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Synthesis and Biodistribution of Bowman-Birk Soybean Protease Inhibitor Conjugate with Amphiphilic Polyester

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ABSTRACT

The modification of Bowman-Birk soybean protease inhibitor (BBI) with the monoaldehyde derivative of block copolymer of ethylene oxide and propylene oxide (PE), M_r 2000 is described. The conjugate contains five covalently bound polymer chains per protein molecule, and retains the ability to inhibit trypsin and chymotrypsin-like proteinases. The distribution of native BBI and the BBI-PE conjugate was examined in mice. After iv injection of [^{125}I]BBI and [^{125}I]BBI-PE, both inhibitors distributed very rapidly to the liver, kidney, and lungs, and more slowly to the brain. At the same time-points (up to 24 h), radioactivity in the blood and organs of mice injected with modified inhibitor was higher than that of the native inhibitor. The blood concentration time profile following iv administration of two BBI preparations at a dose 3 mg/kg was reasonable well described by a two-compartment open model with first-order elimination kinetics. The total clearance of BBI-PE decreased by a factor of 8, body mean residence time increased by a factor of 5 in comparison with BBI. A physiological pharmacokinetic model was developed to describe the tissue-to-blood distribution of two inhibitors. One-compartment physiological organ model (flow limited) was used to describe of time-course profiles of BBI concentration in organs. A two-compartment physiological organ model (membrane limited) was used to predict tissue-to-blood distribution of conjugated BBI in some organs of mice

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(liver, lungs). The predicted concentration curves of BBI and BBI-PE in blood and organs in mice (with the exception of kidney) showed good agreement with the observed values.

Index Entries: Soybean Bowman-Birk protease inhibitor; conjugate; mice; pharmacokinetics; physiological model.

INTRODUCTION

The soybean Bowman-Birk protease inhibitor (BBI) is currently receiving particular attention because of its anticarcinogenic activity (*1,2*). The in vivo transport of BBI to target tissues is limited by epithelial and endothelial barriers, as well as by a rapid clearance from the kidney (*3*). One of the ways to alter the transport pattern and biodistribution of drugs is to couple them with biocompatible polymers (*4*). Although the anticarcinogenic activity of BBI has been investigated for about 10 years, there are a few works on the pharmacokinetics of BBI and its conjugates (*3,5,6*). Unfortunately, the results reported there do not describe the biodistribution of BBI-containing drugs quantitatively.

The first goal of the present study was, to synthesize a conjugate of BBI and the block copolymer of ethylene oxide and propylene oxide (PE), an amphiphilic surfactant capable of penetrating through biological membranes (*7*). The second goal was to perform a comparative study of the pharmacokinetics of native BBI and the BBI-PE conjugate. We developed a simple physiologically based model that allows the quantification of the distribution of two inhibitors in target tissues. Based on anatomical and physiological characteristics of animals and on drug physicochemical parameters, these models greatly facilitate pharmacokinetic data interpretation. Their potential was illustrated in detail for anticancer drugs (*8–9*).

MATERIALS AND METHODS

Materials

BBI was isolated from soybeans, strain VNIIS-2, as described previously (*10*). The water-soluble block copolymer of ethylene oxide and propylene oxide of the formula $H_3C(CH_2)_3O(CH[CH_3]CH_2O)_{14}(CH_2CH_2O)_{20}CH_2OH$ was obtained from the MNPO "NIOPIK" Experimental Plant. The monoaldehyde derivative of the block copolymer was obtained by its oxidation with MnO_2 (*11*).

Synthesis of BBI-PE Conjugate

The monoaldehyde derivatives of PE (120 mg) and BBI (40 mg) (2 Eq of the copolymer/amino group of the inhibitor) were dissolved in minimal volumes of 0.05*M* borate buffer, pH 8.0, mixed, and stirred for 2 h at

room temperature. The Schiff bases formed were reduced by adding a few portions of concentrated sodium cyanoborohydride (3 mg) in borate buffer while stirring at 4°C for 1 h. The mixture was applied on a trypsin-agarose column (total volume 200 mL) pre-equilibrated with 0.05*M* Tris-HCl, pH 6.0. Inactive fraction was eluted by the same buffer. To eliminate Tris buffer from the column, it was washed with acetic acid, pH 4.0. The BBI-polyester conjugate was eluted with 0.1*M* acetic acid, containing 20% isopropanol, adjusted to pH 2.0 with HCl. The absence of noncovalently bound polymer in the conjugate was shown by thin-layer chromatography. The protein content in the conjugates was quantified by the Bradford method (*12*) using native BBI as a standard and by the amino acid analysis of a 24-h hydrolysate.

