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Research paper

Influence of the spacer on the inhibitory effect of different polycarbophil–protease inhibitor conjugates

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Abstract

Within the present study various polycarbophil (PCP)–serine protease inhibitor conjugates were synthesized and the influence of different spacers on their inhibitory efficacy was evaluated in vitro. Results demonstrated that 4.2 ± 0.15 units ($n = 3$; ±SD) of α-chymotrypsin were inhibited by 50% utilizing 0.86% (w/v) of a PCP–tetramethylenediamine (TMDA)-chymostatin 20:1 conjugate. In contrast, only 0.6 ± 0.05 units ($n = 3$; ±SD) of α-chymotrypsin were inhibited by a corresponding PCP–poly(ethylene glycol) (PEG)-chymostatin conjugate. Inhibitory effects of PCP–TMDA-antipain and -elastatinal conjugates towards trypsin and elastase, respectively, were also significantly higher ($P < 0.05$) than those of corresponding PCP–PEG-inhibitor conjugates. Hence, the great impact of the molecular size as well as the structure of the spacer on resulting polymer-inhibitor conjugates could be demonstrated. The small and rigid C4-spacer TMDA (molecular weight (MW) 161.1) was thereby shown to be highly advantageous over a long, hydrophilic and flexible PEG-diamine spacer (MW 3400). Results obtained should provide helpful basic knowledge for the development of mucoadhesive polymer–inhibitor conjugates used as auxiliary agents for the oral administration of peptide drugs.

Keywords: Antipain; Chymostatin; Elastatinal; Mucoadhesive drug-carrier matrix; Oral peptide drug delivery; Poly(ethylene glycol); Polycarbophil; Protease inhibitor

1. Introduction

In recent years, the combination of mucoadhesive polymers with enzyme inhibitors has gained considerable interest in peroral peptide and protein delivery. Mucoadhesive polymers, on the one hand, are supposed to provide an intimate contact with the intestinal mucosa, thereby reducing the degradation of orally administered peptide and protein drugs between the delivery system and the absorbing membrane. Additionally, they provide a prolonged residence time of the delivery system in the intestine and increase drug concentration at the surface of the mucous membrane, thereby leading to improved drug absorption [1]. Enzyme inhibitors, on the other hand, are able to protect therapeutic (poly)peptides from enzymatic attack caused by proteases penetrating in the mucoadhesive polymer.

Various in vivo studies could already demonstrate that the bioavailability of orally administered therapeutic (poly)peptides was significantly increased by protease inhibitors [2–5]. However, a major drawback is generally the toxicity and side effects of these auxiliary agents, if co-administered [6]. Even if the inhibitor is unabsorbable, a disturbed digestion of nutritive proteins and pancreatic hypersecretion caused by luminal feedback regulation must be taken into consideration [7]. To overcome these problems, we focused our research work in recent years on the immobilization of protease inhibitors to unabsorbable mucoadhesive drug-carrier matrices (e.g. Refs. [8–10]). Easy accessibility of the immobilized inhibitor for the corresponding protease is thereby a prerequisite in the development of such polymer–inhibitor conjugates. The use of an appropriate spacer should satisfy for this requirement. To substantiate our knowledge for the development of very effective bioadhesive polymers displaying a protective effect toward enzymatic attack, it was the aim of the present study to perform a comparative study of different spacer conjugates, which should contribute to our understanding concerning structure-function relations. The linear and low molecular weight C4-chain spacer tetramethylenediamine (TMDA, molecular weight (MW) 161.1), and the high molecular weight diami-

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nated poly(ethylene glycol) (PEG, MW 3400), were chosen as the model spacers to immobilize the protease-inhibitors antipain, chymostatin and elastatinal, respectively, on the mucoadhesive anionic polymer polycarbophil (PCP). Both spacers are easily soluble in water and therefore should not have a negative influence on the mucoadhesive properties of PCP. Moreover, PEG was recently reported as acting as an adhesion promoter when it was added to poly(acrylic acid) [11]. In contrast, linear C-chain spacers with more than 10-C are often too hydrophobic, thereby leading to a strongly reduced mucoadhesiveness of the resulting polymer-conjugates. PEG-chains should also be more flexible, thereby enabling better accessibility of the immobilized inhibitors for corresponding proteases. Hence, the influence of the molecular size and consequently the structure of the spacer on the inhibitory efficacy of the resulting polymer–inhibitor conjugates was evaluated in vitro. Data obtained from this study should give helpful basic information for the design of peroral (poly)peptide delivery systems based on bioadhesive polymer–inhibitor conjugates.

