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Preparation and Preliminary Characterization of Poly(ethylene Glycol)-Pepstatin Conjugate

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ABSTRACT

The carboxyl function of pepstatin has been coupled, through an amide bond, to methoxypoly(ethylene glycol) (5 kDa), to which an amino function had been previously grafted. The mPEG-pepstatin conjugate inhibits hog pepsin (aspartic proteinase) in vitro as pepstatin itself, however, with a 400 times higher apparent K_i. The conjugate apparently does not inhibit proteinases belonging to other proteinase families such as serine (trypsin, carboxypeptidase Y), cysteine (Papaya proteinase III), or metallo (collagenase) proteinases.

Index Entries: Aspartic proteinases; inhibitors; polyethylene glycol; covalent attachment.

INTRODUCTION

The rapid spread of human immunodeficiency virus (HIV), as causative agent of acquired immunodeficiency syndrome (AIDS), has prompted, throughout the world, an intense search for antiretroviral therapeutics.

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Among the key steps identified to date, proteolytic processing of the polyprotein encoded by the *gag pol* gene of the retrovirus has drawn much attention. This step, which yields the virally encoded enzymes HIV protease (HIVP), reverse transcriptase and integrase is known to be effected by HIVP itself since mutant systems in which the catalytic sequence is modified are noninfectious (*1*). Owing to its role in viral replication and infectivity, HIVP has thus become a major target in the search for an effective antiviral agent (*2,3* and references therein). Several series of different chemical inhibitors of HIVP have been described (*3–8*). Although very efficient in vitro, in most cases, these inhibitors are unable to inhibit viral replication in infected cells. This low antiviral activity has been attributed to a poor penetration of these inhibitors across the cell membranes (*7*).

On the other hand, the covalent modification of enzymes with methoxypoly(ethylene glycol) (mPEG) chains is, in some cases, followed by an increased uptake of these enzymes by various types of cells. This was examined in the cases of PEG-superoxide dismutase (*7*), PEG-catalase (*9,10*), PEG-sphingomyelinase (*11*), and PEG-asialofetuin (*12*).

Although the mechanism of this process remains under debate, several main lines suggest that PEG facilitates binding (interaction) to cell membranes; this step being then followed by endocytosis of the PEG-protein conjugate (*9*). Evidence that PEG interacts with cell membranes is now well documented since the demonstration of the PEG-induced fusion of cells and liposomes (*13–20*). Noteworthy, the interpretation of some two-phases partitioning results requires the concept that PEG interacts with cell membranes (*21*).

Thus, the chemical modification of known powerful inhibitors of HIVP by PEG chains is potentially an efficient way to increase their uptake by infected cells and as a consequence their ability to inhibit viral replication. The inhibitory strength of such mPEG-proteinase inhibitor conjugates, remains however to be evaluated since such cases have not yet been examined and/or reported. This prompted us to covalently attach mPEG to pepstatin, an inhibitor of most proteases belonging to the aspartic proteinase family and to evaluate the conjugate as an inhibitor of pepsin. In addition to pepsin, pepstatin has also been reported to inhibit HIVP (*22–24*).

MATERIALS AND METHODS

Reagents

Pepsin (from hog stomach) was purchased from Fluka (Buchs, Switzerland). Pepstatin A, Trypsin (from bovine pancreas), carboxypeptidase Y, collagenase Typ XI (from *Clostridium histolyticum*), *N*-alpha-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA), *N*-alpha-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) and 2-furanacroyl-L-leucylglycyl-L-propyl-L-alanine

(FALGPA) were all Sigma (St. Louis, MO) products. *N*-acetyl-L-phenylalanine-3,5-diiodotyrosine was purchased from I.C.N. Flow (High Wycombe, England). Fluorescamine, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) and mPEG (M_r: 5 kDa) were Aldrich (Milwaukee, WI) reagents.

Preparation of the mPEG-Pepstatin Conjugate

Pepstatin A (73 μmol) and amino-mPEG (M_r: 5.123 kDa-73 μmol) were dissolved in dry tetrahydrofuran (50 mL). EEDQ (1 mmol) was then added and the reaction allowed to proceed for 24 h at 35–40°C under magnetic stirring. The reactional mixture was concentrated by rotaevaporation and then poured in diethylether (100 mL). The conjugate was collected by filtration, dried, and redissolved in water. The aqueous solution was dialyzed exhaustively against water and lyophilized. Amino-mPEG was obtained as previously described (*25*).

