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CYSTATIN C BASED PEPTIDYL DIAZOMETHANES AS CYSTEINE PROTEINASE INHIBITORS: INFLUENCE OF THE PEPTIDYL CHAIN LENGTH

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The peptidyl diazomethanes Cbz-Gly-CHN_2, Boc-Val-Gly-CHN_2, H-Leu-Val-Gly-CHN_2, Cbz-Leu-Val-Gly-CHN_2 and Cbz-Arg-Leu-Val-Gly-CHN_2, with peptidyl portions modelled after the proposed cysteine proteinase interacting N-terminal segment of human cystatin C, were synthesized. Their efficiency as cysteine proteinase inhibitors was tested against papain, human cathepsin B and bovine cathepsin B. All, except Cbz-Gly-CHN_2, were found to be irreversible inhibitors of the tested enzymes. Each addition of an amino acid residue to their peptidyl portions resulted in an increased inhibition rate of all three enzymes. These data suggest that the arginyl residue of the tetrapeptidyl diazomethane, and also the corresponding arginyl residue in native cystatin C, interact with a S_4 substrate pocket subsite of both papain and cathepsin B. The most efficient inhibitor, Cbz-Arg-Leu-Val-Gly-CHN_2, inhibited papain and cathepsin B with rate constants of the same order of magnitude as those for L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64). The high water-solubility of Cbz-Arg-Leu-Val-Gly-CHN_2 allowing it to be dissolved to molar concentrations without use of non-physiological additives, makes it suitable for *in vitro* and *in vivo* cysteine proteinase inhibition studies.

KEY WORDS: Cystatin C, papain, cathepsin B, peptidyl diazomethanes, irreversible inhibitors.

ABBREVIATIONS: Boc, *t*-butyloxycarbonyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; FAB-MS, fast atom bombardment-mass spectrometry; NHMec, 7-(4-methyl)coumarylamide; pNA, *p*-nitroanilide.

INTRODUCTION

Cysteine proteinases participate in the extra- and intra-cellular catabolism of proteins and peptides,[1] in the processing of proproteins,[2] and are assumed to play a role not only in the normal penetration of human tissues by cells like macrophages and lymphocytes, but also in the abnormal tissue penetration by malignant cells and some microorganisms.[3,4,5] Cysteine proteinases are in addition probably required for the replication of some bacteria, viruses and protozoans.[6,7] It is apparent that regulation of cysteine proteinase activity in higher organisms is required, since a systemic excess of such activity produces detrimental effects like shock and multiple hemorrhages in several mammals, including man.[8] It is therefore not surprising that a large number of naturally occurring proteins, which inhibit cysteine proteinase activities, have been

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described. Several of these inhibitors are homologous in sequence and constitute the cystatin superfamily of proteins. Cystatin C is a dominating inhibitor among these, being synthesized in most human tissues and with a resulting widespread distribution in human extracellular fluids.[9,10] Studies of the inhibitory properties of native and N-terminally truncated forms of human cystatin C[11,12] and chicken cystatin[11,13] together with data from crystallographic studies of chicken cystatin[14] and a complex between human cystatin B and papain[15] strongly indicate that amino acid residues on the N-terminal side of a glycine residue, conserved in all cystatins, closely interact with the active site of cysteine proteinases on inhibition. Peptidyl diazomethanes, in which the peptidyl parts are modelled after good natural cysteine proteinase substrates, have earlier been shown to be specific and efficient irreversible cysteine proteinase inhibitors.[16] The objective of the present work was to synthesize peptidyl diazomethanes with their peptidyl portions modelled after the proposed cysteine proteinase interacting N-terminal segment of human cystatin C, and to investigate their inhibitory properties in an attempt to obtain efficient synthetic inhibitors as well as to gain more information on the cystatin C-cysteine proteinase interaction.