2. Materials and methods

2.1. Synthesis and isolation of the polycarbophil (PCP)–protease-inhibitor conjugates

The protease inhibitors antipain, chymostatin, and elastatinal (Sigma, St. Louis, MO) were immobilized on the mucoadhesive polymer sodium polycarbophil (Na-PCP) by use of either the low molecular weight spacer tetramethylenediamine·2HCl (TMDA; putrescine dihydrochloride; MW 161.1; Sigma) or the high molecular weight spacer t-BOC-NH-PEG-NH_2 (diaminated poly(ethylene glycol); MW 3400; Shearwater Polymers Inc., Huntsville, AL). The inhibitors were thereby covalently attached in a two step condensation reaction which was mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma) as described below. The presumptive structures of the resulting polymer-inhibitor conjugates are shown in Fig. 1.

2.2. Synthesis of the PCP–TMDA-inhibitor conjugates

(I) Antipain (3.5 mg) and elastatinal (3.5 mg) were dissolved in 1.75 ml of demineralized water, while chymostatin (3.5 mg) was suspended in a mixture of tetrahydrofuran (THF, 0.7 ml) and demineralized water (1.75 ml) in order to provide sufficient solubility. EDAC was added to each preparation in order to activate the carboxylic acid group of the inhibitor, and the reaction mixtures were stirred for 15 min at room temperature. TMDA (molar ratio 1:1 to the inhibitors, see Table 1) was dissolved in 1.75 ml of demineralized water and the pH was adjusted to 6 with 0.1 M NaOH. This solution was added to each activated inhibitor. The resulting reaction mixtures were then incubated for 6 h at room temperature under stirring. Controls were prepared for each inhibitor-solution; however, EDAC was omitted during the coupling reaction. An overview concerning the amounts of reagents used for the coupling reaction is given in Table 1. Additionally, final concentrations of reagents are given in brackets (Table 1).

(II) The resulting inhibitor-spacer conjugates were directly coupled to Na-PCP by the same condensation reaction as described above. The remaining NH_2-group of the spacer was thereby covalently bound to a carboxylic acid group of the polymer by the formation of an amide bond. Na-PCP (40 mg) was hydrated in 16 ml of demineralized water, EDAC was added in a final concentration of 100 mM and mixtures were preincubated for 15 min at room temperature in order to activate the carboxylic acid groups of the polymer. The inhibitor-spacer reaction mixture was added to each sample in amounts as listed in Table 2, and the reaction was allowed to proceed for at least 12 h with stirring at room temperature. The resulting polymer–inhibitor conjugates were isolated by dialyzing against 5 mM NaOH containing 1% NaCl four times each for 8–12 h at 10°C and then exhaustively against demineralized water for 12 h at room temperature.

Control polymers were prepared and isolated in the same way as described for the PCP–TMDA-inhibitor conjugates, however, EDAC was omitted during the coupling reaction. The isolated PCP–TMDA-inhibitor conjugates were lyophilized by drying frozen aqueous polymer solutions at −30°C and 0.01 mbar (Christ Beta 1-8 K; Osterode am Harz, Germany) and stored at −20°C.

2.3. Synthesis of the PCP–PEG-inhibitor conjugates

Coupling of the PEG-derivative to the inhibitors followed the same procedure as described above for the TMDA-inhibitor conjugates. An overview concerning the amounts of reagents used for the coupling reaction is given in Table 1.

To induce cleavage of the protective t-BOC group from PEG, the reaction mixture was brought to pH 1 after 6 h by adding 5 M HCl under stirring. After 3 h of incubation the pH was readjusted to 6 with 5 M NaOH. Removal of t-BOC was monitored by taking 20-μl aliquots from the reaction mixtures prior and after pH shifting. The aliquots were diluted with 180 μl of demineralized water and transferred to the wells of a microtitration plate. Increase in available primary amino groups was then determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma). Thereby 100 μl of 0.1% TNBS containing 4% $NaHCO_3$ were added to each diluted aliquot and absorbance at 450 nm was measured after 2 h incubation at 37°C using a microtitration plate reader (Anthos reader, 2001, Salzburg, Austria).