Iodination of BBI and BBI-PE Conjugate

BBI and BBI-PE were iodinated with $Na^{125}I$ using chloramine-T (*13*) and purified by gel filtration on a Sephadex G-25 column (0.9 × 30 cm) pre-equilibrated with 0.01*M* phosphate-buffered saline (pH 7.4). The iodination did not affect the biological activity of BBI.

Pharmacokinetic Studies

Animals

Male albino mice, 20 g mean body wt, were used. The mice were allowed free access to water and laboratory mice chow, and were housed in a room with 12 h light–12 h dark cycle for at least 2 wk before the day of experiment. Animals were injected into the tail vein with 10 μL working solution/10 g body wt (protein dose 3.0 mg/kg). The working solutions contained 3 mg protein/mL, and their specific radioactivities were 19,100 and 31,800 cpm/μL for BBI and BBI-PE, respectively. At certain time intervals following drug administration, the anesthetized animals were sacrificed. Blood samples and organs (brain, lungs, liver, and kidney) were collected, and radioactivity was measured on a "Delta-300" counter (TRAKOR, USA). Data were expressed in μg protein/mL blood or g tissue, assuming that only unchanged and unbound forms of BBI and BBI-PE were present in blood and organs. Urine was collected via the urinary catheter for 24 h after iv injection of BBI-PE.

Data Analysis

Dependence of the inhibitor concentration in blood ($C_p[t]$) vs time was analyzed using Eq (1) for the two-compartment open-model system after iv administration (*14*):

$$C_p(t) = A_1 \cdot \exp\{-\alpha t\} + A_2 \cdot \exp\{-\beta t\} \quad (1)$$

where A_1 and A_2 are the macroconstants, and α and β are the rate distribution constants.

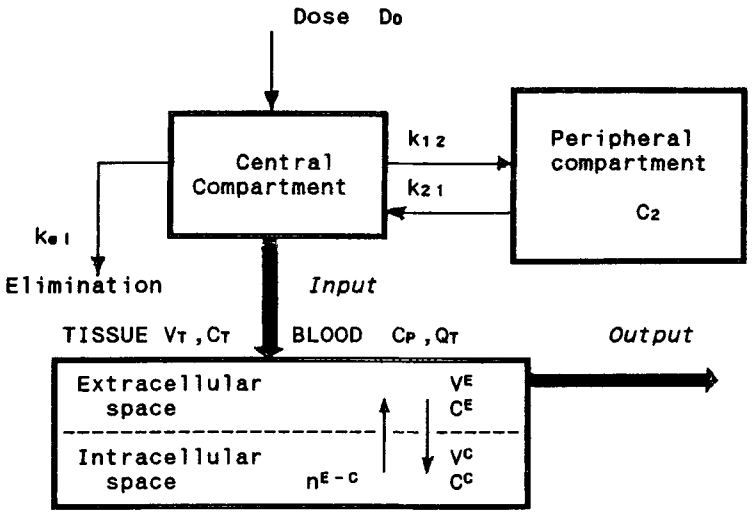

Fig. 1. Schematic diagram of the hybrid three-compartmental physiologically based model developed for BBI and BBI-PE (D_0, dose; Q_T, blood flow rate; C_P, concentration in blood; C_2, concentration in peripheral compartment; C^E, concentration in extracellular space; C^C, concentration in intracellular space; C_T, total concentration in tissue; V^E and V^C volume of extracellular and intracellular spaces, respectively; V^T, total tissue volume; k_{12} and k_{21}, rate constants for intercompartmental clearance; k_{el}, rate constant of elimination; $n^{E\text{-}C}$, the flux across the cell membrane).

The hybrid three-compartment model (*15,16*) was used to describe the behavior of the inhibitors in various tissues. The compartments of each organ were independently connected with the blood compartment by apparent diffusion clearance. Figure 1 illustrates the major components of the model and shows the mass-flow pathways of drugs in the system. A flow-limited (one-compartment) physiologically based pharmacokinetic model was used to describe the distribution of BBI in the organs of mice and BBI-PE in brain and kidney. A two-compartment organ model subdivided anatomically into two fluid compartments (membrane limited) was used to describe the distribution of BBI-PE in liver and lungs. This model is based on the nonlinear transport mechanism inside the cell with a nonspecific carrier (*17,18*). Blood concentration of experimental data vs time was fitted to theoretical Eq (1) with a nonlinear regression analysis program (*19*). A set of pharmacokinetic parameters that describe a common distribution of drugs in the bodies of mice was also determined (*14*). Using the known physiological parameters of mice (*18,20*), the observed drug concentration in each organ was fitted to a three-compartment model as shown in Fig. 1. To solve the equations of the model, the program was written in Turbo Pascal for an IBM PC computer.