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MATERIALS AND METHODS

Materials

The synthetic chromogenic substrate Bz-DL-Arg-pNA and the fluorogenic substrate Cbz-Phe-Arg-NHMec were obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. L-3-carboxy-2,3-*trans* -epoxypropionyl- leucylamido(4-guanidino)butane (E-64), azocoll, azocasein and hemoglobin were from Sigma Chemical Co., St. Louis, MO, USA. Papain (EC 3.4.22.2) isolated from crude papaya latex by affinity chromatography on Gly-Gly-Tyr(Bzl)-Arg-Sepharose,[17] activatable to at least 70%, was a gift from Dr. I Björk, Uppsala, Sweden. Bovine cathepsin B (EC 3.4.22.1) and clostridial collagenase (EC 3.4.24.3) were obtained from Sigma. Human cathepsin B (EC 3.4.22.1), thermolysin (EC 3.4.24.2) and pepsin (EC 3.4.23.1) were from Calbiochem, La Jolla, CA, USA. Trypsin (EC 3.4.21.4) was from Worthington Biochemical Co., New Jersey, NJ, USA. The activities of the papain and the cathepsins were determined by active site titration with E-64,[18] and was determined every time before use. All chemicals used were of analytical grade and obtained from Sigma.

Synthesis of Peptidyl Diazomethanes

All amino acid derivatives used in the described syntheses were obtained from Nova-Biochem. Melting points are uncorrected. Optical rotations were determined on a Higler-Watts polarimeter with an accurancy of 0.01°. Samples for analytical purposes were dried over phosphorous pentoxide *in vacuo* for 24 h. The structures of synthesized products were confirmed by NMR (Varian XL-200) and IR (Specord 71 IR spectrometer) spectra. The structures of H-Leu-Val-Gly-CHN_2 and Cbz-Arg-Leu-Val-Gly-CHN_2 were additionally confirmed by FAB-MS.

N-Benzyloxycarbonyl-valyl-glycine tert-butyl ester, (Z-Val-Gly-OBu^t) This was obtained (on 20 mmoles scale) by coupling of Z-Val-OH with H-Gly-OBu^t in methylene chloride by activation with dicyclohexylocarbodiimide. The product was isolated by precipitation with n-hexane from ethyl acetate solution. Yield = 5.78 g (79.3%), m.p. = 143–144°C, $[\alpha]_D^{20} = -26.2°$ (c = 1, MeOH).

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N-Fluorenylmethyloxycarbonyl-leucyl-valyl-glycine tert-butyl ester, (Fmoc-Leu-Val-Gly-OBu^t) Z-Val-Gly-OBu^t was deprotected by hydrogenolysis in tetrahydrofuran with 10% Pd/C as catalyst followed by coupling with Fmoc-Leu-OH (14 mmoles) with 1-hydroxybenztriazole/dicyclohexylcarbodiimide activation. The product was isolated by precipitation with n-hexane. Yield = 5.73 g (73.9%), m.p. = 161–162°C, $[\alpha]_D^{20} = -45.2°$ (c = 1, MeOH).

N-Fluorenylmethyloxycarbonyl-leucyl-valyl-glycine, (Z-Leu-Val-Gly-OH)
N-Fluorenylmethyloxycarbonyl-leucyl-valyl-glycine *tert*-butyl ester (2.85 g, 5 mmoles) was mixed with 20 ml of 50% trifluoroacetic acid in methylene chloride and 1 ml of anisole during 1.5 h. The residue obtained was triturated with ethyl ether and the precipitate was collected on a filter. The product was recrystallized from a methanol-water system. Yield = 2.39 g (93.8%), m.p. = 184–187°C, $[\alpha]_D^{20} = -44.7°$ (c = 1, MeOH).