The inhibitor–PEG-amine conjugates resulting from the coupling reaction described above were then directly coupled to Na-PCP as described for the PCP–TMDA-inhibitor conjugates. An overview concerning the amounts of reagents used is given in Table 2. The resulting polymer-

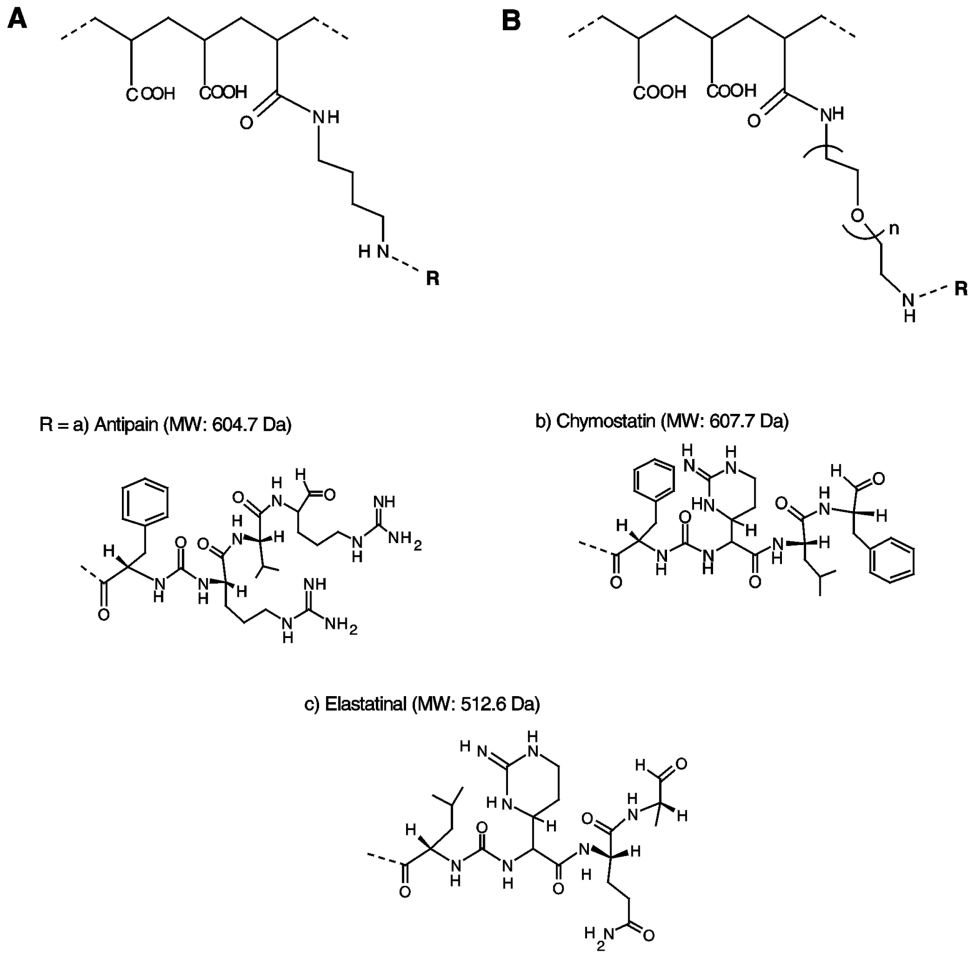

Fig. 1. Presumed chemical structure of the PCP–TMDA (A)- and PCP–PEG (B)-inhibitor conjugates; covalent attachment was achieved by the constitution of an amide bond of the available amino residue exhibited by the inhibitor–spacer conjugate with a carboxylic acid residue of the polymer.

inhibitor conjugates were isolated by dialyzing as described for the PCP–TMDA-inhibitor conjugates.

Control polymers were prepared and isolated in the same way as described for the PCP–PEG-inhibitor conjugates; however, EDAC was omitted during the coupling reaction. The isolated PCP–PEG-inhibitor conjugates were lyophilized by drying frozen aqueous polymer solutions at −30°C and 0.01 mbar (Christ Beta 1-8 K) and stored at −20°C.

2.4. In vitro evaluation of the inhibitory effect of the conjugates towards enzymatic degradation

2.4.1. Trypsin (EC 3.4.21.4)

Polymer-antipain conjugates, control or unmodified Na-PCP (0.70 mg) were hydrated in 0.4 ml of 100 mM phosphate buffered saline (PBS), pH 7.1. Trypsin (45 spectrophotometric BAEE units; type I, from bovine pancreas, Sigma), dissolved in 100 μl of 100 mM PBS (pH 7.1), was added to the hydrated polymer and the samples were incubated for 30 min at room temperature. The synthetic trypsin substrate *N*-α-benzoylarginine ethyl ester (BAEE, Sigma) was dissolved in 100 mM PBS, pH 7.1 (1 mg/ml). To each sample 200 μl of substrate stock were added and the change in absorbance at 253 nm caused by the hydrolysis of this substrate to *N*-α-benzoylarginine (BA) was recorded at 1-min intervals for 10 min using a UV/Vis-spectrophotometer (Lambda-16, Perkin-Elmer, USA).

