H]serine. Labeled PG in the medium and in the Zwittergent-extracted cell layer was isolated by chromatography on prepacked PD-10 columns followed by DEAE-Sephacel chromatography. Rabbit CMP was concentrated 10-fold on an Amicon YM 5 filter and then diluted to 1–2 times its original concentration with either 50 mM Tris buffer containing 10 mM calcium chloride, pH 7.4, or with test medium or serum. Fetal calf serum was heat-inactivated at 56° for 30 min prior to use. Inhibitors were dissolved in DMSO and diluted in buffer, medium, or serum prior to enzyme additions. The final concentration of DMSO never exceeded 1%, which does not interfere with CMP activity in this assay (unpublished observation). Core protein degradation was monitored as follows. A volume of 10 μl of radiolabeled PG monomer, which contained 400,000 dpm of [^{35}S], without carrier PG, was added to the mixture of 20 μl of the 10-fold concentrated CMP and 80 μl of test compound in buffer, medium, or serum. For some experiments these volumes were reduced by half. Endogenous inhibitors of CMP in serum were inactivated by treating rabbit serum with 3 M sodium thiocyanate for 6 hr at 4° and then dialyzing the serum to remove excess thiocyanate. The digestion mixtures were incubated at 37° for 1 hr. The entire digest was applied to 1 ml of DEAE. Two milliliters of 50 mM Tris buffer was applied to the column, and 0.5 ml of the collected fraction was counted for radioactivity. Degradation was calculated as the percent of total [^{35}S] radioactivity that did not bind DEAE after digestion with CMP.

The log of the dose of each inhibitor was plotted against the log of the percent inhibition of enzyme activity. The linear regression fit to the data, r^2 values, and IC_{50} values were calculated.

Radiolabeled proteoglycan trapped in polyacrylamide beads. In one study, degradation of labeled proteoglycan in solution, as described above, was compared with degradation of labeled PG trapped in polyacrylamide beads. Beads were prepared and assayed as described previously [23], except that 100 mg of non-labeled rat chondrosarcoma PG monomer was dissolved in 5.6 ml of labeled PG (1.7×10^7 dpm of [^{3}H] and 0.9×10^8 dpm of [^{35}S]). Tris was added to the labeled PG solution to a final concentration of 1 M, and the solution was titrated to pH 8.5. To this mixture 20 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 1.6 ml of acrylamide solution and 2.4 ml of ammonium persulfate solution were added. Other beads were made using only non-labeled rat chondrosarcoma PG monomer, with the same protocol as used for labeled PG. Radiolabeled PG released from beads was assayed on DEAE columns as described above.

RESULTS

Rabbit CMP released 20.5 to 22.6% of the total substrate radioactivity in 1 hr. This amount was decreased in the presence of inhibitors. Inhibition by thiol compounds resulted in steep dose–response

