Water-soluble fatty acid derivatives as acylating agents for reversible lipidization of polypeptides

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Abstract **A novel method allowing the conjugation of a fatty acid to a peptide or protein in aqueous buffer is described in this paper. L-Cysteinyl 2-pyridyl disulfide (CPD) (III), which was obtained by reacting L-cysteine (I) with 2,2-dithiopyridine (II), was reacted with the *N*-hydroxysuccinimide ester of palmitic acid (IV) to yield a water-soluble derivative of palmitic acid, termed Pal-CPD (V). Pal-CPD (V) could be reacted with a sulfhydryl-containing peptide or protein in aqeous buffer to yield the palmitic acid-derivatized conjugate (VI). The palmitic acid-derivatized Bowman–Birk protease inhibitor (BBI), synthesized using this conjugation method, was demonstrated to have 140-fold higher uptake into Caco-2 cell monolayers compared to native-BBI. The biological activity of the conjugate, as assessed using an in vitro transformation assay, was retained.**

Key words. Palmitic acid; Conjugation; Peptide; Protein; Cell-uptake

1. Introduction

The effective delivery of therapeutic peptides and proteins to their site of action in vivo is an area of great importance in pharmaceutics and biotechnology. Peptides and proteins, as a class of macromolecules, are generally poorly transported across biological membranes. Hydrophilicity, the lack of stability due to enzymatic or chemical degradation, and the lack of transport carriers capable of shuttling proteins across cell membranes, all play a part in precluding proteins from uptake into and transport across biological barriers such as the blood brain barrier and the gastrointestinal mucosa [1].

One attractive approach for improving the transport properties of peptides and proteins is to conjugate with membrane-binding carrier ligands. Hydrophobic carrier ligands, such as fatty acids, represent potentially one of the most useful types of carriers, and evidence suggests that fatty acid-conjugated peptides and proteins may be able to cross cell membranes, including the blood brain barrier [2]. Furthermore, several fatty acid-peptide conjugates, due to their amphipathic character, have been shown to possess important biological properties such as the immunostimulating activity [3,4].

However, the conjugation of fatty acids to peptides and proteins is a challenging task with unique problems. The major difficulties in conjugating fatty acids to peptides or proteins are the lack of solubility of both the fatty acid and the peptide or protein in the same solvent (organic or aqueous), and the loss of biological activity of peptides or proteins after fatty acid acylation [5,6]. In this paper, we present novel conjugation method which uses a water-soluble derivative of palmitic acid and allows an efficient and selective conjugation of a protein to a fatty acid, via a reversible disulfide linkage. Using this acylation method, we conjugated palmitic acid to Bowman–Birk protease inhibitor (BBI), an 8 kDa soybean polypeptide with potential cancer chemopreventive uses in human [7].

2. Experimental

2.1. Synthesis of L-cysteinyl 2-pyridyl disulfide (CPD) (III)

L-Cysteine (3.0 g) (**I**) and 2,2-dithiopyridine (**II**) (7.5 g) were mixed in ethanol, and the reaction was allowed to proceed at 25°C for 18 h. The solution was centrifuged in order to remove any precipitate, and CPD (**III**) in the supernatant was crystallized by the addition of cold benzene. The final product after recrystallized in benzene was a white solid with a m.p. (with decomposition) of 158°C. The molar yield of the product using this procedure was approximately 60%.

2.2. Synthesis of Pal-CPD (V)

Triethylamine (100 μl) was added to a solution of CPD (100 mg) (**III**) in 5 ml of dimethylformamide (DMF) and the resultant suspension was reacted with the *N*-hydroxysuccinimide ester of palmitic acid (**IV**) (250 mg) in DMF (5 ml) at 25°C for 24 h, during which time the suspension turned clear. This solution was diluted with 40 ml of ice-cold water and the precipitate, which contained Pal-CPD (**V**) and palmitic acid, was isolated by centrifugation. Pal-CPD (**V**) was separated from palmitic by suspension of the precipitate in water at pH 7.0, which dissolved Pal- CPD (**V**), but not palmitic acid. Pal-CPD (**V**) was purified by repeating precipitation at pH 3 and was analyzed by using NMR and TLC techniques.

2.3. Synthesis of SPDP-modified BBI (BBI-PDP)

BBI (20 mg) was reacted with 3-(2-pyridyldithio) propionic acid *N*-hydroxysuccinimide ester (SPDP, 5 mg in 100 μl of DMF) in 1 ml of sodium bicarbonate buffer (0.3 M, pH 8.0) for 2 h at 25°C. After purification of BBI-PDP using Sephadex G-50 gel-filtration chromatography, the extent of modification of BBI was estimated by measuring the release of the thiopyridine after the reduction of BBI-PDP with dithiothreitol (DTT). Using this procedure, approximately 4 amino groups per BBI molecule were modified with SPDP.