Leucyl-valyl-glycyl-diazomethane (H-Leu-Val-Gly-CHN_2)
N-Fluorenylmethylenoxycarbonyl-leucyl-valyl-glycine (1.53 g, 3 mmoles) was dissolved in 50 ml of methylene chloride, containing 0.303 g (3 mmoles) of triethylamine. The solution obtained was cooled to −30°C and isobutyl chloroformate (0.414 g, 3 mmoles) was added. The reaction mixture was stirred for 30 min after which a diazomethane solution, in diethyl ether, was added in about twofold excess. The reaction was carried out overnight at 4°C. Solvents were removed under reduced pressure. The residue was then dissolved in methanol and precipitated with water. The crude N-fluorenylmethylenoxycarbonyl-leucyl-valyl-glycyl-diazomethane (1.30 g) was stirred with 30 ml of 20% piperidine in dimethylformamide (v/v) during 30 min and the reaction mixture was evaporated to dryness *in vacuo*. The residue was triturated three times with 100 ml portions of n-hexane. The crude leucyl-valyl-glycyl-diazomethane (0.890 g, brown oil) was purified by preparative RP-HPLC (Peptide & Protein Vydac C-18, 25–35 μm, 35 × 500 mm column), using 3% isopropanol in 0.1 M triethylammonium phosphate buffer, pH 7.0. Fractions containing leucyl-valyl-glycyl-diazomethane were collected and concentrated under reduced pressure. The product was desalted by sorption on XAD-7 (25 × 200 mm column), washing of the bed with 1 M NaCl (100 ml) and water (80 ml). The leucyl-valyl-glycyl-diazomethane was eluted with about 700 ml of 80% methanol. The eluate was evaporated under reduced pressure and the oil obtained was dried *in vacuo* over calcium chloride, giving a crystalline yellow mass. Yield = 0.396 g (42.4%), $[\alpha]_D^{20} = -33.7°$ (c = 1 MeOH), FAB-MS (M + H) = 312.1 Da.

N-Benzyloxycarbonyl-arginyl-leucyl-valyl-glycl-diazomethane acetate (Z-Arg(AcOH)-Leu-Val-Gly-CHN_2) N-Benzyloxycarbonyl-arginine hydrochloride (0.690 g, 2 mmoles) and 1-hydroxybenztriazole (0.552 g, 4 mmoles) were dissolved in 20 ml of dimethylformamide and cooled in an ice bath. Dicyclohexylcarbodiimide (0.412 g, 2 mmoles) was added in small portions during 30 min to the solution. The mixture was next stirred on ice for 3 h. Precipitated dicyclohexylurea was filtered off and washed with 5 ml of dimethylformamide. Combined filtrates were added to 0.311 g (1 mmole) of leucyl-valyl-glycl-diazomethane. The pH of the reaction mixture was adjusted to 7–8 with triethylamine and left in room temperature for 24 h. Solvents were removed *in vacuo*. The residue was then dissolved in 20% methanol and injected on an S-Sepharose FF (50 × 200 mm column, Na^+ form). The column was washed with

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20% methanol (500 ml) and the product was then eluted by a sodium chloride gradient in 20% methanol (from 0 to 0.5 M in 2000 ml). Fractions containing the N-benzyloxycarbonyyl- arginyl-leucyl-valyl-glycyl-diazomethane were collected and evaporated under reduced pressure. The residue was treated with 50 ml of anhydrous methanol and the resulting mixture was filtered. The filtrate was evaporated under reduced pressure and was finally purified by preparative RP-HPLC (Peptide & Protein Vydac C-18, 25–35 μm, 35 × 500 mm column) using 15% isopropanol in 0.1 M triethylammonium phosphate buffer, pH 7.0, and desalted by sorbtion on XAD-2 resin, washing with 1 M sodium acetate (1000 ml) and water (80 ml). The N-benzyloxycarbonyl-leucyl-valyl-glycyl-diazomethane was eluted with about 700 ml of 80% methanol. The eluate obtained was evaporated under reduced pressure, diluted with water and lyophilized. Yield = 177 mg (26.6%), $[\alpha]_D^{20} = -44.9°$ (c = 1, MeOH), FAB-MS [M+H] = 602.3 Da.

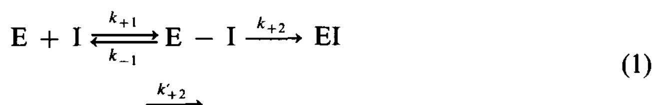
N-Benzyloxycarbonyl-glycyl-diazomethane (Cbz-Gly-CHN_2), t-Butyloxycarbonyl-valyl-glycyl-diazomethane (Boc-Val-Gly-CHN_2) and N-Benzyloxycarbonyl-leucyl-valyl-glycyl-diazomethane (Cbz-Leu-Val-Gly-CHN_2) were prepared as described in references 19 and 20.