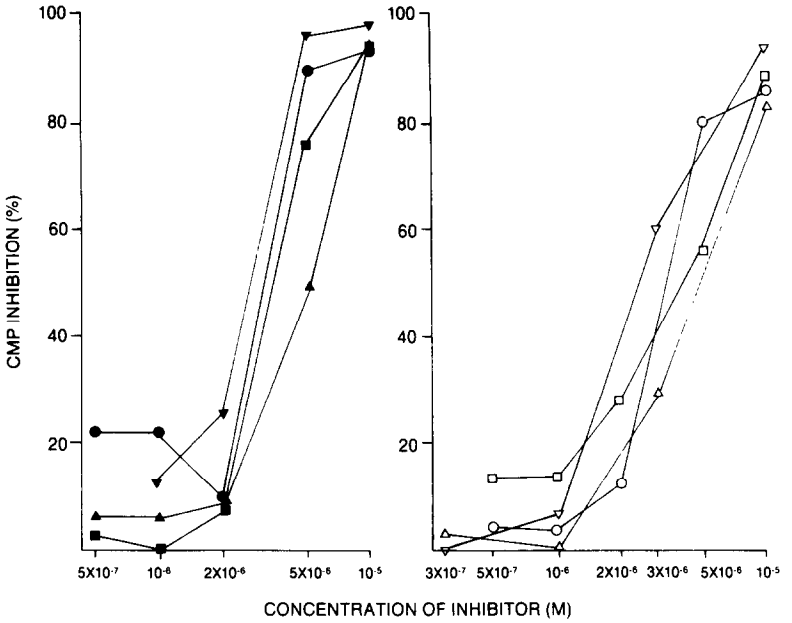

Fig. 1. Chondrocyte metalloprotease activity measured in the presence of thiol di- and tripeptides. In the absence of inhibitors, CMP released 22.6 ± 0.8% of [^{35}S] from [^{35}S]methionine-labeled proteoglycan in 1 hr. The relative inhibition of this activity in the presence of various thiol peptides is illustrated. Key: (▼) U18598; (●) U11866; (■) U11790; (▲) U18857; (▽) U22136; (○) U19345; (□) U19346; (△) U22203.

curves (Fig. 1), compared to the slopes for the dose–response curves for carboxyalkyl peptides (Fig. 2). The slopes of the dose–response curves for the hydroxamic acids were similar to the slopes for the carboxyalkyl peptides, although the hydroxamic acids were more potent inhibitors (Fig. 3). The IC_{50} values are listed in Table 1. The r^2 values ranged from 0.89 to 0.99.

The reproducibility of CMP activity and inhibitor activity in this assay was assessed. Three inhibitors were assayed in five assays run on separate days, using the same CMP preparation in each assay. The enzyme activity varied from 16.7 to 18.7% of substrate degraded in 1 hr (Table 2). Consistent levels of inhibition over the five experiments were produced, with no value more than 7 percentage points from the mean level of inhibition for each inhibitor.

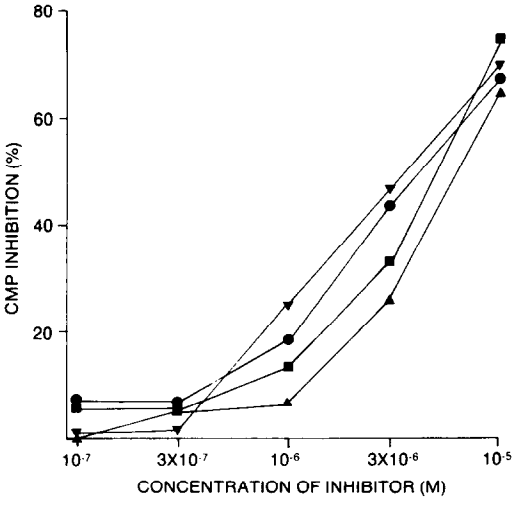

Fig. 2. Chondrocyte metalloprotease activity measured in the presence of *N*-carboxyalkyl peptides. In the absence of inhibitors, CMP released 20.4 ± 0.6% of [^{35}S] from [^{35}S]methionine-labeled proteoglycan in 1 hr. The relative inhibition of this activity in the presence of various *N*-carboxyalkyl peptides is illustrated. Key: (▼) U22312; (●) U22932; (■) U22989; and (▲) U23161.