RESULTS AND DISCUSSION

Synthesis and Characterization of BBI–Polyester Conjugate

To create a potent BBI preparation for application in vivo, we took into account that the anticarcinogenic activity of BBI is provided by its antichymotrypsin site Leu43-Ser44 (*21,22*). We used reductive alkylation to produce the BBI–copolymer conjugate.

The homogeneity of the conjugate obtained was demonstrated by reverse-phase HPLC. There were difficulties in determining the composition of the conjugate. The presence of copolymer chains complicates both the titration of amino groups in protein by the standard method of Fields (*23*) and the assay of protein by Lowry (*24*). Therefore, the copolymer content in the conjugate was calculated by subtracting the weight of protein in the conjugate determined as described in the Materials and Methods from the weight of the preparation. The conjugate contains five chains of the copolymer per protein molecule. The modified BBI inhibited effectively α-chymotrypsin ($K_i = 4.0 \times 10^{-8} M$) and human leukocyte elastase ($K_i = 1.2 \times 10^{-7} M$) shown elsewhere (*25*). As purified BBI, BBI-PE suppressed radiation transformation in vitro (*25*).

Biodistribution of BBI and BBI-PE in Mice

The blood and tissue profiles of BBI and BBI-PE in mice after iv administration obtained with the previously defined model are presented in Figs. 2 and 3. They show that the theoretical curves (solid lines) are in good agreement with the observed data.

The experimentally estimated pharmacokinetic parameters are listed in Tables 1 and 2. The following parameters were calculated for blood and each organ:

AUC_P is the area of C_P vs time;
V_1 is the central volume of distribution;
V_{SS} is the steady-state volume of distribution;
V_T is the tissue volume of distribution;
CL_P is the plasma clearance;
CL_D is the distribution clearance;
CL_E is the elimination clearance;
MRT_B is the body mean residence time;
MRT_S is the central mean residence time;
MRT_P is the peripheral mean residence time;
$T_{1/2,\alpha}$ is the half-life time on α-phase;
$T_{1/2,el}$ is the elimination half-life time;
$C_{T,max}$ and t_{max} are the maximal concentration in tissue and the corresponding time, respectively;

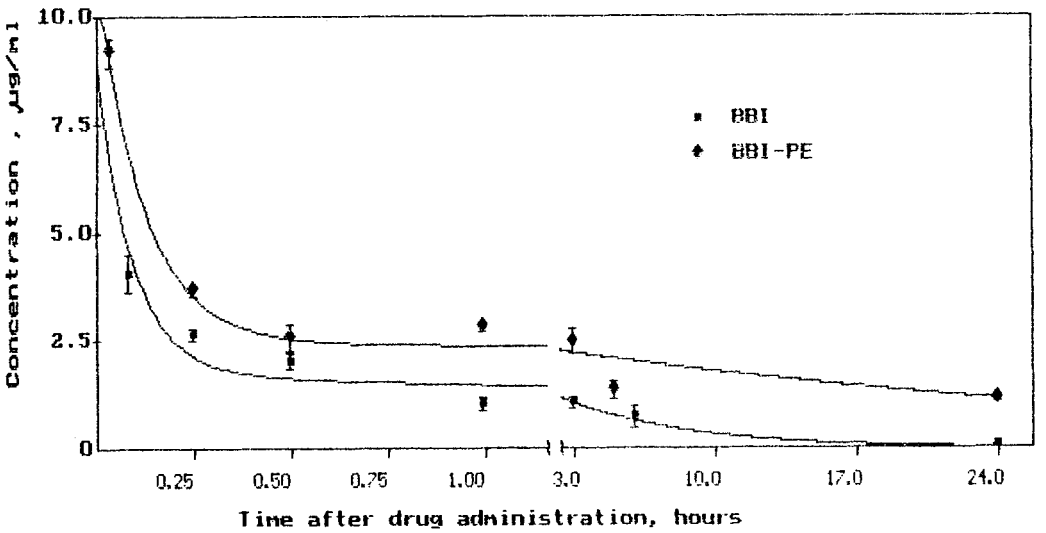

Fig. 2. Kinetics of BBI and BBI-PE elimination from the blood of mice after a single iv administration of 3 mg/kg. The solid lines are the theoretical distributions. Each point and vertical bar represent the mean value and standard deviation of four to seven mice, respectively.