Fluorimetric Assay of Primary Amine

Primary amine functions were determined fluorimetrically after reaction with fluorescamine (*26*). Solutions of PEG conjugates were prepared in 100 m*M* phosphate buffer at pH 8. Each test tube containing the PEG derivatives was adjusted to 1.5 mL. Fluorescamine (0.3 mg/mL) in acetone (0.5 mL) was added to each test tube while vortexing. After 10 min, the fluorescence of the solutions was measured at 25°C, on a Shimadzu RF 5001-PC using an excitation wavelength of 390 nm. Emission intensities were recorded at 475 nm.

Pepsin Assay

Pepsin activity was measured at pH 2 at 37°C using *N*-acetyl-L-phenylalanine-L-3,5-diiodotyrosine as substrate (*27*). Each test tube (total volume: 3 mL) contained 150 μ*M* substrate, variable concentrations (from 0 to 1 μ*M*) of pepstatin or of the mPEG-pepstatin conjugate, 0.5 μ*M* pepsin, and dimethylsulfoxide (final concentration: 5% v/v) dissolved in a buffer at pH 2. The pH 2 buffer referred to a solution containing 1 m*M* acetic acid and HCl to adjust the pH to its final value. Stock solutions of substrate (10 m*M*), of pepstatin (50 μ*M*), and of the mPEG-pepstatin conjugate (50 μ*M*) were prepared in dimethylsulfoxide (DMSO). Peptic hydrolyses were initiated by adding the enzyme and stopped after 5 min by adjusting the mixtures to pH 8 by addition of 300 μL of a 100 m*M* Na_3PO_4 solution.

The extent of hydrolysis was determined by measuring the fluorescence generated by the reaction between fluorescamine and the L-diiodotyrosine formed (*26*). In the absence of inhibitor, about 10% of the dipeptide substrate was hydrolyzed during the 5 min incubation. The production of L-diidotyrosine, on the other hand, has been reported to be a linear function of time until about 30% of the substrate was hydrolyzed (*27*).

Other Enzyme Assays

Trypsin and Papaya proteinase III were assayed at 37°C by using BAPNA as substrate (*28*). Papaya proteinase III was isolated and purified as previously described (*29*). Similarly, carboxypeptidase Y was assayed at 22°C by using BTPNA as substrate (*30*). Each test tube (total volume: 2 mL) contained 1 m*M* BAPNA or 250 μM BTPNA, various concentrations (1.2, 12, or 120 μM) of the mPEG-pepstatin conjugate and the enzyme: trypsin (7.5 μg), Papaya proteinase III (50 μg), or carboxypeptidase Y (105 μg) dissolved in buffers and containing DMSO (10% v/v). The buffers used were 100 m*M* Tris-HCl at pH 8.5 for trypsin and 100 m*M* phosphate containing 1 m*M* EDTA and 2 m*M* dithiothreitol at pH 6.8 for Papaya proteinase III and for carboxypeptidase Y. Stock solutions of BAPNA (20 m*M*), BTPNA (5 m*M*), and of the mPEG-pepstatin conjugate (2.4 m*M*) were prepared in DMSO.

Hydrolysis was initiated by adding the substrate and stopped by addition of 500 μL of an aqueous acetic acid solution. The extent of hydrolysis was determined by measuring spectrophotometrically at 410 nm, the release of 4-nitroaniline (*28*) using a molar absorption coefficient of 8800 $L \cdot mol^{-1} \cdot cm^{-1}$.

As previously described (*31*), collagenase was assayed at 22°C with FALGPA as substrate.