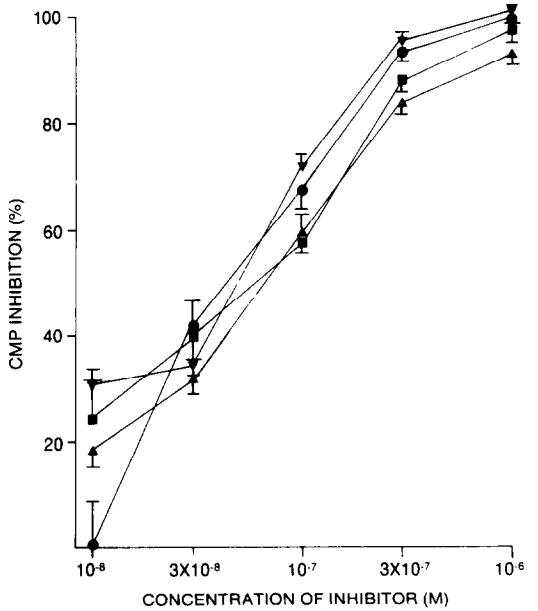

Fig. 3. Chondrocyte metalloprotease activity measured in the presence of hydroxamic acid peptides. In the absence of inhibitors, CMP released 13.9 ± 1.3% of [^{35}S] from [^{35}S]methionine-labeled proteoglycan in 1 hr. The relative inhibition of this activity in the presence of various hydroxamic acid peptides is illustrated. Data are means ± SE for triplicate samples. Key: (▼) U24279; (●) U24631; (■) U24278; and (▲) U24522.

Table 1. Abbreviated structures for the test compounds and IC_{50} values calculated from the data shown in Figs. 1–3

Class	Compound No.	Structure*	IC_{50} (M)
Thiol	U11790	AcetylPhe-LeuSH	4×10^{-6}
	U11866	AcetylSer-LeuSH	3×10^{-6}
	U18598	AcetylTrp-LeuSH	2×10^{-6}
	U18857	AcetylPhe-Phe-LeuSH	4×10^{-6}
	U19345	$HSCH_2CH$(i-Butyl)$COPheNH_2$	4×10^{-6}
	U19346	$HSCH_2CH$(i-Butyl)COLeu-$PheNH_2$	3×10^{-6}
	U22136	AcetylTrp-IleSH	3×10^{-6}
	U22203	AcetylPhe-IleSH	6×10^{-6}
Carboxylic acid	U22312	HOOCCH(i-Butyl)Leu-Leu-$LeuOCH_3$	4×10^{-6}
	U22932	HOOCCH(i-Butyl)Leu-Leu-$AlaNH_2$	4×10^{-6}
	U22989	HOOCCH(i-Butyl)Leu-Leu-$PheNH_2$	5×10^{-6}
	U23161	HOOCCH(i-Butyl)Leu-Leu-Leu-$AlaNH_2$	7×10^{-6}
Hydroxamic acid	U24278	$HONHCOCH_2CH$(n-Pentyl)COLeu-$PheNH_2$	4.1×10^{-8}
	U24279	$HONHCOCH_2CH$(n-Pentyl)COLeu-$AlaNH_2$	3.2×10^{-8}
	U24522	$HONHCOCH_2CH$(i-Butyl)COLeu-$PheNH_2$	6.0×10^{-8}
	U24631	$HONHCOCH_2CH$(n-Pentyl)COVal-$AlaNH_2$	4.4×10^{-8}

* For complete structures, see Materials and Methods.

The amount of degradation product released by CMP was compared in the DEAE and bead assays. The radiolabeled substrate used for the DEAE assay was used in beads. The bead assay was then performed as usual, and the products released after 24 hr were analyzed in the DEAE assay. The amount of chondroitin sulfate (the usual end point in the bead assay) was 42 μg from non-labeled beads and 36 μg from the radiolabeled beads. The percent of (non-DEAE bound) [^{35}S] radioactivity released in the DEAE assay was 15.8% in 1 hr and from the radiolabeled beads was 15.5% in 24 hr. (Total substrate radioactivity in the beads was estimated from papain digests of the beads). At 10^{-5} M, U11866 completely inhibited CMP activity measured at 1 and 6 hr on DEAE and at 6 and 24 hr measured with beads.

When tested in a cartilage autolysis assay in which retinoic acid stimulates PG loss, several synthetic inhibitors failed to inhibit PG release [24]. To determine whether these compounds retain their CMP inhibitory activity in culture medium, we assayed CMP with thiols and carboxyalkyl peptide inhibitors in DMEM, the medium used in the autolysis assay. CMP degraded 29.0% of the substrate in 1 hr in buffer and 19.2% in DMEM. The inhibitors also lost substantial activity in DMEM (Table 3). These findings agree with unpublished results of Perry, Dea, and DiPasquale. We assume that cystine in DMEM inactivates thiol inhibitors.

