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Soybean Bowman–Birk Inhibitor Conjugates with Clinical Dextran. Synthesis and Antiproteolytic Activity

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Abstract—Conjugates of the classical soybean Bowman–Birk inhibitor (BBI) with clinical dextran were synthesized. Clinical dextran was preliminarily oxidized with periodate to dialdehydedextran (DAD). The effect of the degree of oxidation of DAD on coupling of the inhibitor was evaluated. The binding of the protein was shown to increase with increasing degree of DAD oxidation (5, 10, 20%). Total coupling of the inhibitor occurred when the degree of oxidation of the dextran was 20%. The BBI–DAD (20%) conjugate contained 13% protein with BBI/DAD molar ratio 1 : 1. The conjugates retained the ability to inhibit trypsin (K_i = 0.2-0.3 nM) and α-chymotrypsin (K_i = 15-30 nM). Thus, the coupling of BBI with the polymeric carrier caused practically no decrease in the antiproteolytic activity of the inhibitor.

Key words: Bowman–Birk inhibitor, dextran, conjugate

Dextran is a common name for polysaccharides obtained by microbiological synthesis; their molecular chain consists of anhydro-D-glucopyranose units linked mainly by α-1,6-glycoside bonds [1]. Dextrans have been used for years in medical practice in Russia [1], the United States, and Japan as an effective plasma expander after blood loss and shock [2]. The use of biocompatible, biologically inert dextrans whose molecules can be relatively easily modified and decomposed by dextranases to easily clearable low-molecular-weight products is very promising for preparation of high-molecular-weight water-soluble forms of biologically active compounds. This is an advantage of dextran over carbon-chain polymers (derivatives of polyvinylpyrrolidone and polyvinyl alcohol). Therefore, with the dextrans the danger of accumulation of the polymer in the body is excluded even though the rate of enzymatic decomposition of the modified dextrans is decreased [3].

Modification of Bowman–Birk soybean proteinase inhibitor (BBI) by the clinical dextran polyglukin for preparation of a derivative with prolonged action is described in this work. BBI efficiently inhibits hydrolysis of the most important components of the extracellular matrix catalyzed by leucocyte enzymes [4, 5]. The ability of BBI to actively suppress the cell transformation *in vitro* and its antitumor activity in model systems have also been directly demonstrated in animals [6].

MATERIALS AND METHODS

The following reagents were used in this study: bovine trypsin from Spofa (Czech Republic); α-chymotrypsin and Tris-HCl from Olaine (Latvia); ethyl ester of N-benzoyl-L-tyrosine (Bz-Tyr-EE), ethyl ester of N-benzoyl-L-arginine (Bz-Arg-EE), *p*-nitroanilide of N-benzoyl-L-arginine (BApNA), and dimethylsulfoxide (DMSO) from Sigma (USA); Ultragel 54 from LKB (Sweden); Sephadex G-25, G-50 from Pharmacia (Sweden); Biogel P-20, P-60, and P-300 from BioRad (USA); TSK-Gel Toyopearl HW-40, 50, 55, 60, 75 from ToyoSoda MFG (Japan); AB-17 resin from Azot (Cherkassk, Russia); polyglukin with molecular mass 60 kD was an experimental sample from the Institute of Technology of Medicinal Preparations (Russia). Other reagents were of extra pure, chemically pure and analytically pure grade.

The active site content in α-chymotrypsin determined according to Shonbaum et al. [7] was 65%, that in trypsin determined according to Chase and Show [8] was 63%.

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Isolation of BBI. Classical BBI was isolated from soybean strain VNIIS-2 by the method developed by us earlier [9, 10] that included extraction of proteins with ethyl alcohol and subsequent precipitation of the BBI-containing fraction with acetone. The inhibitor was purified using gel-filtration and ion-exchange chromatography.

Preparation of dialdehydedextran (DAD) from polyglukin. DAD was obtained from polyglukin by periodate oxidation in the dark for 20-30 min at room temperature. Changes in periodate concentration were monitored spectrophotometrically at 235 nm. At this wavelength, the ratio of molar absorptivities of IO_4^- and IO_3^- ions is 7.5 [11].