Solubility and Activity Tests of Peptidyl Diazomethanes

For enzyme inhibition tests, the peptidyl diazomethanes were dissolved in 100% DMSO and the solutions were then diluted to 1% DMSO with 0.01% Brij-35. Cbz-Arg-Leu-Val-Gly-CHN_2 was easily dissolved in water and was therefore dissolved directly in 0.01% Brij-35. In order to estimate the activity of inhibitors in the solutions, they were titrated against papain which itself had been active site titrated with E-64.[18] A study of the solubility of the two most rapidly inhibiting peptidyl diazomethanes in 0.15 M NaCl, distilled water and 0.01% Brij was also carried out. Portions of 32 mg of Cbz-Arg-Leu-Val-Gly-CHN_2 were added to 50 μl of each solvent. The solutions were then centrifuged and the supernatants titrated for inhibitory activity against papain. For Cbz-Leu-Val-Gly-CHN_2, 5 mg portions were suspended in 5 ml of each solvent. The solutions were incubated for 30 min in a sonication bath, centrifuged at 27 000 × g for 10 min and the resulting supernatants were titrated against papain.

Enzyme Assays and Kinetic Methods

For the irreversible peptidyl diazomethane inhibitors, apparent second-order rate constants of inactivation (k'_{+2}) (eqn 1) were determined by continuously monitoring enzyme activity against a flourogenic substrate before and after addition of the inhibitor.[21]



$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E - I \xrightarrow{k_{+2}} EI \qquad \xrightarrow{k'_{+2}} \qquad (1)$$

The program used to calculate a substrate dependent rate constant (k''_{+2}), FLUSYS[22], was kindly provided by Neil Rawlings and Alan Barrett, Strangeways Research Laboratory, Cambridge, England. The program calculates a pseudo-first-order rate constant, k_{obs}, by the Guggenheim method and gives the apparent second-order rate constant, k''_{+2}, by dividing with [I]. K_m for the reaction between enzyme and substrate

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under the given conditions was then used to calculate the substrate independent apparent second-order rate constant, k'_{+2}, according to eqn (2).

$$k'_{+2} = k''_{+2}(1 + [S]/K_m) \quad (2)$$

Cysteine proteinase activities against Cbz-Phe-Arg-NHMec (10 μM) were assayed at 37°C in a Perkin Elmer LS 50 fluorimeter at an excitation wavelength of 360 nm and emission monitored at 460 nm. The enzyme concentration in the assays was 0.01–0.3 nM. Papain was dissolved in 0.1 M Na-phosphate buffer, pH 6.5, containing 1 mM dithiothreitol and 2 mM EDTA. Human cathepsin B and bovine cathepsin B were dissolved in the same buffer but adjusted to pH 6.0 and was preincubated for 20 min at room temperature before use. All determinations of k''_{+2} were based on assays with less than 2% substrate hydrolysis and $[I] > 5 \times [E]$. A number of 3.5–4.5 enzyme half-lives and non-linear correlation coefficient greater than 0.990 was obtained in all assays used to calculate k''_{+2}. K_m values for papain and bovine cathepsin B hydrolysis of Cbz-Phe-Arg-NMec were estimated by plotting k''_{+2}, in reactions with Cbz-Arg-Leu-Val-Gly CHN_2, against substrate concentration (5–30 μM), at constant enzyme concentration. The k'_{+2} value for Cbz-Arg-Leu-Val-Gly-CHN_2 was then determined by extrapolation to zero substrate concentration and K_m calculated from eqn (2). A K_m value of 250 μM for human cathepsin B hydrolysis of Cbz-Phe-Arg-NMec was taken from the literature.[23] The non-cysteine proteinases clostridial collagenase, thermolysin, pepsin and trypsin were assayed with azocoll, azocasein, hemoglobin and Bz-DL-Arg-pNA as substrates, respectively, as previously described.[18,24,25]

RESULTS

The eleventh residue in the single polypeptide chain of human cystatin C corresponds to the glycine residue, which is conserved in all inhibitory cystatins.[11] Crystallographic data for chicken cystatin[14] and a human cystatin B-papain complex,[15] as well as enzyme inhibitory studies of truncated forms of human cystatin C,[11,12] strongly indicate that this residue is in the P_1-position when cystatin C interacts with papain. We therefore decided to synthesize a series of peptidyl diazomethanes based on the schematic model for the cystatin C-papain interaction given in Figure 1.