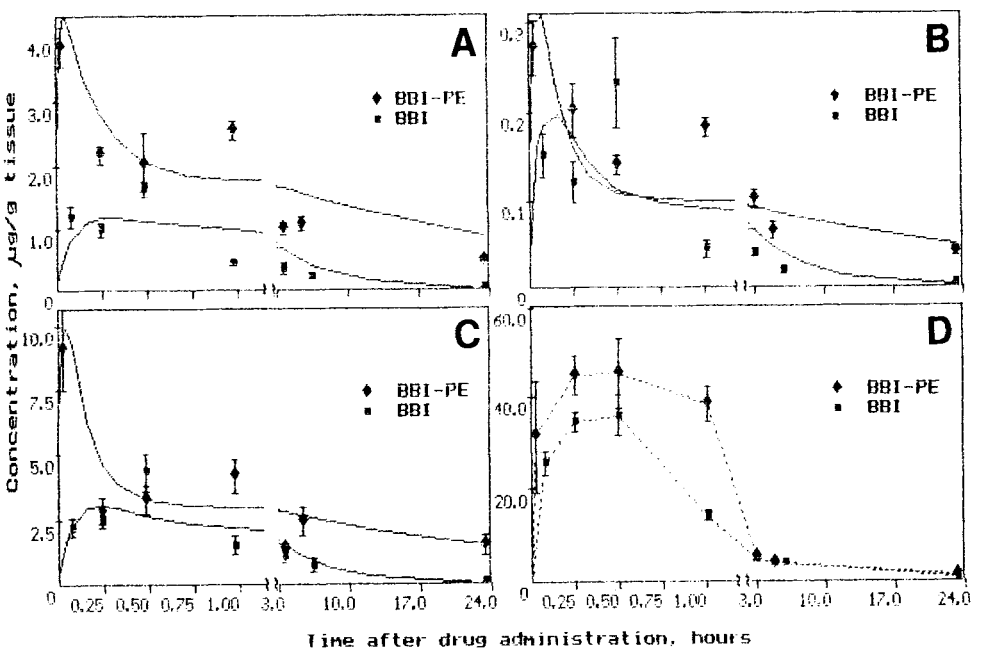

Fig. 3. Distributions of BBI and BBI-PE in (**A**) liver, (**B**) brain, (**C**) lung, and (**D**) kidney after a single iv administration of 3 mg/kg. The solid lines are the theoretical distributions.

Table 1
Pharmacokinetic Parameters of BBI and BBI-PE for Mice After iv Bolus Administration [^{125}I]-Labeled Drugs at a Dose of 3 mg/kg

Parameters	Calculated value	
	BBI	BBI-PE
A_1, μg/mL	7.0 (72%)[a]	8.7 (12%)
A_2, μg/mL	1.8 (35%)	2.5 (15%)
α, min^{-1}	0.2 (88%)	0.14 (32%)
β, min^{-1}	0.003 (93%)[a]	0.0005 (68%)
AUC_P, μg·min·mL^{-1}	703.0	4794.0
V_1, mL	6.7	5.4
V_{SS}, mL	29.7	23.7
V_P, mL	23.1	18.4
CL_P, mL/min	0.085	0.013
CL_D, mL/min	0.869	0.562
CL_E, mL/min	0.954	0.575
MRT_B, min	350.0	1897.0
MRT_S, min	78.0	430.0
MRT_P, min	272.0	1467.0
$T_{1/2,\alpha}$, min	4.0	5.0
$T_{1/2,el}$, min	54.0	298.0

[a]Relative error is given in parentheses.