DAD was then purified using ion-exchange chromatography on AB-17 resin equilibrated with 0.05 M sodium acetate, pH 5.5. The efficiency of separation was checked spectrophotometrically at 235 nm.

The degree of oxidation of DAD (the number of aldehyde groups per 100 glucoside units in the molecule of oxidized dextran) was determined by iodometric titration [12].

Preparation of the BBI–DAD conjugate. The BBI–DAD conjugate was synthesized via reductive alkylation. The pH of the freshly prepared DAD solution was brought to 8.6 with borax. The lyophilized BBI was added to the resulting solution (DAD/BBI = 8 : 1 w/w), and the mixture was incubated with stirring at room temperature. After 2 h, dry sodium borohydride was slowly added with stirring to the conjugate solution (molar ratio $NaBH_4$/aldehyde groups of the initial DAD = 1 : 1), and the mixture was incubated for 1 h. To remove excess $NaBH_4$, the reaction mixture was acidified to pH 3.0 with concentrated hydrochloric acid and kept for 3 h.

Purification of the BBI–DAD conjugate. The BBI–DAD conjugate was purified from unbound protein by gel filtration on a Sephadex G-50 column equilibrated with 0.05 M NH_4HCO_3, pH 8.0. The elution of protein was monitored spectrophotometrically by the optical density at 280 nm and that of polysaccharide by the optical density at 485 nm according to Dubois [13]. The first peak was collected and lyophilized.

Determination of the polysaccharide concentration in the modified BBI preparations. The polysaccharide concentration in the BBI conjugates was determined according to Dubois [13]. Aqueous phenol solution (5%, 0.25 ml) and concentrated sulfuric acid (1.25 ml) were added to 0.25 ml of aqueous solution of the modified BBI (concentration 0.1-0.2 mg/ml). After cooling the resulting solution, its optical density was measured at 485 nm. The polysaccharide concentration was calculated using a standard curve plotted on the same day for solutions of clinical dextran of known concentrations.

Determination of the protein concentration in the native and modified BBI preparations. The protein concentration in the modified BBI preparations was determined according to Lowry [14] using classical BBI samples with the known protein concentration as the standard.

Determination of the active BBI concentration in the inhibitor preparations and its conjugates with DAD. The inhibitory activity of BBI in the isolated preparation and in the BBI–DAD conjugates was judged from suppression of the amidase activity of trypsin and the esterase activity of α-chymotrypsin using as the substrates BApNA and Bz-Tyr-EE, respectively [15, 16]. Tris-HCl (0.05 M) containing 0.02 M $CaCl_2$ (pH 8.0) was used as a buffer. The enzyme–inhibitor (conjugate) mixture was incubated in the working buffer for 10 min, and then the reaction was initiated by addition of substrate solution. The concentration of the active protein was determined from the plot of the remaining enzymatic activity versus concentration of the inhibitor preparation. Based on the calculated concentration of the active protein and the protein concentration according to Lowry, the percent concentration of the active inhibitor in the conjugate preparations was determined.

Determination of the inhibition constants of proteinases by the BBI–DAD conjugates. The inhibition constants (K_i) of α-chymotrypsin were determined according to Bieth [17]. To determine the inhibition constants of trypsin, the establishment of equilibrium was investigated in the simultaneous presence of the enzyme, inhibitor, and substrate [18-20]. To calculate the constants by this method, the following conditions must be met: 1) at the initial moment, the enzyme–inhibitor complex is absent; 2) $[S_o] >> K_m$; 3) $[I]_o/K_i > S/K_m$. Calculations were performed using the equation:

$$K_i = [I]_o/(v_o/v_s - 1)/(1 + [S]_o/K_m), \quad (1)$$

where v_o is the initial reaction rate, which is determined graphically as the slope of the asymptote to the beginning of the full kinetic curve, and v_s is the reaction rate after the equilibrium in the enzyme–inhibitor–substrate system is established. In this case, the kinetic curve approaches the asymptote with slope equal to v_s.