The rate constants, k'_{+2}, for the inhibition of the three cysteine proteinases papain, bovine cathepsin B and human cathepsin B by the peptidyl diazomethanes were calculated from fluorometric assays with Cbz-Phe-Arg-NHMec as substrate. K_m values at 37°C for papain at pH 6.5 and bovine cathepsin B at pH 6.0 were determined experimentally to be 60 μM and 79 μM, respectively, in order to enable corrections of obtained values for substrate competition. The results (Table I) show that the Cbz-Gly-CHN_2 does not inhibit any of the three cysteine proteinases. The dipeptidyl diazomethane, Boc-Val-Gly-CHN_2, irreversibly inhibits the proteinases with a low rate constant, the tripeptidyl diazomethane Cbz-Leu-Val-Gly-CHN_2, has a considerably higher rate constant for all proteinases and the tetrapeptidyl diazomethane, Cbz-Arg-Leu-Val-Gly-CHN_2, displays a very rapid inhibition of all tested proteinases with rate constants of the same order of magnitude as those determined for E-64. All inhibiting peptidyl diazomethanes were more rapid inhibitors of papain than of human or bovine cathepsin B. The results also demonstrate that addition of the N-terminal protecting group, benzyloxycarbonyl-, to the unblocked tripeptidyl diazomethane H-Leu-Val-Gly-CHN_2, results in a significantly increased inhibition rate of the resulting derivative for all three cysteine proteinases.

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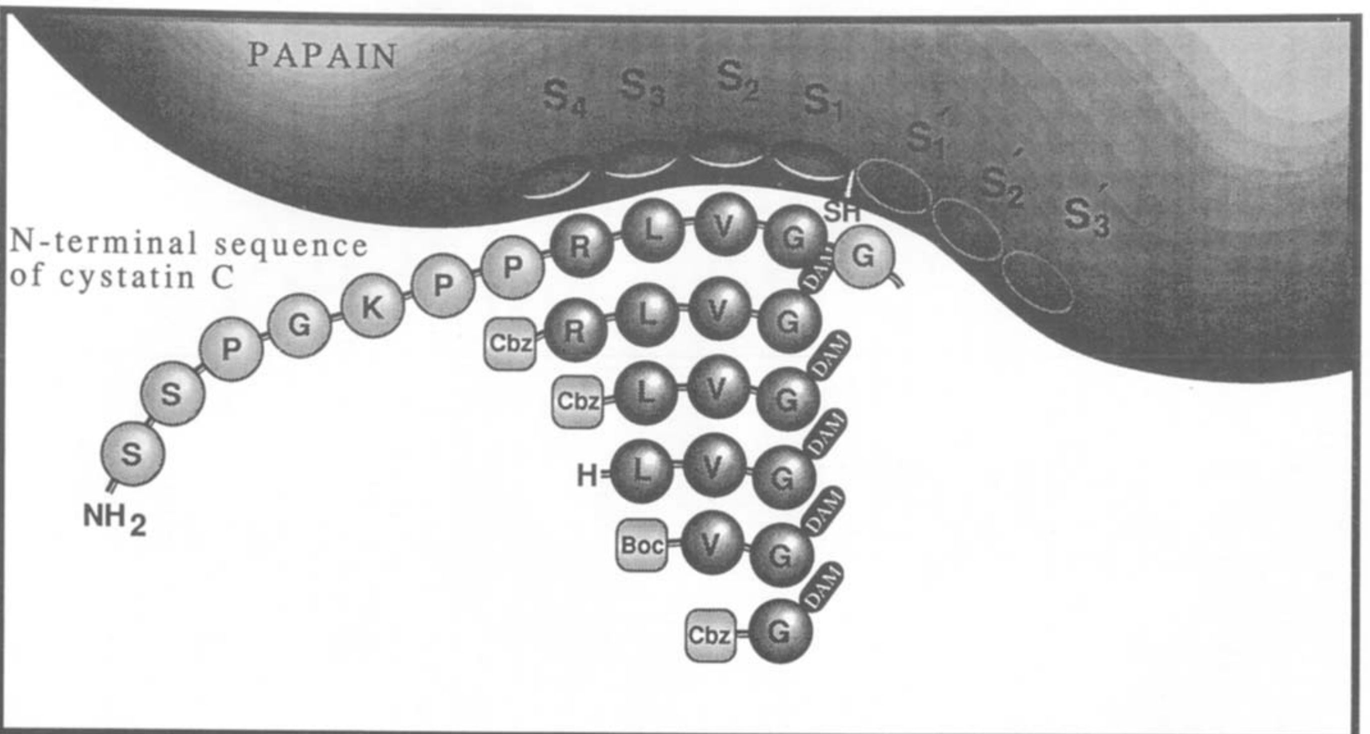