2.4.2. α-Chymotrypsin (EC 3.4.21.1)

Polymer-chymostatin conjugates, control or unmodified Na-PCP (0.60 mg) were hydrated in 0.3 ml of 100 mM PBS, pH 7.1. Increasing amounts of α-chymotrypsin (52 BTEE units/mg; type II, from bovine pancreas, Sigma) dissolved in 100 μl of the same buffer were added in steps of 0.05 units to determine the amount of α-chymotrypsin which is 50% inhibited by the tested polymers. After an incubation period of 30 min at room temperature, 0.3 ml of the substrate solution (BTEE, *N*-benzoyl-L-tyrosine ethyl ester (Sigma); 18.5 mg substrate dissolved in 31.7 ml of methanol and 18.3 ml of demineralized water) were added and the change in absorbance at 260 nm was recorded at 1-min intervals for 10 min using a UV/Vis-spectrophotometer (Lambda-16, Perkin-Elmer). It was impossible to show more informative

Table 1
Total amounts of reagents used for reaction mixtures in the preparation of protease-inhibitor-spacer conjugates 1:1 (molar ratio), with resulting final concentrations in the solution given in parentheses

Protease inhibitor-spacer conjugates	Antipain	Chymostatin	Elastatinal	EDAC	Tetramethylenediamine (TMDA)	t-BOC-NH-PEG-NH_2 (PEG)
Antipain-TMDA control	0.5 mg (1 mg/ml)	–	–	–	0.14 mg (0.27 mg/ml)	–
Antipain-TMDA conjugate	3.5 mg (1 mg/ml)	–	–	70 mg (20 mg/ml)	0.95 mg (0.27 mg/ml)	–
Antipain-PEG control	0.5 mg (1 mg/ml)	–	–	–	–	2.81 mg (5.62 mg/ml)
Antipain-PEG conjugate	3.5 mg (1 mg/ml)	–	–	70 mg (20mg/ml)	–	19.67 mg (5.62 mg/ml)
Chymostatin-TMDA control	–	0.5 mg (0.83 mg/ml)	–	–	0.13 mg (0.22 mg/ml)	–
Chymostatin-TMDA conjugate	–	3.5 mg (0.83 mg/ml)	–	84 mg (20 mg/ml)	0.91 mg (0.22 mg/ml)	–
Chymostatin-PEG control	–	0.5 mg (0.83 mg/ml)	–	–	–	2.8 mg (4.67 mg/ml)
Chymostatin-PEG conjugate	–	3.5 mg (0.83 mg/ml)	–	84 mg (20 mg/ml)	–	19.6 mg (4.67 mg/ml)
Elastatinal-TMDA control	–	–	0.5 mg (1 mg/ml)	–	0.16 mg (0.32 mg/ml)	–
Elastatinal-TMDA conjugate	–	–	3.5 mg (1 mg/ml)	70 mg (20 mg/ml)	1.12 mg (0.32 mg/ml)	–
Elastatinal-PEG control	–	–	0.5 mg (1 mg/ml)	–	–	3.33 mg (6.66 mg/ml)
Elastatinal-PEG conjugate	–	–	3.5 mg (1 mg/ml)	70 mg (20 mg/ml)	–	23.28 mg (6.66 mg/ml)

Table 2
Amounts of reagents used for reaction mixtures in the preparation of PCP–protease inhibitor conjugates with increasing amounts of antipain, chymostatin and elastatinal, respectively

PCP–protease inhibitor conjugate	Na-PCP (mg; 2.5 mg PCP/ml demineralized water)	EDAC (mg)	Protease inhibitor-TMDA conjugates (ml of solutions according to Table 1)	Protease inhibitor-PEG conjugates (ml of solutions according to Table 1)
PCP–TMDA-antipain control	10	–	0.5 (control)	–
PCP–TMDA-antipain 20:1	40	360	2	–
PCP–TMDA-antipain 40:1	40	340	1	–
PCP–TMDA-antipain 80:1	40	330	0.5	–
PCP–PEG-antipain control	10	–	–	0.5 (control)
PCP–PEG-antipain 20:1	40	360	–	2
PCP–PEG-antipain 40:1	40	340	–	1
PCP–PEG-antipain 80:1	40	330	–	0.5
PCP–TMDA-chymostatin control	10	–	0.6 (control)	–
PCP–TMDA-chymostatin 20:1	40	370	2.4	–
PCP–TMDA-chymostatin 40:1	40	345	1.2	–
PCP–TMDA-chymostatin 80:1	40	330	0.6	–
PCP–PEG-chymostatin control	10	–	–	0.6 (control)
PCP–PEG-chymostatin 20:1	40	370	–	2.4
PCP–PEG-chymostatin 40:1	40	345	–	1.2
PCP–PEG-chymostatin 80:1	40	330	–	0.6
PCP–TMDA-elastatinal control	10	–	0.5 (control)	–
PCP–TMDA-elastatinal 20:1	40	360	2	–
PCP–TMDA-elastatinal 40:1	40	340	1	–
PCP–TMDA-elastatinal 80:1	40	330	0.5	–
PCP–PEG-elastatinal control	10	–	–	0.5 (control)
PCP–PEG-elastatinal 20:1	40	360	–	2
PCP–PEG-elastatinal 40:1	40	340	–	1
PCP–PEG-elastatinal 80:1	40	330	–	0.5

