Amino Acids and Peptides. XXVIII.[1] Preparation of Poly(Ethylene Glycol) Hybrids of Gln–Val–Val–Ala–Gly Analogs and Their Inhibitory Effect on Papain

Koichi KAWASAKI,* Sachie INOUE, Yoshio OKADA, Shin IGUCHI, and Hiroshi OKAMOTO

Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Ikawadani-cho, Nishi-ku, Kobe 651–21, Japan.
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Gln–Val–Val–Ala–Gly is a sequence which is common in cysteine protease inhibitors. Poly(ethylene glycol) hybrids of Gln–Val–Val–Ala–Gly analogs were prepared by the solid phase method by fluorenylmethyloxycarbonyl chemistry. The hybrid formation of Gln–Val–Val–Ala–Gly resulted in improvement of the solubility and also in enhancement of the inhibitory effect of the peptide on papain. In addition, the hybrid formation of Gln–Val–Val–Ala–Gly analogs resulted in the enhancement of the inhibitory effect of the peptide analogs.

Key words cysteine protease inhibitor; poly(ethylene glycol); poly(ethylene glycol) hybrid; peptide hybrid

Poly(ethylene glycol) (PEG) is low toxic, low immunogenic and has good solubility in both aqueous and organic solvents. It thus seems to be a promising canditate as a drug carrier. We already reported that the hybrid formation of an oligopeptide with PEG was effective to potentiate the effect of the peptide. The inhibitory effects of fibronectin-related peptides (Arg–Gly–Asp and Arg–Gly–Asp–Ser) and a laminin-related peptide (Tyr–Ile–Gly–Ser–Arg) on experimental metastasis were potentiated by hybrid formation with PEG.[2]

The Gln–Val–Val–Ala–Gly (QVVAG) sequence was found to be a common sequence in cystein protease inhibitors, and peptides containing the Gln–Val–Val–Ala–Gly sequence were found to be inhibitors of cysteine protease.[3] Here we describe the preparation and inhibitory effect of PEG hybrids of Gln–Val–Val–Ala–Gly derivatives. Of the synthetic Gln–Val–Val–Ala–Gly analogs reported already, the inhibitory effect of benzyloxycarbonyl (Z) derivative (Z–Gln–Val–Val–Ala–Gly–OH) was more potent than that of H–Gln–Val–Val–Ala–Gly–OH, furthermore, the inhibitory effect of *p*-nitroanilide (*p*NA) analog (Z–Gln–Val–Val–Ala–Ala–*p*NA) was more potent than that of Z–Gln–Val–Val–Ala–Ala–OMe.[3] Z and *p*NA are aromatic moieties, therefore, we planned to prepare a Gln–Val–Val–Ala–Gly derivative which has an aromatic moiety at amino- and/or carboxyl terminals. As an aromatic moiety, *p*-aminobenzoic acid (PABA) was selected. Peptides were prepared by the manual solid phase method using fluorenylmethyloxycarbonyl (Fmoc)[4] chemistry on Rink resin.[5] PEG was oxidized to give a carboxylic acid derivative (cPEG)[6] and it was reacted with the peptide-resin. The following peptides were prepared: H–Gln–Val–Val–Ala–Gly–NH_2 (I), cPEG–Gln–Val–Val–Ala–Gly–NH_2 (II), H–Gln–Val–Val–Ala–Gly–PABA–NH_2 (III), cPEG–Gln–Val–Val–Ala–Gly–PABA–NH_2 (IV), H–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 (V), cPEG–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 (VI).

MATERIALS AND METHODS

For amino acid analysis, synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h and PEG–peptide hybrids were hydrolyzed for 48 h. The amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. Solvent systems for ascending TLC on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = *n*-BuOH–AcOH–water (4 : 1 : 5, upper phase), Rf^2 = *n*-BuOH–pyridine–AcOH–water (4 : 1 : 1 : 2), Rf^3 = $CHCl_3$–MeOH–water (8 : 3 : 1, lower phase), Rf^5 = $CHCl_3$–AcOH–MeOH (90 : 2 : 8). Reverse phase (RP)-HPLC was conducted with a Waters 600 on a YMC Pack AQ-ODS-5 column using gradient systems of CH_3CN–H_2O containing 0.1% trifluoroacetic acid (TFA). FAB-MS were measured on a VG Analytical ZAV-SE spectrometer. Poly(ethylene glycol) #4000 (molecular weight 2700—3500, average molecular weight 3100. Nacalai Tesque, Inc.) was converted to its carboxylic acid derivative (cPEG) according to the method reported by Ueyama *et al.*[6] Rink amide resin[4(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin] was purchased from Watanabe Chemical Industries, Ltd.