FIGURE 1 A schematic model for the interaction between papain and the N-terminal part of human cystatin C. The four papain substrate pocket subsites, S_1-S_4, which are assumed to interact with the N-terminal part of cystatins are shown in a darker shade than the subsites, S_1'-S_3', which do not interact with the N-terminal part of cystatins. The five peptidyl diazomethanes indicated at the bottom were synthesised and studied in the present work. The procedures for synthesis are given in the Materials and Methods section. Cbz: Benzyloxycarbonyl; Boc: *t*-Butyloxycarbonyl; DAM: diazomethane.

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TABLE I
Apparent second order rate constants (k'_{+2}, $M^{-1}s^{-1}$) for inhibition of cysteine proteinases by synthetic peptidyl diazomethanes modelled after the N-terminal segment of human cystatin C

P_5-P_4-P_3-P_2-P_1	papain	bovine cathepsin B	human cathepsin B
Cbz-Gly-CHN_2	< 100 (2)	no inhib.	no inhib.
Boc-Val-Gly-CHN_2	16600 ± 1000 (5)	1200 ± 100 (3)	2800 ± 500 (3)
H-Leu-Val-Gly-CHN_2	22600 ± 1100 (3)	1500 ± 100 (3)	2600 ± 200 (3)
Cbz-Leu-Val-Gly-CHN_2	448000 ± 17000 (5)	24000 ± 1200 (3)	28100 ± 1200 (3)
Cbz-Arg-Leu-Val-Gly-CHN_2	559000 ± 23000 (5)	39600 ± 800 (4)	75600 ± 1200 (3)
E-64	813000 ± 27000 (14)	53500 ± 2000 (3)	85700 ± 3600 (3)

Mean rate constant values and standard errors of the means are given. The number of assays, used for each calculation, are given in parentheses.

The proteolytic activity of the metalloproteinases thermolysin and clostridial collagenase, the aspartic proteinase pepsin and the serine proteinase trypsin was measured before and after addition of each of the synthesized peptidyl diazomethanes. No influence on the activity of any of the proteinases could be registered, not even in the presence of high molar excesses (100–6000 fold) of the peptidyl diazomethanes.

Solubility studies were performed concerning the two most rapidly inhibiting peptidyl diazomethanes, Cbz-Leu-Val-Gly-CHN_2 and Cbz-Arg-Leu-Val-Gly-CHN_2. Whereas the tetrapeptidyl derivative, Cbz-Arg-Leu-Val-Gly-CHN_2, was easily dissolved to at least 0.5 M in distilled water, 0.15 M NaCl and 0.01% Brij-35 at room temperature, the tripeptidyl derivative, Cbz-Leu-Val-Gly-CHN_2, had maximal solubilities of only 0.05–0.15 mM in these solvents. The solubility of the tripeptidyl derivative could be increased, but only twofold, by addition of DMSO to the solvents to final concentrations of 1% (v/v).