reaction kinetics of enzyme inhibition, as differences in the inhibitory effect of polymer–TMDA/PEG–chymostatin conjugates could not be evaluated at just one enzyme level for this comparison.

2.4.3. Elastase (EC 3.4.21.36)

Polymer–elastatinal conjugates, control or unmodified Na-PCP (0.80 mg) were hydrated in 160 μl of 100 mM PBS (pH 7.1) and transferred to the wells of a microtitration plate (96-well, Greiner Labortechnik, Austria). Elastase (670 μg; type II-A, from porcine pancreas; Sigma) was dissolved in 1 ml of 100 mM PBS (pH 7.1) and 10 μl of this stock solution were added to each sample followed by 30 min incubation at 37°C. Thereafter, 130 μl of the substrate medium (0.2 mg of succinyl-(L-alanyl)$_3$-4-nitroanilide (Sigma)/ml of 100 mM PBS (pH 7.1); filtered before use) were added and the change in absorbance at 405 nm caused by the enzymatic reaction at room temperature was recorded at 1-min intervals for 10 min with a microtitration plate reader (Anthos reader). The concentration of the hydrolyzed substrate was calculated by interpolation from a standard curve.

2.5. Statistical data analysis

Statistical data analysis was performed using the Student *t*-test with $P < 0.05$ as the minimal level of significance unless indicated otherwise.

3. Results and discussion

The serine protease inhibitors antipain, chymostatin and elastatinal – their chemical structures are depicted in Fig. 1 – exert their inhibitory activity through the terminally located aldehyde function [12,13]. In order to keep this moiety uninfluenced, the inhibitors were bound to polycarbophil at the opposite end of the molecule.

3.1. PCP–TMDA-inhibitor conjugates

Fig. 2 shows the results of the evaluation of the inhibitory effects of 0.1% PCP–TMDA-antipain conjugates. Inhibition kinetics of all conjugates – nominal ratios of polymer/inhibitor were 20:1, 40:1 and 80:1 – were significantly different from those of the control polymer and unmodified Na-PCP, respectively. In contrast, 0.1% control polymer and 0.1% unmodified Na-PCP did not show significant differences in the enzyme assay, verifying the efficacy of the purification method.

Recently, it could be demonstrated, that 0.045% of a chitosan–antipain 20:1 conjugate displayed a more than 70% inhibitory activity after an incubation period of 9 min with trypsin (82 units/ml) [14]. In contrast, 0.1% of the PCP–TMDA-antipain 20:1 conjugate showed only 25% inhibitory activity towards trypsin (70.7 units/ml) within the same time period in the present study (Fig. 2). The reason for this comparatively weak protective effect

