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Gastric retention and stability of lipidized Bowman–Birk protease inhibitor in mice

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Abstract

Bowman–Birk protease inhibitor (BBI) was modified with a reversible lipidizing agent. The palmitoylated product, Pal-BBI, and BBI were iodinated and orally administered to mice using a gavage needle. A prolonged retention of Pal-BBI was found in the stomach. Furthermore, a significant amount of Pal-BBI was detected as intact polypeptide in the stomach of mice fed with Pal-BBI, while only degradation products were detected with BBI. There was also a significant increase of radioactivity in the blood and liver in mice 1.5 h post-administration of Pal-BBI. These results indicate that lipidized polypeptide can have a longer retention and lower digestion in the stomach. They also suggest that the Pal-BBI may have a higher gastrointestinal absorption than the original polypeptide.

Keywords: Bowman–Birk protease inhibitor; Polypeptide lipidization; Gastric retention; Gastrointestinal absorption

1. Introduction

Bowman–Birk protease inhibitor (BBI), an inhibitor for both trypsin and chymotrypsin, is a soybean polypeptide with a molecular weight of 8 kDa (Birk, 1985). Our interest in this polypeptide comes from the findings by others that BBI is a very potent chemopreventive agent for cancer (Kennedy, 1994). It has been shown that this polypeptide at ng/ml levels can effectively prevent transformation of cultured mammalian cells by chemical carcinogens or radiation (Yavelow et al., 1985). However, like many other peptide drugs (Lee, 1991), the investigation and application of BBI for cancer prevention suffers from its short plasma half-life and poor gastrointestinal absorption. In order to develop BBI into an effective cancer chemopreventive agent, a method for preparing a reversibly conjugated BBI with palmitic acid (Pal-BBI) was developed in our laboratory (Ekrami et al., 1995; Shen et al., 1998). It was found that the pharmacokinetic parameters of Pal-BBI were very different from those of native BBI (Honeycutt et al., 1996). Briefly, a prolonged plasma half-life and a decrease of the volume of distribution (V_d) of BBI were observed

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when this polypeptide in the lipidized form of Pal-BBI was injected intravenously to mice (Honeycutt et al., 1996). Consequently, a more than 11-fold increase in the area under the curve (AUC) was obtained in Pal-BBI (Honeycutt et al., 1996). In addition, the biodistribution of BBI was also significantly altered upon lipidization (Honeycutt et al., 1996). Due to the reversibility of the modification, Pal-BBI was demonstrated as equally potent as the native BBI in preventing carcinogen-induced transformation of C3H10T1/2 cells in cultures (Ekrami et al., 1995).

Since cancer prevention requires a chronic administration of chemopreventive agents, it is most desirable that these chemopreventive agents can be administered non-invasively, preferentially by the oral route. As the first step to develop an orally administrable BBI, we compared the stability and retention of Pal-BBI with those of BBI in the gastrointetsinal (GI) tract of mice.

2. Materials and methods

2.1. Materials

BALB/c mice, female, 7–8 weeks old, were purchased from Harlen Sprague–Dawley, Inc. (Indianapolis, IN). Animal experiments were compliant with the 'Principles of Laboratory Animal Care' (NIH Publication # 85-23) and approved by the IACUC at USC.

Chemicals, including BBI and reagents used in Pal-BBI preparation were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY).