DISCUSSION

A glycine residue, present in the N-terminal segment of the polypeptide chains of all inhibiting cystatins, is assumed to be in the vicinity of the active site sulfhydryl group in cystatin-cysteine proteinase complexes.[11] X-ray cystallographic data for chicken cystatin[14] and a human cystatin B-papain complex,[15] as well as enzyme inhibitory studies of truncated forms of human cystatin C and chicken cystatin,[12,13] strongly indicate that residues on the N-terminal side of this conserved glycine residue are of crucial importance in the cystatin-cysteine proteinase interaction. Although the two residues, occupying the tentative P_2- and P_3-positions, on the immediate N-terminal side of the invariant residue in human cystatins B and C and chicken cystatin, clearly contribute considerably in the cystatin-cysteine proteinase interaction,[12,13,15] no systematic studies have been addressing the question of the possible importance of amino acid residues in positions preceding the P_3-position. One way to elucidate this question is to synthesise peptidyl diazomethanes, in which the peptidyl portions vary in length and are modelled after the relevant N-terminal segements of cystatins, and investigate the kinetics of cystein proteinase inhibition for these peptidyl diazomethanes.
E-64. All inhibiting peptidyl diazomethanes were more rapid inhibitors of papain than of human or bovine cathepsin B. The results also demonstrate that addition of the N-terminal protecting group, benzyloxycarbonyl-, to the unblocked tripeptidyl diazomethane H-Leu-Val-Gly-CHN_2, results in a significantly increased inhibition rate of the resulting derivative for all three cysteine proteinases.

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C and contained from one to four amino acid residues, and investigated their properties as cysteine proteinase inhibitors. The shortest peptidyl diazomethane, containing only the glycine residue in the tentative P_1-position, did not inhibit any of the tested cysteine proteinases, whereas all peptidyl diazomethanes containing two, three of four amino acid residues inhibited all three investigated proteinases and with an inhibition rate that increased with the number of residues. These results are in agreement with earlier studies indicating that the residues in the tentative P_2- and P_3-positions contribute significantly in the cystatin-cysteine proteinase binding, and also suggest that the arginyl-residue in the P_4-position migh be of importance for the binding of papain and cathepsin B by human cystatin C. It might be of interest to investigate whether residues in the corresponding P_4-positions in other cystatins also contribute in cystatin-cysteine proteinase interactions, and if cystatin C-based peptidyl diazomethanes with more than four amino residues would be still more efficient for irreversible inhibition of certain cysteine proteinases. Attempts are presently being made to synthesise the peptidyl diazomethanes required for such investigations.

The rate for papain inactivation by E-64 has earlier been determined, with values ranging from 638 000 to 1 160 000 $M^{-1}s^{-1}$ (e.g. refs. 18, 26). This rather wide range could probably be explained by differences in activity and purity between enzyme preparations used. In this study we used affinity purified papain in 14 separate determinations of the inactivation rate constant for E-64, and we found that the rate constant varied with the activity of the papain preparation used, which in turn depends on the time passed since activation of the enzyme. Consequently, comparison of rate constants for synthetic inhibitors should preferentially be performed within a series of measurements using the same enzyme preparation. In this study we also intended to investigate if a species variation between human and bovine cathepsin B could be demonstrated by differences in inactivation rates by synthetic active site directed inhibitors. We could not demonstrate any such differences, however. This suggests that inhibition data for synthetic inhibitors determined against bovine cathepsin B are comparable to these obtained using human cathepsin B.

The amino acid sequences of the peptidyl parts of the synthetic inhibitors investigated in this study are all based upon that of an amino-terminal segment in the polypeptide chain of a natural cysteine proteinase inhibitor, cystatin C. Since this segment is the part of the molecule that presumably interacts with the substrate binding pockets of cysteine proteinases and has been evolutionary selected, it is of considerable interest to compare inhibition data for the cystatin C-based synthetic inhibitors with those determined for other peptidyl diazomethanes, some of which have been synthesized to meet the substrate requirements of certain cysteine proteinases. Kinetic data for inactivation of papain and cathepsin B by the rather few previously synthesized peptidyl diazomethanes that we judge have been studied using methods comparable to ours, are summarized in Table II. The cystatin C-based dipeptidyl inhibitor displays an inhibition rate, which is not exceptionally high with, for example, the commonly used inhibitor Cbz-Phe-Ala-CHN_2 being a considerably faster papain inhibitor. The cystatin C-based-tri- and tetrapeptidyl inhibitors however, display rate constants for inactivation of both enzymes that not only are one order of magnitude higher than those of the fastest of all dipeptidyl inhibitors, but also of virtually all previously synthesized tripeptidyl diazomethanes. Thus, from the data available it appears that the amino-terminal cystatin C segment has a sequence that can interact comparably well with the substrate pockets of both papain and cathepsin B. It therefore seems to be a fruitful approach in the design of peptidyl diazomethanes for

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TABLE II
Comparison of inhibition rate constants ($M^{-1}s^{-1}$) for peptidyl diazomethanes.