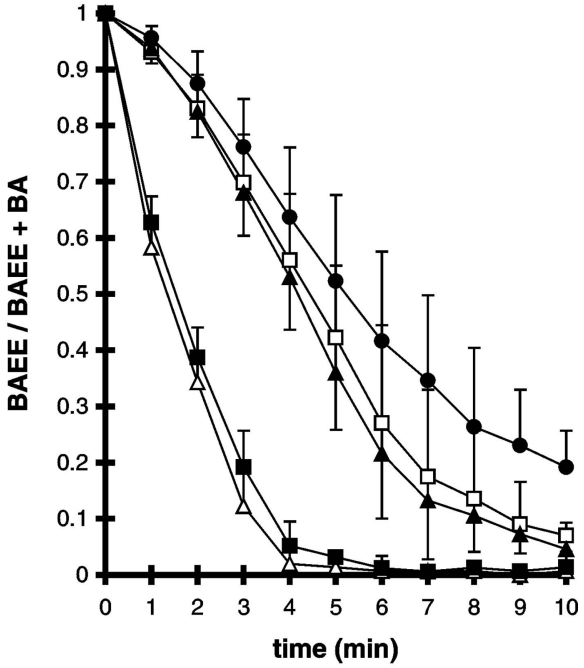

Fig. 2. Comparison of the inhibitory effect of 0.1% PCP–TMDA-antipain conjugates 20:1 (●), 40:1 (□), 80:1 (▲), control (△), and 0.1% unmodified Na-PCP (■) towards trypsin (70.7 spectrophotometric BAEE units/ml). Hydrolysis of the substrate *N*-α-benzoylarginine ethyl ester (BAEE) to *N*-α-benzoylarginine (BA) was determined at pH 7.1 and 20°C. Indicated values are means of at least three experiments (±SD).

might be due to an ionic interaction of the immobilized inhibitor antipain with polycarbophil. Antipain exhibits two guanidino-residues in its structure (Fig. 1). As one of them is located near the aldehyde function of the molecule, interaction of the carboxylic acid moieties of the polymer with the cationic guanidino-residues of the inhibitor might have led to a sterical hindrance decreasing the accessibility of the immobilized inhibitor for the corresponding protease. In contrast, such interactions may not occur in case of the cationic chitosan-conjugate.

Chymostatin is a lipophilic agent, due to an iso-butyl and two benzyl-residues (as shown in Fig. 1) exhibiting poor solubility in water. Therefore, the coupling reaction was performed in a mixture of THF/water (2:5; v/v) to guarantee sufficient solubility of chymostatin during the coupling reaction and consequently to obtain a high coupling rate of the inhibitor on the polymer. Results of the evaluation of the inhibitory activity of PCP–TMDA-chymostatin conjugates towards α-chymotrypsin are shown in Fig. 3. The protective effect was determined by units of α-chymotrypsin (BTEE), which had to be added to 0.6 mg of conjugates in order to obtain a 50% inhibition of proteolytic activity within 10 min. The protective effect of the conjugates significantly increased under the conditions of the study with raising the ratio of the inhibitor, whereas control polymer and unmodified PCP exhibited only a negligible inhibitory effect against α-chymotrypsinic degradation. Recently, 1 mg of a similar poly(acrylic acid)–chymostatin

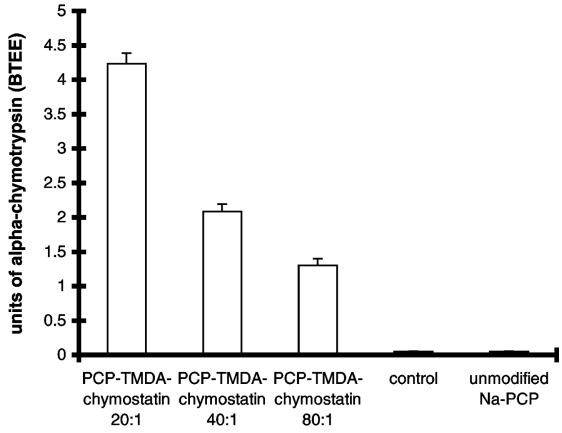

Fig. 3. Comparison of the inhibitory activity of PCP–TMDA-chymostatin conjugates towards α-chymotrypsin. The protective effect was determined by units of α-chymotrypsin (BTEE), which had to be added to 0.6 mg of derivative in order to obtain a 50% inhibition of proteolytic activity within 10 min. The assay was performed at room temperature. Each bar represents the mean protective effect of at least three experiments ± SD.

conjugate also resulting from a nominal polymer/inhibitor ratio of 20:1 but using 1,8-diaminooctane as spacer was shown to be capable of inhibiting the proteolytic activity of 4.0 ± 0.7 units ($n = 3$; ±SD) of α-chymotrypsin by 50% [15]. The inhibitory efficacy of PCP–TMDA-chymostatin 20:1 was determined to be even 1.8-fold higher than that of the C8-spacer conjugate, as 0.6 mg of this conjugate inhibited 4.2 ± 0.15 units ($n = 3$; ±SD) of the enzyme by 50%.