We selected U22136 to test whether other types of culture media would also interfere with CMP inhibition by this compound, since it was one of the most active of the series and was one of the most sensitive compounds to DMEM. Two of the enriched media, BGJ and RPMI, produced substantial CMP

Table 2. Reproducibility of CMP assay (same enzyme preparation was used to assay three compounds at 3×10^{-6} M on five occasions)

	Percent of [^{35}S]met-PG released* in 1 hr				
Compound	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
CMP	17.79 ± 0.64	17.79 ± 0.51	16.73 ± 0.67	17.65 ± 0.70	18.65 ± 0.84
+U22136	3.47 ± 0.17	3.89 ± 0.10	4.27 ± 0.18	4.56 ± 0.27	4.83 ± 0.51
+U22312	8.05 ± 0.13	8.09 ± 0.40	7.47 ± 0.31	8.55 ± 0.31	10.32 ± 0.34
+U23161	11.49 ± 0.26	12.53 ± 0.83	10.80 ± 0.45	12.12 ± 0.55	12.75 ± 0.33
No CMP	0.72 ± 0.03	0.67 ± 0.05	0.86 ± 0.03	0.97 ± 0.06	0.96 ± 0.02

	% Inhibition of CMP activity					
Compound	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Mean ± SE
U22136	83.9	81.2	78.5	78.5	78.1	80.0 ± 1.1
U22312	57.1	56.7	58.3	54.6	47.1	54.8 ± 1.8
U23161	36.9	30.7	37.4	33.2	33.4	34.3 ± 1.3

* Means ± SE; N = 5–6.

Table 3. Activities of test compounds tested in buffer and DMEM culture medium

Compound (10^{-5} M)	% Inhibition of CMP activity	
	Buffer*	Medium*
U22136	91.1	22.5
U22203	83.3	19.1
U22312	68.5	46.2
U22932	73.9	36.4
U22989	66.5	38.7
U23161	54.5	24.3

* CMP alone released 29.0% of [^{35}S]-met-PG in buffer and 19.2% in medium. Percent inhibition was calculated based on CMP activity in the same solvent (i.e. buffer or medium).

inhibition (Table 4). Both of these media, along with DMEM, interfered with U22136 activity, as well. Salt solutions provided minimal interference with either CMP or U22136 activity. The remaining media all produced some inhibition of both activities. At 5%, a concentration adequate for autolysis assays, heat-inactivated fetal calf serum did not interfere with CMP activity.

The hydroxamic acid inhibitors were the most potent CMP inhibitors when assayed in buffer. They were tested in Ham's F10 nutrient mixture since this medium supports autolysis, unlike the salt solutions. All four compounds tested retained maximum activity in Ham's F10 medium at 10^{-4}–10^{-5} M (Table 5), the concentrations required for activity in the *in vitro* autolysis system [24]. This activity was maintained even after incubation at 37° for 1–2 days, prior to assay. It is unknown whether the activity of doses that produce partial inhibition would be retained under these incubation conditions.

Five CMP inhibitors were evaluated for stability in serum. These compounds were incubated in thiocyanate-treated rabbit serum for up to 3 days at 37°. They were diluted to 5×10^{-6} M (U11866) or 3×10^{-7} M (remaining four compounds) with CMP and assayed for inhibition of CMP activity. All compounds showed less activity in serum than in buffer when assayed prior to incubation in serum (Table 6). The thiol compound U11866 lost activity in serum within 1 hr. The four hydroxamic acid derivatives retained some activity in serum for 71 hr.