Inhibitors	Papain	Cathepsin B (bovine)
Boc-Val-Gly-CHN$_2$	**17 000[a]**	**2 800[a]**
Cbz-Phe-Ala-CHN$_2$	43 000[b]	1 300[c]
Cbz-Phe-Gly-CHN$_2$	12 000[b]	700[c]
Cbz-Gln-Gly-CHN$_2$	50[b]	
Cbz-Leu-Val-Gly-CHN$_2$	**448 000[a]**	**24 000[a]**
Cbz-Leu-Gly-Gly-CHN$_2$	< 50[b]	
Cbz-Leu-Phe-Gly-CHN$_2$	62 000[b]	
Cbz-Ala-Phe-Ala-CHN$_2$		1 200[c]
Cbz-Arg-Leu-Val-Gly-CHN$_2$	**559 000[a]**	**40 000[a]**

[a]Data from the present investigation. [b]Selected data from ref. 26. [c]Selected data from ref. 16. Kinetic constants for inactivation of papain and cathepsin B are given when available, for di- and tripeptidyl diazomethanes reported in the literature. Inhibitors with blocked amino-termini and unmodified amino acid sidechains were selected for comparison with the cystatin C based inhibitors, marked in boldface.

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cysteine proteinase inhibition to base the structure of their peptidyl portions on the sequence of appropriate segments of naturally occurring protein inhibitors.

In a recent study[12] it has been shown that a truncated cystatin C form, lacking the 10 most N-terminal amino acids residues, is considerably less efficient as a cathepsin B or L inhibitor than native cystatin C. The cathepsin H inhibition is hardly affected by N-terminal truncation, however. The cystatin C inhibition of cathepsin B is also slower and has a much higher K_i than its inhibition of papain or human cathepsin L.[12,27] This data indicates that the N-terminal portion of cystatin C is important for the inhibitory specificity of the molecule. It is of interest to note that these differences are reflected in the inhibition characteristics of all cystatin C based peptidyl diazomethanes, since their rate constants for inhibition of papain in all cases are one order of magnitude higher than their corresponding constants for inactivation of human or bovine cathepsin B. However, despite this analogy, it should be stressed that studies of inhibitory properties of cystatin based peptidyl derivatives only give indirect evidence for the interaction of native cystatins and cysteine proteinases and that the results of studies involving only peptidyl derivatives must be interpreted with caution. This is, for example, illustrated by the significant difference in inhibition rate displayed by the N-terminally blocked and unblocked tripeptidyl derivatives Cbz-Leu-Val-Gly-CHN$_2$ and H-Leu-Val-Gly-CHN$_2$, respectively. The inhibition constants of Boc-Val-Gly-CHN$_2$ and H-Leu-Val-Gly-CHN$_2$ are of the same order of magnitude which also suggests a possible interaction of the N-terminal blocking group and the subsites S_3 and S_4. Cbz does not seem to interact with the papain or cathepsin B S_2 subsite in Cbz-Gly-CHN$_2$, again stressing that the peptidyl derivatives only can give indirect evidence for the interaction of native cystatin C with cysteine proteinases.

The tripeptidyl diazomethane, Cbz-Leu-Val-Gly-CHN$_2$, has been synthesized before[19] and shown to block the replication of group A streptococci[6] and of herpes simplex virus type 1[7], presumably by inhibition of vital cysteine proteinases of these microorganisms. The low solubility of the tripeptidyl derivative in physiological fluids is, however, an obstacle for further investigations of its effects in biological systems.

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It is therefore of considerable interest that the tetrapeptidyl diazomethane, Cbz-Arg-Leu-Val-Gly-CHN_2, described in this work, can easily be dissolved to high concentrations in water and physiological buffers without addition of organic solvents like DMSO, and that it is a still more rapid inhibitor of the three tested cysteine proteinases than the tripeptidyl diazomethane. Indeed, preliminary investigations show that the tetrapeptidyl derivative not only blocks the replication of group A streptococci as efficiently as the tripeptidyl derivative, but also curbs osteoclast mediated bone degradation (U. Lerner and A. Grubb, unpublished).

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