2.2. Preparation of Pal-BBI

Thiol-containing BBI was prepared by reducing endogenous disulfides in BBI with sodium borohydride ($NaBH_4$) (Hogle and Liener, 1973). Pal-BBI was prepared by modifying the reduced BBI with a lipidizing agent, *N*-palmitoyl cysteinyl 2-pyridyl disulfide (Pal-CPD) as previously reported (Ekrami et al., 1995). Since there are seven disulfide bonds in BBI (Birk, 1985), a maximum of 14 fatty acid moieties could be incorporated into each BBI molcule. However, the more disulfide bonds are reduced and modified, the less likely the original BBI structure can be regenerated (Hogle and Liener, 1973). Therefore, a mild condition was used to reduce an average of 1.5 disulfide bonds, thus adding approximately two or four fatty acid moieties to each BBI molecule. BBI (10 mg, Sigma) was dissolved in 1 ml of 0.1 M borate buffer, pH 9, and then 7.8 mg of $NaBH_4$ was added slowly at 25°C. A small aliquot of the reaction mixture was pipetted at different time intervals, diluted in 20% acetone solution, and the number of free sulfdydryl groups was determined by using Ellman's reagent. When the Ellman reaction indicated that an average of 3 sulfhydryl groups were generated per each BBI, the reaction was stopped by the addition of 5% acetone, and, subsequently, a 3-fold molar excess of Pal-CPD was added to the reduced BBI solution. The reaction was monitored by measuring the increase of 2-thiopyridine generation at 343 nm. The final product was purified by two chromatographic procedures, first using an LH-20 column eluted with DMF to remove excess Pal-CPD and other small molecules, and subsequently, a Sephadex G-50 column to separate Pal-BBI from BBI. The final yield of Pal-BBI was 7.5 mg.

2.3. Biodistribution of orally administered BBI and Pal-BBI in mice

Oral delivery of BBI and Pal-BBI was performed in mice. Eighteen female Balb/c mice, 7–8 weeks old, were fasted for 16 h and, subsequently, were dosed orally with either ^{125}I-BBI or ^{125}I-Pal-BBI using a gavage needle. The doses and radioactivities of BBI and Pal-BBI were identical, i.e. 3 mg/kg in 1% intralipid in phosphate-buffered saline, pH 7 (PBS) and 5×10^6 cpm per mouse. Animals were sacrificed at 0.5, 1.5 and 3 h after the administration, and the following organs were removed and counted in a gamma counter: blood, liver, kidneys, small intestine, large intestine, and stomach.

2.4. Identification of Pal-BBI in gastric mucosal fluids

Stomachs, isolated from mice that were fed orally with either ^{125}I-BBI or ^{125}I-Pal-BBI, were cut to open and stomach-associated mucosal fluid was collected. The mucosal fluid was diluted with PBS containing 20% fetal bovine serum (FBS) as carrier proteins and subsequently precipitated with 10% trichloroacetic acid (TCA) at 0°C. The efficiency of TCA precipitation under these conditions, i.e. 10% TCA with 20% FBS as a carrier at 0°C, was determined to be 91.6% and 99.1% for BBI and Pal-BBI, respectively. Therefore, the TCA-precipitate fractions were considered as intact polypeptides and the TCA-soluble fractions as degradation products.

The radioactive TCA-precipitate from the stomach mucosal fluid in Pal-BBI administered mice was subjected to further characterization. The TCA-precipitates from mice at 0.5 h time point were combined and re-dissolved in 1 ml of PBS by adjusting pH to 7 with 1 N NaOH. The re-dissovled solution was loaded onto a Sephadex G-50 gel filtration column and, subsequently, eluted with PBS. It was anticipated that the lipophilic Pal-BBI would be bound to serum proteins and separated from BBI and other degradation products (Honeycutt et al., 1996). Partial reduction of the disulfide bond in the conjugate was carried out by incubation with 25 mM of dithiothreitol (DTT) at 37° for 30 min.