DISCUSSION

Compounds that inhibited chondroitin sulfate (CS) release from PG in polyacrylamide beads [12] by CMP are shown here to block PG core protein degradation with the same potencies. The assay for core protein degradation used in these studies differs from previously reported assays in several key ways. With this assay, substrate is defined as protein that is covalently bound to glycosaminoglycan and prod-

Table 4. Effect of medium on CMP activity and on U22136 inhibition of CMP activity measured as release of radiolabeled PG from DEAE bound substrate in 1 hr

Type of medium		% Inhibition of CMP activity in buffer	
		CMP only	CMP + 10^{-5} M U22136
Buffer (Expt. 1)		(0)	91
Fetal calf serum	20%	71	83
	5%	6	60
	1%	9	71
Complete media	DMEM	30	43
	RPMI	65	72
	F12	27	75
	NCTC	26	79
Salt solutions	Hank's		88
	Earle's	30	87
	Geys	12	89
	RPMI	1	92
RPMI vitamins		30	96
Phenol red, glucose		50	90
Buffer (Expt. 2)		(0)	96
Complete media	BGJ	65	74
	F10	24	78
	BME*	30	78
	MEM*	28	78
MEM vitamins		23	92
MEM amino acids		20	87
MEM salts and phenol red		39	91

CMP released 11.6% of [^{35}S]-met-PG in Expt. 1 and 12.5% in Expt. 2.
* BME, Basal Medium Eagles; and MEM, Minimal Essential Medium.

Table 5. CMP inhibition by test compounds in buffer or Ham's F10 medium

Compound	Percent inhibition of CMP activity by test compounds					
	Inhibitor concn (M)	Buffer	Inhibitor concn (M)	Medium	Inhibitor concn (M)	Medium-37°*
U24278			10^{-4}	100	10^{-4}	100
			10^{-5}	100	10^{-5}	100
	10^{-6}	100	10^{-6}	88.9		
	10^{-7}	69.5	10^{-7}	2.8		
U24279			10^{-4}	100	10^{-4}	100
			10^{-5}	100	10^{-5}	100
	10^{-6}	100	10^{-6}	98.8		
	10^{-7}	57.0	10^{-7}	55.8		
U24522	10^{-6}	92.3	10^{-4}	100	10^{-4}	100
	3×10^{-7}	83.5	3×10^{-5}	99.9	3×10^{-5}	97.0
	10^{-7}	58.5	10^{-5}	100	10^{-5}	97.7
U24631	10^{-6}	99.6	10^{-4}	100	10^{-4}	100
	3×10^{-7}	93.6	3×10^{-5}	100	3×10^{-5}	100
	10^{-7}	67.6	10^{-5}	100	10^{-5}	100

Compounds were incubated for up to 2 days at 37° at the concentrations that reduce PG release from cartilage *in vitro*. CMP in buffer released 15.3% of radioactivity from PG in 1 hr and was inhibited 33.1% when assayed in F10.

* U24278 and U24279 were incubated in medium at 37° for 1 day; U24522 and U24631 were incubated under the same condition for 2 days.

Table 6. Stability of CMP inhibitors in rabbit serum at 37°

Treatment	Incubation period (hr) prior to assay	Inhibition of CMP activity (%)	
		Buffer	SCN-Serum
U11866 (5×10^{-6} M)	0	75.7 ± 4.5	51.8 ± 1.4
	1		7.6 ± 1.3
	3.5		7.3 ± 1.2
	27		0
U24278 (3×10^{-7} M)	0	62.5 ± 1.3	36.3 ± 4.1
	1		34.7 ± 1.9
	3.5		38.5 ± 2.9
	27		38.9 ± 5.0
	40		35.8 ± 4.6
	71		27.6 ± 2.8
U24279 (3×10^{-7} M)	0	84.1 ± 1.0	70.2 ± 2.1
	1		73.1 ± 1.9
	3.5		74.0 ± 0.7
	27		63.8 ± 3.8
	40		30.4 ± 2.8
	71		20.7 ± 4.0
U24522 (3×10^{-7} M)	0	84.3 ± 0.6	69.6 ± 4.0
	1		69.1 ± 3.5
	3.5		76.5 ± 0.6
	27		49.3 ± 4.1
	40		52.8 ± 2.5
	71		47.6 ± 0.8
U24631 (3×10^{-7} M)	0	82.1 ± 0.5	70.1 ± 0.8
	1		73.7 ± 1.8
	3.5		73.1 ± 3.1
	27		67.6 ± 1.2
	40		55.3 ± 3.5
	71		52.7 ± 4.1