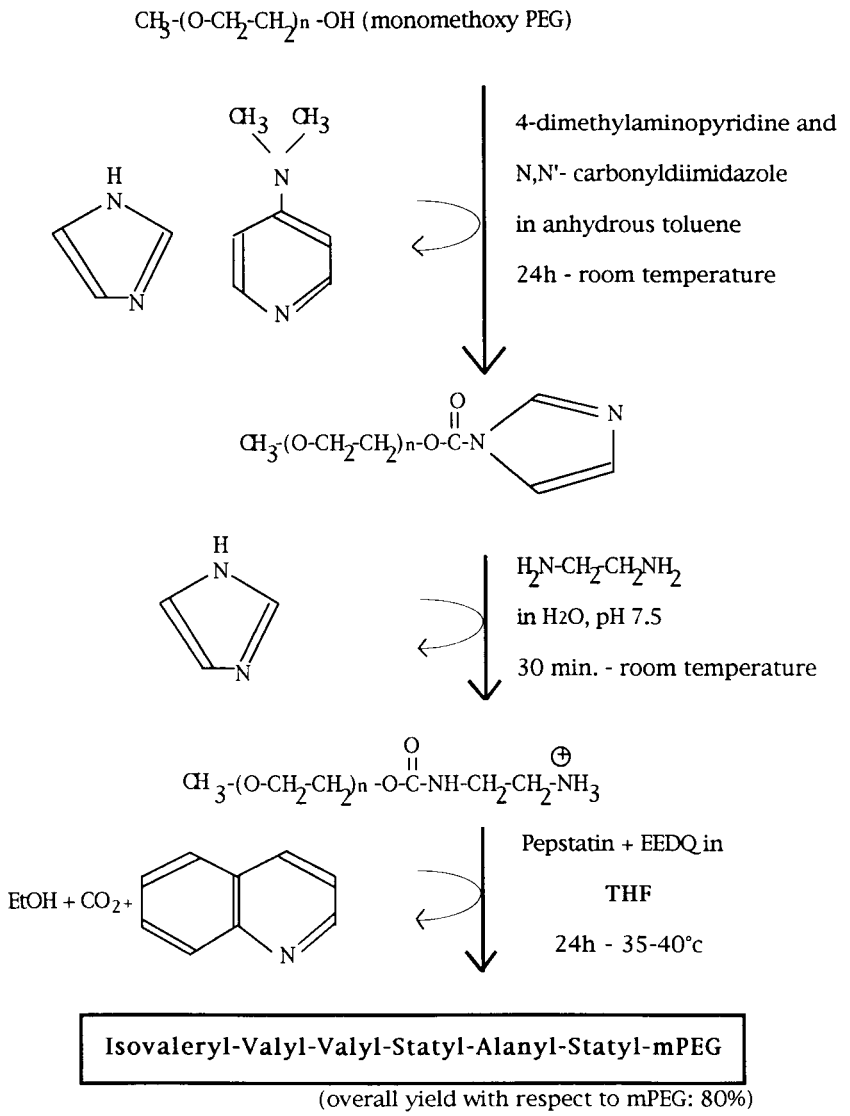
RESULTS AND DISCUSSION

Synthesis and Characterization of the mPEG-Pepstatin Conjugate

The mPEG-pepstatin conjugate was prepared as summarized in Scheme 1. The detailed procedure to obtain carbonylimidazol-1-yl-mPEG and amino-mPEG has been described previously (*25*).

EEDQ was chosen to couple the amino function of amino-mPEG to the carboxyl function of pepstatin. This choice was dictated by previous observations indicating that EEDQ selectively activates carboxyl functions even in the presence of other nucleophiles (pepstatin also contains two alcoholic functions) without introducing racemization in peptides (*32*).

The infrared spectrum of the mPEG-pepstatin conjugate is shown in Fig. 1 and is compared to that of the amino-mPEG. Both compounds are characterized by an absorption band at 1717 cm^{-1} owing to the presence of the carbamate function. In addition to this absorption band, the mPEG-pepstatin conjugate shows three additional bands at 1613, 1632.5, and 1653.5 cm^{-1} that reveal the presence of amide functions. The Infrared spectrum of the mPEG-pepstatin conjugate also shows the absence of carboxylic function. This indicates that unreacted pepstatin does not contaminate the preparation containing the conjugate.



Scheme 1. Schematic route to the conjugate PEG-pepstatin.

To detect the possible presence of unreacted amino-mPEG, the mPEG-pepstatin conjugate was reacted with fluorescamine and the reaction mixture was analyzed fluorimetrically (*26*). As shown in Fig. 2, this method is able to detect μM amounts of amino-mPEG and give rise to fluorescence signals proportional to the concentration of amino-mPEG up to at least 30 μM (correlation coefficient: 0.999). Figure 2 also shows that no amino-mPEG could be detected in the preparation of the mPEG-pepstatin conjugate (even at concentration up to 250 μM).

Finally, it was observed that the mPEG-pepstatin conjugate was quite transparent in the UV range above 240 nm. This indicates that the preparation was free from UV absorbing reaction by-products resulting from Step 3 in Scheme 1.