Fmoc–Gly–PABA–OH Thionyl chloride (0.73 ml, 10.1 mmol) was added to a solution of Fmoc–Gly–OH (1 g, 3.36 mmol) dissolved in a mixture of dioxane (5 ml) and dichloromethane (DCM) (20 ml) and the whole was stirred for 1 h at 40 °C. The solvent was removed and the residue was dried *in vacuo*. The residue was dissolved in DCM–dioxane (1 : 1, 10 ml) and the solution was combined with a PABA solution [1.38 g (10 mmol) in dioxane (25 ml)]. Precipitation occurred immediately and the mixture was stirred for 2 h. The solvent was removed *in vacuo* and 0.5 N HCl (50 ml) was added to the residue. The mixture was stirred for 1 h and the precipitate was collected by filtration. The precipitate was washed with water repeatedly and dried. The material was suspended in MeOH, boiled for 5 min and collected by filtration. This operation was repeated 3 times. Yield 614 mg (44%), mp 270—272 °C (dec.), Rf^5 0.49. *Anal.* Calcd for $C_{24}H_{20}N_2O_5$: C, 69.22; H, 4.84; N, 6.73. Found: C, 68.99; H, 4.79; N, 6.54.

Peptide Synthesis The peptides and cPEG–peptide hybrids were prepared by the solid phase method using Fmoc chemistry.[6] Fmoc–Gly–PABA–OH, Fmoc–Ala–OH, Fmoc–Val–OH, and Fmoc–Gln–OH were allowed to react according to the dicyclohexylcarbodiimide (DCC) method in the presence of 1-hydroxybenzotriazole (HOBt)[7]

* To whom correspondence should be addressed.

in *N*,*N*-dimethylformamide (DMF). The Fmoc group was removed by 20% piperidine–DMF treatment. Cleavage of peptides and cPEG-peptide hybrids from the resin was performed by 10% phenol–TFA treatment for 1 h. The resin was removed by filtration and ether was added to the filtrate to give a precipitate. The precipitate was collected by filtration or centrifugation, washed with ether and dried. Synthetic peptide analogs and cPEG hybrids were purified by RP-HPLC.

H–Gln–Val–Val–Ala–Gly–NH_2 · HCl (I) Amino acid ratios in an acid hydrolysate: Glu 1.03, Val 1.77, Ala 0.99, Gly 1.00 (average recovery 90%). Rf^1 0.08, Rf^2 0.13, $[\alpha]_D^{25}$ −56.6° (c=1.0, 50% AcOH), FAB-MS m/z 472 $(M+1)^+$.

cPEG–Gln–Val–Val–Ala–Gly–NH_2 (II) Amino acid ratios in an acid hydrolysate: Glu 1.06, Val 1.71, Ala 1.01, Gly 1.00 (peptide content: 484 μmol/g). Rf^2 0.30, Rf^3 0.54, Rf^5 0.20.

H–Gln–Val–Val–Ala–Gly–PABA–NH_2 · HCl (III) Amino acid ratios in an acid hydrolysate: Glu 0.98, Val 1.81 Ala 0.96, Gly 1.00 (average recovery 87%), Rf^1 0.14, Rf^2 0.47, $[\alpha]_D^{25}$ −45.0° (c=1.0, 50% AcOH), FAB-MS m/z 592 $(M+1)^+$.

cPEG–Gln–Val–Val–Ala–Gly–PABA–NH_2 (IV) Amino acid ratios in an acid hydrolysate: Glu 0.96, Val 1.74, Ala 1.02, Gly 1.00 (peptide content: 434 μmol/g). Rf^2 0.28, Rf^3 0.55, Rf^5 0.18.

H–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 · HCl (V) Amino acid ratios in an acid hydrolysate: Glu 0.93, Val 1.77, Ala 0.97, Gly 1.00 (average recovery 87%), Rf^1 0.05, Rf^2 0.05, $[\alpha]_D^{25}$ −40.3° (c=1.0, 50% AcOH), FAB-MS m/z 768 $(M+1)^+$.

cPEG–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 (VI) Amino acid ratios in an acid hydrolysate: Glu 0.93, Val 1.77, Ala 0.95, Gly 1.00 (peptide content: 398 μmol/g). Rf^2 0.24, Rf^3 0.46, Rf^5 0.11.

Measurement of Inhibitory Effect on Papain Activity The inhibitory effect of synthetic peptides and their hybrids on papain was measured according to the method described previously.[3] Papain activity was assayed by the hydrolysis of a synthetic substrate, N^{α}-benzoylarginine naphthylamide. All peptide samples were dissolved in dimethylsulfoxide (DMSO) and added to papain solution before the addition of the substrate.