Serum was treated with thiocyanate (SCN) and dialyzed prior to use. CMP activity was 11.9% of [^{35}S]PG released in 1 hr assayed in buffer and 11.5% in SCN-treated serum. Serum without SCN treatment inhibited CMP by 89.0%. Means ± SE for triplicate samples are given.

uct as peptide free of glycosaminoglycan. For assays with PG trapped in beads, product is defined as CS that is small enough to diffuse from beads. The maximum size of products that can diffuse out of beads (corresponding to minimum degradation that can be detected) is not known. To compare the extent of degradation in these two assays, we determined the amount of product released from radiolabeled PG in beads in 24 hr. Upon applying the product released from beads to DEAE columns, the amount of product was the same as the amount measured using the same substrate without beads after a 1-hr digest. Therefore, the extent of digestion measured at 24 hr in the bead assay is equivalent to the extent measured with DEAE after a 1-hr digest. Release of PG from beads was progressive, increasing at each of the time points of 1, 2, 6, and 24 hr.

The assay in this study measures proteases directly by measuring protein, rather than indirectly by measuring carbohydrate, which is not evenly distributed over the length of the core protein. The hyaluronic acid-binding region of the core protein is probably the most susceptible region to protease digestion [25] but has no CS bound to it [26]. This assay favors detection of degradation in this region. The isotope used to label PG is methionine, which is preferentially incorporated into the hyaluronic acid-binding region. Degradation in this region is not detected by assays that measure carbohydrate. Since results from the bead assay and this core protein assay are similar for the potencies of the three classes of synthetic CMP inhibitors, they seem to block the action of CMP at both the hyaluronic acid-binding region and the CS binding region.

Along with direct measurement of core protein degradation, the assay described here provides a rapid assay of CMP activity that can be performed easily on large numbers of inhibitors. Results using this assay have confirmed the activities and potencies of all known CMP inhibitors. The assay is reproducible. The reaction time is brief relative to assays that require time for diffusion of enzyme into bead-entrapped substrate and of product out of beads. The assay time of 1 hr may still be too long to detect weak reversible inhibitors. An assay that continuously monitors substrate degradation would be more likely to detect this type of inhibitor.

At non-cytotoxic doses, the hydroxamic acid peptides have blocked cartilage degradation in an *in vitro* assay, consistent with their action as CMP inhibitors [24]. Based on the results of this *in vitro* study and the present study, these inhibitors may be useful as probes of endogenous CMP activity *in vivo*. In addition, the carboxyalkyl peptides, which selectively inhibit CMP over collagenase, provide a way to separate the effects of these two enzymes, which are difficult to separate by other techniques [2]. These compounds retain their activities under the conditions used in these systems.

The present study demonstrates that the hydroxamic acid peptides retained their activity for several days in media that will also promote CMP activity, although low doses that produce partial inhibition in the present assay have not been evaluated yet. However, every medium with amino acids inhibits CMP to some extent. High levels of serum also inhibit CMP. Since thiocyanate reverses this endogenous serum inhibition, stability of CMP inhibitors was evaluated in thiocyanate-treated serum. The thiol compound U11866 lost activity during the first hour in serum. This compound degrades in serum in this time period, based on monitoring this compound by high performance liquid chromatography.* Based on the thiocyanate-treated serum study, thiocyanate did not interfere with the capacity of serum to degrade U11866, and its metabolites did not retain activity as CMP inhibitors. In contrast, the hydroxamic acid peptides were active in serum for several days at 37°.

* K. Monson-Kirkland, personal communication.

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