2.4. Synthesis of BBIssPal (VI)

BBI-PDP (20 mg) dissolved in 1 ml of PBS was adjusted to pH 5.0, reduced with DTT (25 mM) for 30 min, and subsequently eluted from a Sephadex G-50 column. The sulfhydryl-containing BBI fractions, which eluted at column void volume, were identified using Elman's reagent, and then reacted with a 3-fold excess (per sulfhydryl group on BBI) of Pal-CPD (**V**) in pH 7.0 phosphate buffered saline (PBS) for 16 h at 4°C. The reaction mixture was then acidified to pH 3.0 using 1 N HCl and the precipitate, which contained all of BBIssPal (**VI**) and the excess reagent of (**V**), was isolated by centrifugation. The precipitate was dissolved in DMF and eluted from a Sephadex LH-20 column using DMF. BBIssPal (**VI**), was isolated from fractions eluted at

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column void volume. The yield of the conjugate using this procedure was approximately 80% (by weight of BBI). The conjugation and the ratio of fatty acid to BBI in the conjugate was confirmed by using [^{3}H]palmitic acid as a tracer in a separate, but identical, set of experiments as described above.

2.5 The uptake of [^{125}I]BBIssPal by Caco-2 cells

Radioiodination of BBI and BBIssPal was carried out using the chloramine-T method [8]. Confluent, 14-day-old Caco-2 cell monolayers were washed once and then incubated with serum-free Dulbecco medium at 37°C for 30 min. Subsequently, the incubation media was replaced with media containing [^{125}I]BBI (10 μg/ml), either as native-BBI or as BBIssPal, and the monolayers were incubated for a further 60 min at 37°C. The monolayers were then washed three times with ice-cold pH 7.0 PBS, and then exposed to trypsin for 10 min at 37°C. The detached cells were transferred to tubes, isolated by centrifugation, washed three times using ice-cold PBS, assayed for accumulated radioactivity using a gamma counter, and finally assayed for cell protein using the method of Lowry [9] BBIssPal reduction was carried out at 37°C for 30 min using DTT (50 mM).

2.6. In vitro transformation assay

The transformation assays were carried out using C3H10T1/2 (clone 8) cells according to the procedure described by Reznikoff et al. [10]. Briefly, C3H10T1/2 cells were seeded into 60 mm dishes (20 dishes per each group of treatment) at a density of 1,000 cells/dish. After 24 h, cells were treated with 3-methylcholanthrene (MCA) at a final concentration of 1 μg/ml in the medium. The cells were allowed to grow in the presence of MCA for 24 h and the medium in each dish was replaced with fresh MCA-free medium, and subsequently the medium was replaced twice a week for two weeks and then once a week for four more weeks. For the measurement of anti-transformation activity, BBI or BBIssPal (1 μg/ml) was included in the medium in some dishes after the MCA-treatment for the first 3 weeks only. Six weeks after the MCA-treatment, the cells were washed, fixed and stained with Giemsa stain. The transformed foci (> 3 mm in diameter) in the dishes were examined under a microscope to score as type I, II, and III [10]. Only type II and III foci were used to evaluate the anti-transformation activity of BBI and BBIssPal.

3. Results and discussion

It has been demonstrated that the transport properties of peptides and proteins into and across cell barriers in vitro and in vivo can be drastically improved by conjugation with membrane-binding ligands. For instance, polylysine [11], transferrin [12], antibodies [13], folic acid [14] and spermine [15] have been used as carrier ligands, capable of transporting proteins into and even across cell barriers in vitro. Hydrophobic ligands, such as fatty acids, have also been used as transport ligands in vitro [6] and in vivo [2,16], and conceivably, because of high membrane affinity and low toxicity, may represent the most useful carrier ligands for conjugation to peptides and proteins.

The conjugation of fatty acids to peptides and proteins is, however, a challenging task, made particularly difficult by the lack of solubility of peptides and proteins in organic solvents and by the possible loss of biological activity of peptides and proteins after fatty acid acylation [5,6]. Fatty acids have been conjugated to peptides and proteins in organic solutions either directly [6,16,17] or using reverse phase micelles [2,18], and in aqueous solutions using micelluar suspensions [19,20] containing reactive fatty acid derivatives.