Table 2
Pharmacokinetic Parameters of Distribution of BBI and BBI-PE in Tissues After iv Bolus Administration at a Dose of 3 mg/kg in 20-g Mice

Tissues	Drugs	Parameters				
		$C_{T,max}$ μg/g	t_{max}, min	MRT_T, min	F_T	λ_s, min^{-1}
Brain	BBI	0.20	10.0	407	0.04	0.002
	BBI-PE	0.32	5.0	1549	0.04	0.0006
Liver	BBI	1.2	20.0	374	0.38	0.002
	BBI-PE	3.3	5.0	1344	0.45	0.00064
Kidney[a]	BBI	35.6	30.0	519	8.3	0.0013
	BBI-PE	45.3	30.0	495	2.1	0.0011
Lung	BBI	3.0	15.0	292	1.02	0.0026
	BBI-PE	9.9	2.0	2174	1.3	0.00044

[a]The pharmacokinetic parameters of BBI and BBI-PE for kidney were estimated by the model-independent method.

MRT_T is the mean residence time in tissue;
λ_s is the elimination rate constant at the terminal phase; and
$F_T = AUC_{tissue}/AUC_{blood}$ is the tissue bioavailability.

Several features of the results presented are worth comment. Comparing distributions of two preparations and the value of the parameters given in Table 1 indicated that the conjugation changed the inhibitor distribution in mice. Rapid fall of BBI and BBI-PE concentration in blood was observed for the first 15 min. The estimated half-lives for the distribution phase for the two preparations are similar. However, the blood level of BBI-PE is always higher compared to that of the free inhibitor. More hydrophilic BBI-PE remained in circulation much longer than native BBI. As a result, after 24 h only a "trace" of BBI was detectable in the blood, whereas the concentration of BBI-PE was only two times lower than that after 30 min (Fig. 2). Because of a much slower elimination of BBI-PE from the system, its body mean residence time (MRT_B) was 5.5 times higher than that for BBI. Note that the MRT_P (peripheral mean residence time) was the main part of the body mean residence time. Conjugation with the block copolymer resulted in a 7.3-fold decrease in the total clearance (CL_P). The distribution clearance (CL_D) was shown to be the main part of the elimination clearance (CL_E) for two preparations. This fact confirmed the higher tissue bioavailability of BBI-PE. The ratio of areas under pharmacokinetic curves, "concentration in blood vs time" (AUC_P), was about six, characterizing the comparative systemic bioavailability of BBI-PE vs BBI.

The difference in BBI and BBI-PE behavior in blood is a consequence of peculiarity of distribution of the two inhibitors in tissues. The significantly different distributions of the preparations tested were observed in organs. A small amount of inhibitors was detected in the brain. Thus, it would be expected that the transfer of BBI across the blood–brain barrier is very slow. However, the BBI-PE retention in the brain was longer than that of free BBI. The concentration–time profile of two drugs in the kidney were similar. More significant differences were observed for the distribution in liver and lung.

The maximum tissue concentration ($C_{T,max}$) of the native inhibitor was reached in 30 min after injection. At the same time, $C_{T,max}$ was observed for BBI-PE in all tissues (except kidney) in the first measurement (in < 5 min). In contrast to the free inhibitor, BBI-PE had a constant level of concentration in tissues (lung, liver, brain) in the range of 0.25–3 h after drug administration (Fig. 3). The radioactivity level in mice injected with BBI-PE was 3–4 and 2–5 times higher in lungs and liver, respectively, in the terminal phase of elimination compared to that for the free inhibitor. The mean residence time in the tissue compartment (MRT_T in Table 2) for BBI-PE was much longer than these estimates for the native inhibitor.

The differences in kinetics of the two inhibitors in tissues may be attributed to various transport mechanisms across the cell membrane. In the case of free BBI, the usual flow-limited assumption for the mass transfer

was made for all organs. We also assumed the same mechanism for the transfer of BBI-PE in brain and kidney of mice. The mechanism of mass transfer, including a nonspecific carrier, was proposed for other organs (lung and liver). BBI-PE remained presumably fixed at the intracellular subcompartment. Therefore, its retention in the target tissues was longer than that of free BBI.

Urinary recoveries within 24 h for BBI-PE were 45% of the dose. This result indicated that the contribution of urinary excretion to the total clearance of drug was rather great.

In conclusion, we compared the distribution of two Bowman-Birk protease inhibitors in mice. It was shown experimentally and verified by computation that the distribution of the two drugs in the bloodstream and some target tissues differed significantly. The difference was more pronounced for the terminal phase of drug elimination. The physiological models for the native inhibitor and BBI-polyester conjugate in mice were presented in this study. The models provided an operational basis for estimating the important pharmacokinetic parameters. A possible role of the membrane transport limitation for the BBI-PE conjugate should be examined in more detail for improving the model.

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