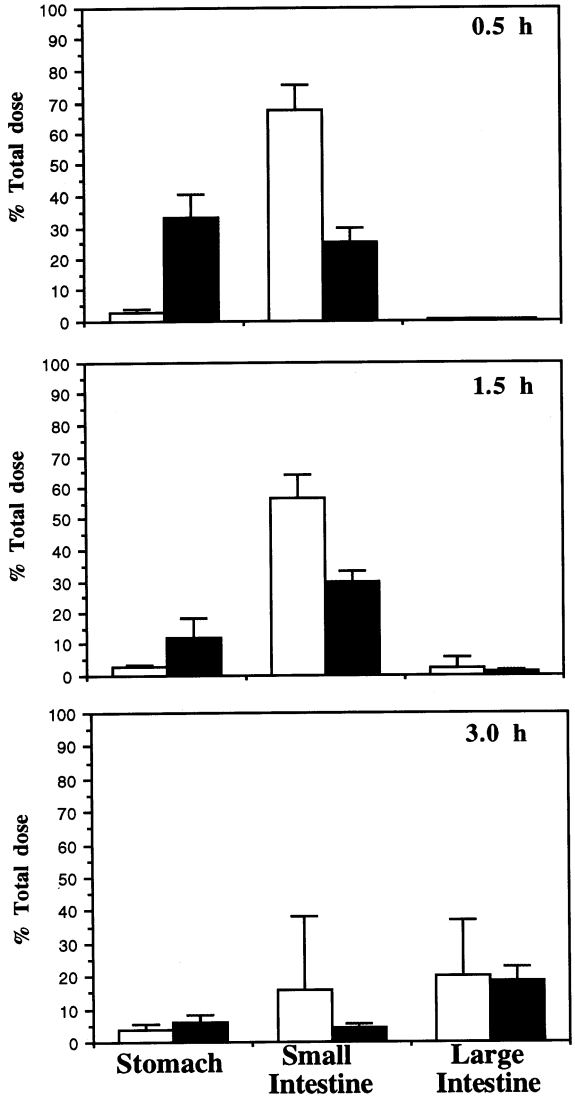

Fig. 1. Biodistribution of orally administered BBI (open column) and Pal-BBI (solid column) in the GI tract of mice. Mice were fed with either ^{125}I-BBI or ^{125}I-Pal-BBI at 3 mg/kg and 5×10^6 cpm/mouse using a gavage needle. Amount of radioactivity localized in stomach, small intestine and large intestine was determined at 0.5 h (top), 1.5 h (center) and 3 h (lower), and was expressed as % of total radioactivity administered in each mouse.

3. Results

There were significant differences between BBI and Pal-BBI in their localization in the GI tract. As shown in Fig. 1, the retention of Pal-BBI in stomach was markedly longer than that of BBI. The prolonged stomach retention appeared to increase the absorption of Pal-BBI in the upper GI tract because there was no corresponding increase of the GI transit time of Pal-BBI in the small and large intestines (Fig. 1).

Since there was a striking difference in stomach retention between the orally administered BBI and Pal-BBI, the composition of the radioactivity in the stomach was further investigated. As shown in Table 1, the difference between the intact polypeptide in the stomach of Pal-BBI-fed mice and of BBI-fed mice was even more noticeable than the total radioactivity. When expressed as

Table 1
Percentage of orally administered BBI or Pal-BBI as intact polypeptide in the stomach of Balb/c mice

Time (h)	% Total dose as intact polypeptide in stomach ($\pm$SD, $n=3$)	
	BBI	Pal-BBI
0.5	0.8 (0.3)	27.5 (6.2)
1.5	0.4 (0.1)	6.7 (5.4)
3.0	0.5 (0.5)	1.5 (0.7)

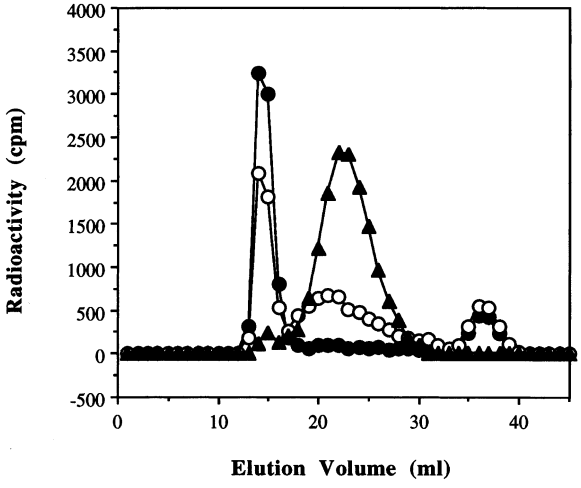

Fig. 2. Identification of Pal-BBI in the gastric mucus fluid of mice. Mice were sacrificed 30 min after oral administration of ^{125}I-Pal-BBI and the mucosal fluids in the stomach were collected. Proteins in the fluid were precipitated with 10% TCA in the presence of 20% FBS as carriers and subsequently redissolved in PBS by adjusting pH to 7 with 1 N NaOH. The solution was added onto a Sephadex G-50 gel filtration column (40-ml). The column was eluted with PBS and 1-ml fractions were collected. The majority of the radioactivity was detected at the void volume of the column indicating a binding of Pal-BBI to serum proteins (●). After partially reduction with DTT, a new radioactive peak appeared (○) that was superimposed with standard BBI peak (▲).

the amount of intact polypeptide in the stomach, Pal-BBI was 34-, 17-, and 3-fold higher than BBI at 0.5, 1.5, and 3 h, respectively. In BBI-fed mice, less than 1% of total radioactivity was detected as the intact BBI in the stomach at the three time points.