In contrast to other fatty acid conjugation methods, the procedure described in this paper allows the selective attachment of a hydrophobic ligand to peptides and proteins in aqueous buffer, avoiding the need to expose peptides and proteins to organic liquids during the conjugation process. Also, the conjugation method presented here is based on the covalent attachment of a fatty acid to a protein via a reversible disulfide linkage. We believe the use of the disulfide linkage offers two major advantages. Firstly, the reversible, biodegradable nature of the bond should allow for the regeneration of the active peptides and proteins after cellular reduction of the disulfide linkage in the conjugate [21]. Secondly, recent data suggest that the transcytosis of a protein-macromolecule conjugate across cell barriers can be achieved only if a reducible, reversible linkage is used in the conjugation of the macromolecule to the protein [22].

As an example of this acylation method, we conjugated palmitic acid to Bowman–Birk protease inhibitor (BBI). BBI is an 8kDa soybean polypeptide with both trypsin and chymotrypsin inhibitory activities [7]. It has been shown that BBI is capable of preventing transformation in vitro [23] and carcinogenesis in vivo [24], and it is currently being investigated as a potential cancer chemopreventive agent for use in humans. It has been suggested that BBI acts intracellularly to prevent the transformation [25–27]. Conceivably, improving the cell uptake properties of BBI may enhance the cancer chemopreventive potential of this polypeptide.

The synthetic route for BBIssPal is shown in Fig. 1. The cell uptake of [^{125}I]BBI, either as the native polypeptide or as BBIssPal, was studied in Caco-2 cell monolayers in the presence and absence of fetal bovine serum (FBS); the results are presented in Table 1. When the uptake studies were carried out in serum-free medium, BBIssPal demonstrated high affinity towards cell monolayers in vitro and was transported into cells by a 140-fold higher level compared to native BBI. The addition of serum to the medium greatly reduced the uptake of BBIssPal into cells in a concentration dependent manner, reducing the uptake by 85% and 98% in the presence of 1% and 10% FBS, respectively. These results can be explained by the affinity of BBIssPal towards not just the cell membrane, but also other components in serum. In particular, binding to fatty acid bind-

Table 1
Uptake of [^{125}I]BBI, either as the native polypeptide or as BBIssPal, by Caco-2 cells[a]

	Uptake (ng BBI/mg of cell protein)[b]		
	Serum-free[c]	1% FBS[c]	10% FBS[c]
BBI	3.9 ± 0.19	2.2 ± 0.17	1.3 ± 0.02
BBIssPal	540.0 ± 24.13	78.5 ± 3.41	12 9 ± 0.02

[a] The cell monolayers were incubated with ^{125}I-labeled conjugates at 10 μg/ml for 60 min at 37°C.
[b] The results presented are the average of three monolayers ± S.E.M
[c] The uptake experiments were carried out in Dulbecco medium, in the presence and absence of added FBS.

Table 2
The uptake of [^{125}I]BBI, either as the native polypeptide or as BBIssPal, by Caco-2 cells before and after reduction with DTT[a]

	Uptake (ng BBI/ mg of cell protein)[b]	
	Untreated[c]	DTT-treated[c]
BBI	4.8 ± 0.00	5.2 ± 0.00
BBIssPal	381.7 ± 0.03	46.5 ± 0.00

[a] The cell monolayers were incubated with ^{125}I-labeled conjugates at 10 μg/ml in serum-free Dulbecco medium for 60 min at 37°C
[b] The results presented are the average of three monolayers ± S.E.M.
[c] [^{125}I]BBI, either as the native protein or as BBIssPal, was incubated at 37°C for 30 min in the presence and absence of DTT (50 mM).