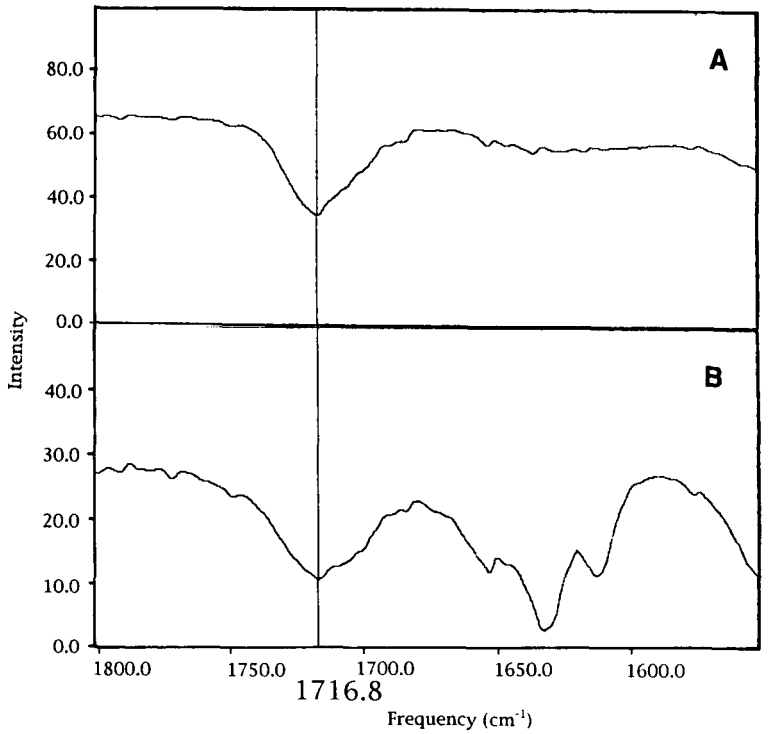


Fig. 1. Infrared spectra of Amino-mPEG (A) and of the mPEG-pepstatin conjugate (B) in the range from 1550 to 1800 cm^{-1}.

Inhibition of Hog Pepsin by the mPEG-Pepstatin Conjugate

Hog pepsin was incubated at 37°C and pH 2 with various concentrations of either pepstatin or of the mPEG-pepstatin conjugate and the residual proteolytic activity was measured using *N*-Acetyl-L-phenylalanine-L-3,5-diiodotyrosine as substrate.

The inhibition constant (K_i) was determined from the IC_{50} value taken from the plots of V_i/V_0 vs inhibitor concentration, where V_i is the inhibited velocity and V_0 the velocity in the absence of inhibitor (calculated from Fig. 3).

The IC_{50} value was converted to K_i by the equation of Cha (33):

$$K_i = (IC_{50} - Et/2)\,(1 + S/K_m)^{-1}$$

where Et is the total enzyme concentration, K_m is the Michaelis constant for the substrate, and S is the substrate concentration. Using a K_m value of 75 μM (27) and the results from Fig. 3, the K_i value of the mPEG-pepstatin conjugate is estimated to be 20 nM.

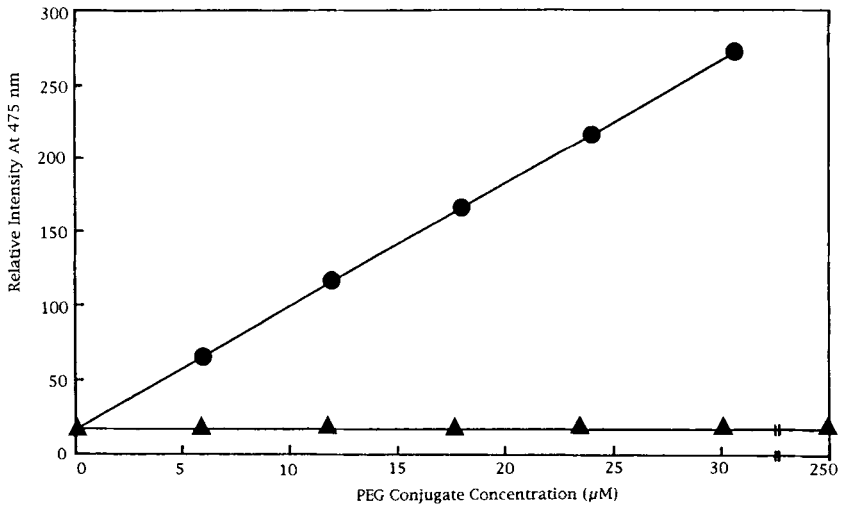


Fig. 2. Fluorescamine standard curves for Amino-mPEG (●) and for the preparation of the mPEG-pepstatin conjugate (▲).

Taking into account the difficulties associated with the determination of inhibition constants for tightly-bound inhibitors (*34*) such as pepstatin or the mPEG-pepstatin conjugate, these values should be regarded as estimated.