RESULTS AND DISCUSSION

Fmoc–PABA–OH was prepared to introduce PABA to the resin, but it is not sufficiently soluble. It did not show good solubility even in DMF, trifluoroethanol, DMSO or mixtures of any of them. Trifluoroacetyl–PABA–OH was also prepared, but it was quite an unstable material. The elimination of a trifluoroacetyl group was observed during storage. Therefore, Fmoc–Gly–OH was prepared first, and it was treated with thionyl chloride to give Fmoc–Gly–Cl. The chloride was coupled with PABA to give Fmoc–Gly–PABA–OH. This was introduced to the resin by the DCC–HOBt method,[7] as shown in Fig. 1.

The Fmoc group was removed with 20% piperidine–DMF, and all coupling reactions were performed by the DCC–HOBt method. Since the introduction reaction of cPEG on the peptide-resin was slow, the coupling reaction

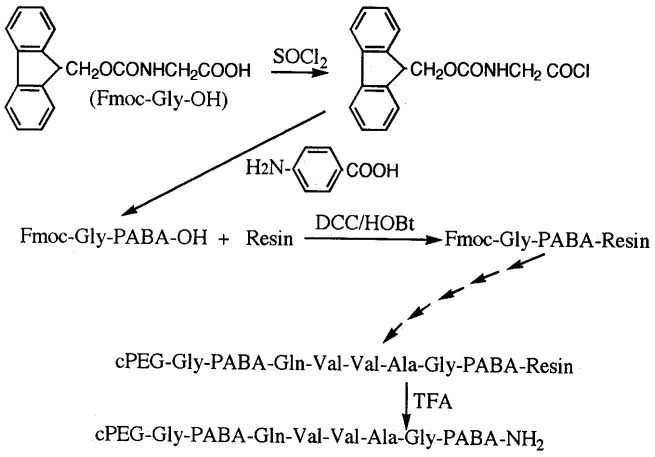

Fig. 1. Synthetic Scheme for cPEG–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2

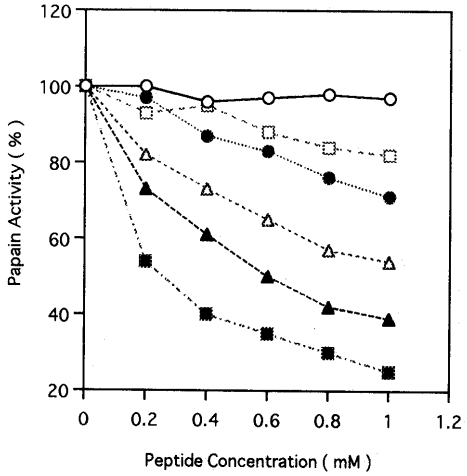

Fig. 2. Inhibition of Papain Activity by cPEG Hybrids of Gln–Val–Val–Ala–Gly Analogs

Papain was preincubated with peptides for 3 min, then the enzyme activity was measured using N^{α}-benzoylarginine naphthylamide as the substrate. Open symbols indicate results of the peptides without cPEG and corresponding closed symbols indicate their cPEG hybrids. Results represent the means of two measurements. —○—, QVVAG (I); ---●---, cPEG-QVVAG (II); ---△---,QVVAG-PABA (III); ---▲---, cPEG-QVVAG-PABA (IV); ---□---, G-PABA-QVVAG-PABA (V); ---■---, cPEG-G-PABA-QVVAG-PABA (VI).

was repeated 3 times using a three-fold molar ratio of cPEG. Synthetic peptides and hybrids were cleaved from resin by TFA treatment and were purified by Sephadex LH 20 column chromatography and RP-HPLC. The solubility of cPEG–Gln–Val–Val–Ala–Gly–NH_2 in water was better than that of H–Gln–Val–Val–Ala–Gly–NH_2 itself. Ten mg of H–Gln–Val–Val–Ala–Gly–NH_2 did not dissolve in 1 ml of water, but cPEG–Gln–Val–Val–Ala–Gly–NH_2 dissolved. A hybrid formation of Gly–PABA–Gln–Val–Val–Ala–Gly–PABA (V) did not result in any remarkable improvement of solubility. The solubility of V was low; 10 mg of the hybrid was soluble in 1 ml of DMSO but was not soluble in DMF and water.