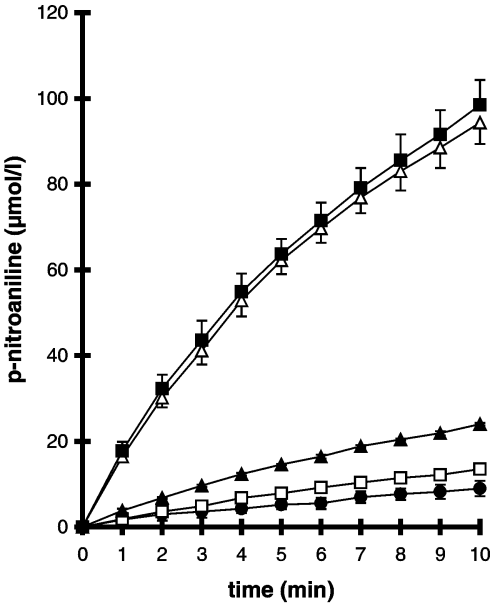

Fig. 4. Comparison of PCP–TMDA-elastatinal conjugates 20:1 (●), 40:1 (□), 80:1 (▲), control (△), and unmodified Na-PCP (■) with regard to their protective effect towards proteolysis caused by elastase. The increase in hydrolyzed substrate (*p*-nitroaniline) was determined at 1-min intervals of solutions containing 6.7 µg of elastase, 0.8 mg of the polymers and 26 µg of succinyl-(L-alanyl)$_3$-4-nitroanilide in 0.3 ml of 100 mM PBS (pH 7.1), at 20°C. Each point represents the mean ± SD of at least three experiments.

Also elastatinal, which is in its structure very similar to antipain and chymostatin (Fig. 1), could be successfully immobilized to PCP via TMDA. The protective effect of the resulting polymer-inhibitor conjugates towards elastase has been evaluated. Results are shown in Fig. 4. All conjugates displayed a significant protective effect, whereas control polymer and unmodified Na-PCP, respectively, showed no inhibitory effect, verifying the efficacy of the purification method.

3.2. PCP–PEG-inhibitor conjugates

To obtain the immobilization of the PEG-inhibitor mixture on polycarbophil, the protective t-BOC group of the diaminated PEG-derivative had to be cleaved prior to adding polycarbophil to the reaction mixture. Determination of the primary amino groups of the PEG-inhibitor reaction mixtures via TNBS-reagent confirmed a successful cleavage. After the pH of the samples was readjusted to 6, the amount of detectable primary amino groups was more than twofold higher. Nevertheless, the inhibitory efficacy of the PEG-conjugates was quite low compared to corresponding TMDA-conjugates. Results of the evaluation of the inhibitory effects of PCP–PEG-inhibitor conjugates towards their corresponding proteases are shown in Figs. 5–7. The inhibitory activity of PCP–PEG-chymostatin 20:1 conjugate, for instance, was determined to be even 7-fold lower than that of the corresponding TMDA conjugate.

The structure of the high molecular weight PEG-derivative

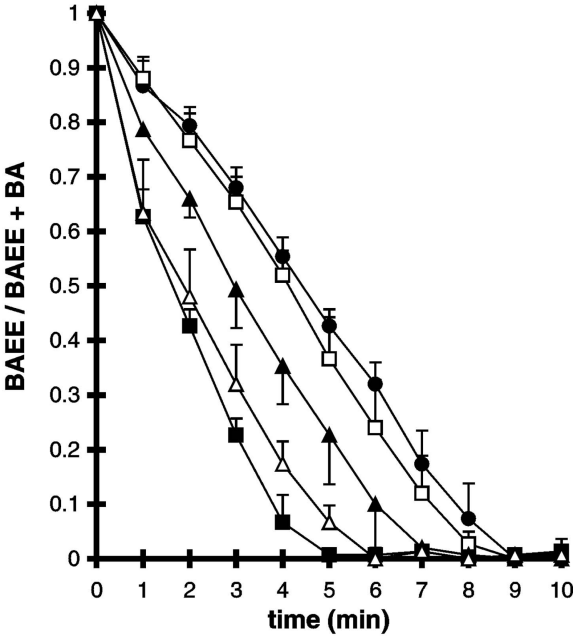

Fig. 5. Comparison of the inhibitory effect of 0.1% PCP–PEG-antipain conjugates 20:1 (●), 40:1 (□), 80:1 (▲), control (△), and 0.1% unmodified Na-PCP (■) towards trypsin (70.7 spectrophotometric BAEE units/ml). Hydrolysis of the substrate *N*-α-benzoylarginine ethyl ester (BAEE) to *N*-α-benzoylarginine (BA) was determined at pH 7.1 and 20°C. Indicated values are means of at least three experiments (±SD).