The stoichiometric inhibition of pepsin by pepstatin, as observed here, is in excellent agreement with the previously reported dissociation constant K_i of 0.05 n*M* (*35*). Thus, the covalent attachment of mPEG to pepstatin appears to reduce its inhibitory power by 2 to 3 orders of magnitude. In the course of this work, it has been firmly established that the activity of the proteinases, investigated here, was unaffected by the presence of mPEG (even at concentration up to 1 m*M*). However, the presence of mPEG in the mPEG-pepstatin conjugate preparation might affect the real inhibitor concentrations and hence the value of K_i might be over-estimated. The presence of mPEG may indeed result from hydrolysis of carbonyl-imidazol-1-yl-mPEG during storage of this material or from step 2 in Scheme 1. Fortunately, the mPEG-pepstatin conjugate is, by far, less water soluble than mPEG. We were thus able to submit the mPEG-pepstatin conjugate preparation to an extraction with water and to show that the results of Fig. 3 were not affected by such a treatment.

Therefore, we concluded that the conjugation of mPEG to pepstatin really reduces the affinity of the inhibitor toward pepsin. Nevertheless, the K_i value of 20 n*M* is still low and, thus, the conjugate preserved a high inhibitory potency. The case of pepstatin, investigated here, might well be not unique and should prompt assays of covalent attachment of PEG chains to other enzyme inhibitors presenting a more relevant character from a therapeutical point of view.

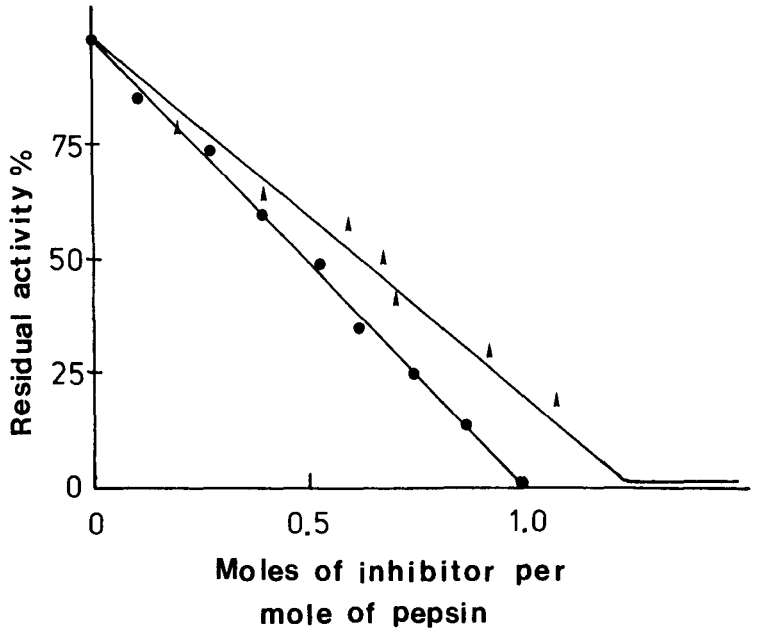


Fig. 3. Inhibition of hog pepsin by pepstatin (●) and by the mPEG-pepstatin conjugate (▲). For experimental details, *see* Material and Methods.

Table 1
Specificity of the Inhibition by the mPEG-Pepstatin Conjugate

Enzyme	Residual activity (%) in the Presence of the mPEG-Pepstatin Conjugate at the Concentration of		
	1.2 μM	12 μM	120 μM
Pepsin	0	0	0
Trypsin	100	65.7	43.6
Papaya proteinase III	100	100	100
Collagenase	n.d.[a]	n.d.	100
Carboxypeptidase Y	n.d.	n.d.	78

[a]Not determined.

Specificity Shown by the mPEG-Pepstatin Conjugate

In order to examine the specificity of the mPEG-pepstatin conjugate, its ability to inhibit trypsin and carboxypeptidase Y (serine proteinase), Papaya proteinase III (cysteine proteinase), and collagenase (metalloproteinase) was also investigated.

As shown in Table 1, at concentrations as high as 120 μM, the conjugate did not inhibit either collagenase nor Papaya proteinase III, whereas

some inhibition of the serine proteinases, trypsin, and carboxypeptidase Y, could be observed. However, such an inhibition could only be measured by using high ratios of inhibitor to enzyme (about 150 mol of inhibitor/mol of enzyme). These conditions basically differ from the nearly stoichiometric complex observed in the case of pepsin.

It may thus be concluded that the mPEG-pepstatin conjugate, as expected, specifically inhibits aspartic proteinases as pepstatin does. It should be important, however, to rule out any possible interference of the inhibitor conjugates with the lysosomal cathepsins, and so avoid any disturbance in the metabolism of noninfected cells. Furthermore, more specific inhibitors of HIVP should be conjugated to mPEG in order to produce a more therapeutically relevant product.

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