The inhibitory effect of all peptides was potentiated by hybrid formation with cPEG (Fig. 2). For example, H–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 did not show a clear inhibitory effect, but its PEG hybrid, cPEG–Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2, did. Modification of the C-terminal with PABA showed a tendency to enhance the inhibitory effect, and modifi-

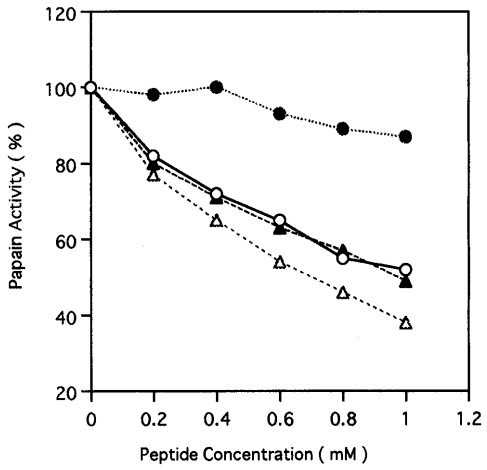

Fig. 3. Effect of Papain-Pretreatment on Inhibitory Activity of Gln–Val–Val–Ala–Gly Analogs

Papain was preincubated with H–Gln–Val–Val–Ala–Gly–PABA–NH_2 or its cPEG hybrid for 3 min (open symbols) or for 30 min (closed symbols), then the enzyme activity was measured. Results represent the means of two measurements. —○—, QVVAG-PABA; ---●---, papain-pretreated QVVAG-PABA; ---△---, cPEG-QVVAG-PABA; ---▲---, papain-pretreated cPEG-QVVAG-PABA.

cation of the N-terminal with PABA showed tendency to reduce the inhibitory effect.

Potentiation of the inhibitory effect of H–Gln–Val–Val–Ala–Gly–NH_2 by the hybrid formation might be explained by the resulting protection of the peptide from papain hydrolysis. To examine the stability of the cPEG-peptide hybrid, H–Gln–Val–Val–Ala–Gly–PABA–NH_2 and its cPEG hybrid were preincubated with papain for 30 min, then the substrate was added to measure papain activity.

As shown in Fig. 3, the inhibitory effect of H–Gln–Val–Val–Ala–Gly–PABA–NH_2 was clearly reduced, but cPEG–Gln—Val–Val–Ala–Gly–PABA–NH_2 maintained its inhibitory effect after the preincubation. Similar results were obtained when Gly–PABA–Gln–Val–Val–Ala–Gly–PABA–NH_2 and its cPEG hybrid were preincubated with papain.

These results suggest that the potentiation by the hybrid formation results from an increased stability in terms of papain hydrolysis. However, the possibility that the hybrid formation increases the intrinsic activity of the peptide could not be excluded. Hybrid formation with PEG may be an interesting topic of research regarding the drug delivery systems of small peptides. A big molecular portion of PEG in a hybrid may not disturb the activity of a small peptide portion since PEG has a flexible conformation. The flexible conformation of PEG may not disturb the binding of the peptide portion of the hybrid to a target molecule. Mechanisms by which the hybrid formation with PEG protects the peptide from enzymatic degradation remain to be determined.

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REFERENCES

1) This paper was reported at 113th Annual Meeting of the Japan Pharmaceutical Society, March 29—31, 1993, Osaka, Japan.
2) *a*) Kawasaki K., Namikawa M., Yamashiro Y., Hama T., Mayumi T., *Biochem. Biophys. Res. Commun.*, **174**, 1159—1162 (1991); *b*) *Idem, Chem. Pharm. Bull.*, **39**, 3373—3375 (1991); *c*) Kawasaki K., Murakami T., Namikawa M., Mizuta T., Iwai Y., Yamashiro Y., Hama T., Yamamoto S., Mayumi T., *ibid.*, **42**, 917—921 (1994).
3) *a*) Okada Y., Teno N., Itoh N., Okamoto H., *Chem. Pharm. Bull.*, **33**, 5149—5152 (1985); *b*) Teno N., Tsuboi S., Okada Y., Itoh N., Okamoto H., *ibid.*, **35**, 3853—3858 (1987); *c*) *Idem, Int. J. Peptide Protein Res.*, **30**, 93—98 (1987).
4) Carpino L. A., Han G. Y., *J. Am. Chem. Soc.*, **92**, 5748—5749 (1970).
5) Rink H., *Tetrahedron Lett.*, **28**, 3787—3790 (1987).
6) Ueyama N., Nakata M., Nakamura M., *Polymer J.*, **5**, 721—727 (1985).
7) König W., Geiger R., *Chem. Ber.*, **103**, 788, 2024, 2034 (1970).