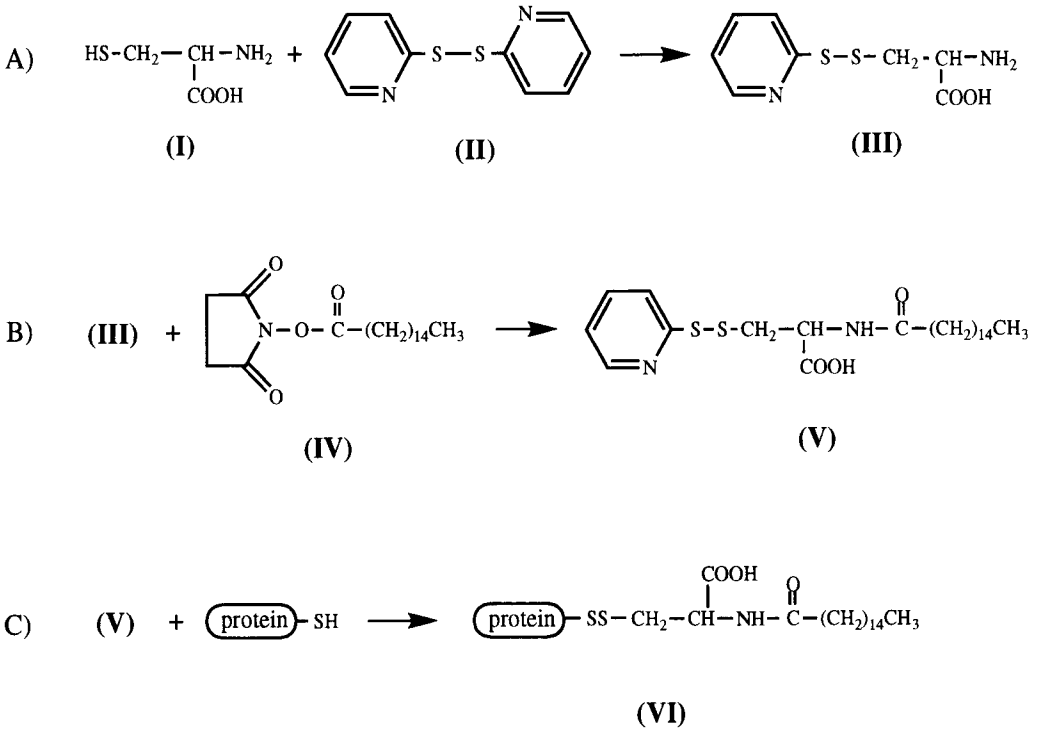

Fig. 1. Protein–palmitic acid conjugation using Pal-CPD (V). Pal-CPD (V) is synthesized in two steps: A and B. A protein containing a sulfhydryl group can then be reacted with Pal-CPD (V) in an aqueous buffer to yield the fatty acid-derivatized conjugate

ing proteins may account for the reduced uptake of BBIssPal into cells in the presence of serum. To demonstrate that the higher uptake of BBIssPal was mediated by the palmitic acid moiety in the conjugate, BBIssPal was reduced with DTT and the cell uptake in serum-free medium are shown in Table 2. The DTT treatment causes the reduction of disulfide linkages in BBIssPal and the detachment of the palmitic acid moiety from the conjugate. As presented in Table 2, the reduction with DTT resulted in a drastic decrease in the uptake of BBIssPal into cells. On the other hand, the uptake of unmodified BBI into cells was not influenced by the DTT treatment (Table 2). We also investigated the biological activity of BBIssPal using an in vitro transformation assay procedure [10], which was have used for the studies of other BBI conjugates [14,20]. The results indicated that BBIssPal is effective in preventing chemical-induced transformations in C3H10T1/2 cells in vitro (Table 3). Similar studies have also been observed in radiation-induced transformation assay (data not shown). Hence, in addition to having improved cell uptake properties, BBIssPal also retains the anticarcinogenic activity of the native BBI. However, since a dose-response relationship for the effectiveness of BBI in suppressing transformation in cell culture systems does not vary with concentrations of BBI ranging over orders of magnitude [28], no attempt was made in this report to correlate the increase of uptake with the anti-transformation activity of BBIssPal at extremely low concentrations C3H10T1/2 cells.

In summary, we conclude that: (a) the conjugation procedure described in this paper is an efficient and selective method of fatty acid conjugation to peptides or proteins; (b) fatty acid-conjugated proteins have high affinity towards cell monolayers in vitro, but can also bind other serum proteins factors; (c) the uptake of fatty acid conjugated polypeptides is mediated by the

Table 3
Effect of BBI and BBIssPal[a] on chemical-induced transformation in C3H 10T1/2 cells

Treatment group	Plating efficiency (%)	No. of dishes with transformed foci/Total no of dishes[c]	Fraction of dishes containing transformed foci
1 Controls–untreated[b]	23 ± 1.5	0/20	0
2. Negative controls–acetone-treated[b]	22 ± 2.0	0/20	0
3. Positive controls–MCA-treated[b]	21 ± 3.0	6/19	0.32
4. Test–MCA-treated + BBI[b]	24 ± 2.0	0/20	0
5. Test–MCA-treated + BBIssPal[b]	23 ± 3.0	1/20	0.05

[a] BBI, either as the free protein or in conjugated form to palmitic acid, was added to the cultures at 1.0 μg/ml for the first three weeks of the transformation assay period starting immediately after the MCA treatment.
[b] MCA-treated cells were exposed to 3-methylcholantherene, dissolved in 25 μl of acetone, at a concentration of 1 μg/ml for 24 h. Acetone-treated cells were exposed to 25 μl of acetone for 24 h only. The test groups were exposed to MCA for 24 h and then to the conjugates for the first three weeks of the assay. Untreated cells were exposed to neither MCA nor acetone.
[c] Statistical analysis (χ square): Group 4 vs. 3, $P < 0.05$; Group 5 vs. 3, $0.05 < P < 0.1$.

lipophilicity of the fatty acid moiety in the conjugate; and (d) the biological activity of the peptide or protein may be retained in the fatty acid conjugate.