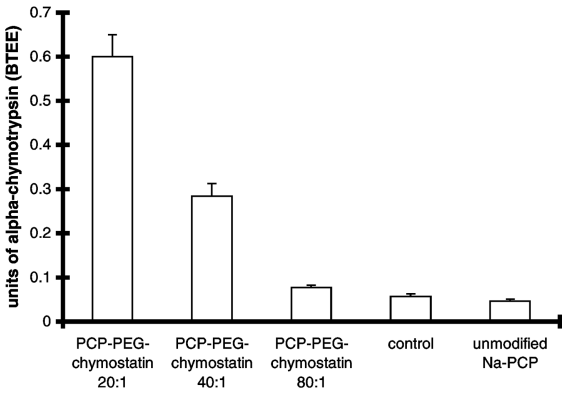

Fig. 6. Comparison of the inhibitory activity of PCP–PEG-chymostatin conjugates towards α-chymotrypsin. The protective effect was determined by units of α-chymotrypsin (BTEE), which had to be added to 0.6 mg of derivative in order to obtain a 50% inhibition of proteolytic activity within 10 min. The assay was performed at room temperature. Each bar represents the mean protective effect of at least three experiments ± SD.

(MW 3400) used in the present study might have an important impact on these results. Although it was believed that the long chains of the high weight PEG molecules could allow the inhibitors attached to the spacer chains ends to act more freely, thus giving it a higher chance of locating and interacting with their corresponding proteases, inhibition studies

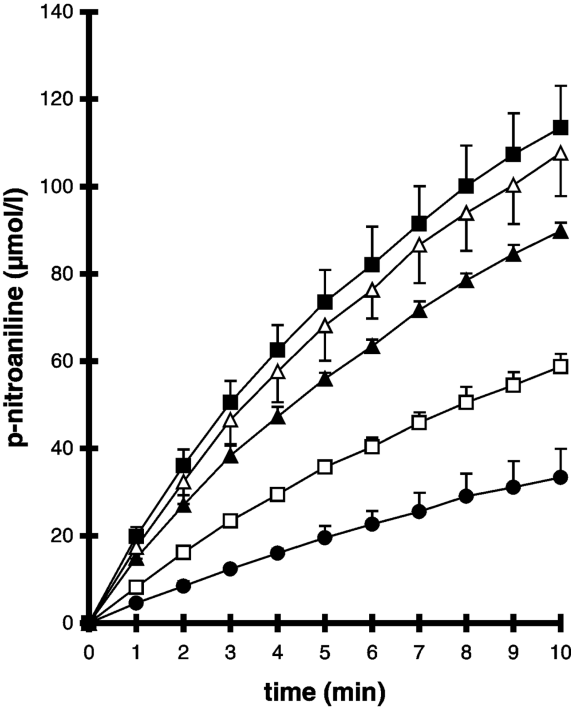

Fig. 7. Comparison of PCP–PEG-elastatinal conjugates 20:1 (●), 40:1 (□), 80:1 (▲), control (△), and unmodified Na-PCP (■) with regard to their protective effect towards proteolysis caused by elastase. The increase in hydrolyzed substrate (*p*-nitroaniline) was determined at 1-min intervals of solutions containing 6.7 µg of elastase, 0.8 mg of the polymers and 26 µg of succinyl-(L-alanyl)$_3$-4-nitroanilide in 0.3 ml of 100 mM PBS (pH 7.1), at 20°C. Each point represents the mean ± SD of at least three experiments.

showed opposite results. DeAscentiis et al. reported recently, that the addition of linear PEG-chains improved mucoadhesion of poly(2-hydroxyethyl methacrylate) (PHEMA) microspheres. This was attributed to a significant increase of the PEG-chains penetrating across the mucosal tissue. However, very long pendant chains had a detrimental effect on interpenetration. This was seen as the result of extensive entanglements by the long PEG-chains and impediment of chain mobility across the PHEMA/mucosal interface [16]. Taking this report into account, results obtained within the present study might indicate a similar phenomenon. It is conceivable that the PEG-chains accommodate a kind of 'mushroom' conformation providing tremendous steric hindrance to the system. In addition, Walker et al. reported recently that inhibition of trypsin by the mucoadhesive polymer carbomer is due to direct enzyme-polymer interaction [17]. With this in mind, it is conceivable that enzyme–polymer interactions only could take place if the inhibitors were immobilized with the C4-spacer TMDA, which should additionally provide evidence for a direct contact between enzyme and inhibitor.

3.3. Conclusion

Within the present study we have immobilized specific serine protease inhibitors on the anionic mucoadhesive polymer polycarbophil. A small rigid C4-spacer was thereby shown to be highly advantageous compared to a long and more flexible PEG-spacer. Further, comparison with former studies dealing with similar inhibitor-conjugates confirmed the potency of the C4-spacer conjugates. The present study might therefore provide helpful information for the design of oral delivery systems for peptide drugs, as overcoming the enzymatic barrier of the gut is still one of the major challenges in oral peptide drug delivery.

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