As shown in Fig. 2, the majority of radioactivity in the TCA-precipitate of the gastric fluids from Pal-BBI-treated mice was associated with the serum proteins in the FBS that emerged from the column at the void volume. Furthermore, a new radioactive peak appeared in the chromatogram of the partially reduced gastric mucosal fluid (Fig. 2). This new peak of radioactivity superimposed with that of free BBI, indicating that the TCA-precipitate contained the intact conjugate of disulfide-linked palmitic acid and BBI, rather than peptide fragments from the degradation of the conjugate.

In addition to the difference in stomach retention of BBI and Pal-BBI, significantly higher concentrations of BBI or its metabolites also appeared in the blood, liver and kidneys in mice at 1.5 h after oral administration of Pal-BBI (Table 2). However, due to the low radioactivity detected in these organs, no attempt was made to identify the nature of this tissue-associated polypeptide.

4. Discussion

Even though BBI is an inhibitor of trypsin- and chymotrypsin-like proteases, it does not inhibit acid proteases such as cathepsins in lysosomes (Shen et al., 1990). Therefore, it is not surprising to find that the small amount of BBI remained in stomach can be degraded by pepsin. Our results from the studies of the stomach-associated Pal-BBI (Fig. 1 and Table 1) suggest that lipidization can increase the retention and the stability of a polypeptide in the GI tract. The prolonged retention of Pal-BBI in the stomach is likely due to the absorption of the lipidized peptide in the hydrophobic mucus lining in the stomach (Lichtenberger, 1995). Another possible reason for the prolonged gastric retention is that Pal-BBI may precipitate in the acidic gastric fluid and, consequently, prolong its deposition in the stomach.

Our findings on the increase of gastric retention time and the decrease of peptic degradation of Pal-BBI are potentially of great significance to the development of peptide drugs for treating gastric disorders (Piazuelo et al., 1998; Konagaya et al., 1998; Jones et al., 1999). For example, epidermal growth factor (EGF), which promotes the healing

Table 2
Biodistribution of orally administered BBI or Pal-BBI in Balb/c mice

Organ	BBI or Pal-BBI	% Total radioactivity ($\pm$ SD, $n = 3$)		
		0.5 h	1.5 h[a]	3.0 h
Blood	BBI	4.6 (0.6)	4.3 (1.2)	6.6 (1.9)
	Pal-BBI	4.1 (1.1)	6.9 (0.9)	6.2 (0.9)
Liver	BBI	1.1 (0.1)	0.7 (0.1)	1.1 (0.3)
	Pal-BBI	1.0 (0.2)	1.3 (0.1)	1.1 (0.3)
Kidneys	BBI	0.5 (0.1)	0.4(0.1)	0.7 (0.2)
	Pal-BBI	0.4 (0.1)	0.7 (0.0)	0.7 (0.1)

[a] Significant difference between the biodistributions of BBI and Pal-BBI at 1.5 h (P-values by t-test): blood, 0.019; liver, 0.003; and kidneys, 0.002.

of ulceration in the stomach (Tarnawski and Jones, 1998), has been shown to be degraded rapidly into small inactive peptides in the gastric juice (Playford et al., 1995). Consequently, formulations for EGF have been developed in attempt to protect this polypeptide from peptic digestion and increase the therapeutic effectiveness (Itoh et al., 1992; Bernkop-Schnürch et al., 1999). The reversible lipidization of BBI described in this paper provides an alternative approach to prolong the localization of active peptide drugs in the stomach. Furthermore, our preliminary results also suggest that there was an increase of the polypeptide or its metabolites in the blood, liver and kidneys following the oral administration of Pal-BBI in mice. However, whether the increase in blood, liver, and kidney localization reflects a higher oral bioavailability of Pal-BBI over native BBI remains to be determined.

Acknowledgements

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