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References

[1] Lee, V.H.L. (1991) Peptide and Protein Drug Delivery, Dekker, New York.
[2] Chekonin, V.P., Kabanov, A.V., Zhirkov, Y.A. and Morozov, G.V. (1991) FEBS Lett. 287, 149–152.
[3] Migliore-Samour, D., Bouchaudon, J., Floc'h, F., Zerial, A., Ninet, L., Werner, G.H. and Jollès, P. (1980) Life Sci. 26, 883–888
[4] Bessler, W.G., Johnson, R.B., Wiesmuller, K. and Jung, G. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 767–770.
[5] Torchilin, V.P., Omel'yaneko, V.G., Kilbanov, A.L., Mikhailov, A.L., Gol'danskII, V.L. and Smirnov, V.N. (1980) Biochim. Biophys. Acta 602, 511–521.
[6] Al-Obeido, F., Hruby, V.J., Yaghoubi, N., Marwan, M.M. and Hadley, M.E. (1992) J. Med. Chem. 35, 118–123.
[7] Birk, Y. (1985) Int. J. Peptide Protein Res. 25, 113–131.
[8] McConahey, P.C. and Dixon, F.J. (1980) Methods Enzymol. 70, 221–247.
[9] Lowry, O.H., Rosebrough, N.S., Farr, A.L. and Randall, R.I. (1951) J. Biol. Chem. 193, 265–275.
[10] Reznikoff, C.A., Bertram, J.S., Brankow, D.W. and Heidelberger, C. (1973) Cancer Res. 33, 3239–3249.
[11] Shen, W.C., Wan, J. and Shen, D. (1990) Biochem. Biophys. Res. Commun. 166, 316–323.
[12] Wan, J., Taub, M., Shah, D. and Shen, W C (1992) J. Biol. Chem. 267, 13446–13450.
[13] Youle, R.J. and Neville, D.M. (1980) Proc Natl. Acad. Sci. USA 77, 5483–5486.
[14] Leamon, C.P. and Low, P.S. (1992) J. Biol. Chem. 267, 24966–24971.
[15] Ekrami, H., Kennedy, A.R., Witschi, H. and Shen, W.C. (1993) J. Drug. Targeting 1, 41–50.
[16] Hashimoto, M., Takada, K., Kiso, Y. and Muranishi, S. (1989) Pharm. Res. 6, 171–176.
[17] Maneta-Peyret, L., Picard, J.P., Bezian, J.H. and Cassagne, C. (1992) Immunol. Lett. 31, 227–232.
[18] Robert, S., Domurado, D., Thomas, D. and Chopineau, J. (1993) Biochem. Biophys. Res. Commun. 196, 447–454.
[19] Martins, M.B.F., Jorge, J.C.S. and Cruz, M.E.M. (1990) Biochimie 72, 671–675.
[20] Huang, A., Huang, L. and Kennel, S.J. (1980) J. Biol. Chem. 255, 8015–8018.
[21] Persiani, S., Yeung, A., Shen, W.C. and Kennedy, A.R. (1991) Carcinogenesis 12, 1149–1152.
[22] Wan, J., Persiani, S. and Shen, W.C. (1991) J. Cell Physiol. 145, 9–15.
[23] Kennedy, A.R. (1993) in. Protease Inhibitors as Cancer Chemopreventive Agents (Troll, W and Kennedy, A.R., eds.) pp. 65–91, Plenum, New York.
[24] Kennedy, A.R. (1993) in: Protease Inhibitors as Cancer Chemopreventive Agents (Troll, W. and Kennedy, A.R., eds.) pp. 9–64, Plenum, New York.
[25] Billings, P.C., Carew, J.A., Keller-McGndy, C.E., Goldberg, A.L. and Kennedy, A.R (1987) Proc. Natl. Acad. Sci. USA 84, 4801–4805.
[26] Billings, P.C., Habres, J.M. and Kennedy, A.R (1990) Carcinogenesis 11, 329–332.
[27] Billings, P.C. and Habres, J.M. (1992) Proc. Natl. Acad. Sci USA 89, 3120–3124.
[28] Yavelow, J., Collins, M., Birk, Y., Troll, W. and Kennedy, A.R. (1985) Proc. Natl. Acad. Sci. USA 82, 5395–5399.