RESULTS AND DISCUSSION

Conjugation of BBI with clinical dextran. For binding of physiologically active substances with dextran, preliminary conversion of the polysaccharide into a chemically active form [21], for example, into dialdehydedextran (DAD), is required. Dextran is most often oxidized with periodates (Fig. 1).

This reaction can proceed with formation of DAD via partial oxidation of one glycol group in the polysaccharide with the consumption of 1 mole of $NaIO_4$. When 2 moles of oxidant are used, as in our case, complete oxidation of a triol group occurs. As shown earlier, almost complete consumption of the oxidant occurs in 20-

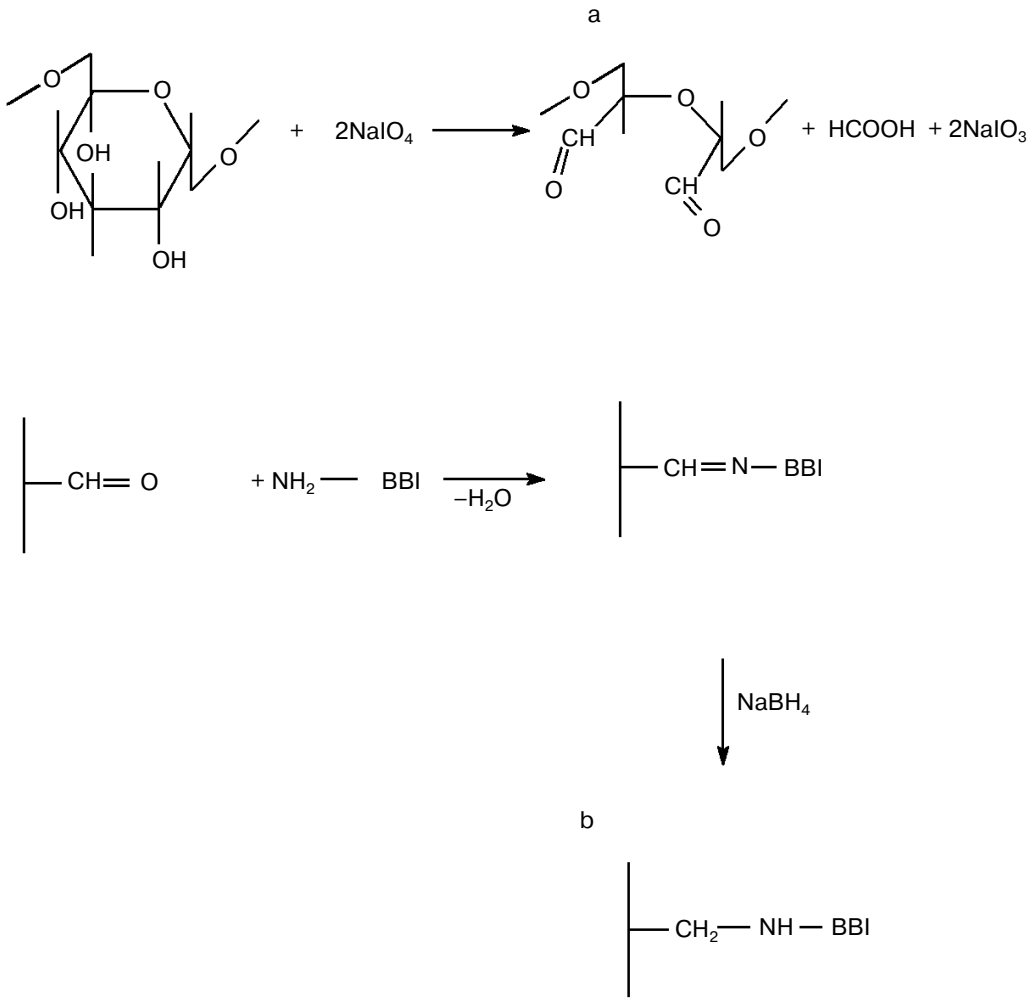

Fig. 1. Scheme for conjugation of BBI with clinical dextran: a) periodate oxidation of dextran to DAD; b) conjugation of DAD with the primary amino groups of BBI.

30 min [22]. To prevent further modification of the carrier as well as the bound protein, it is necessary to remove the excess oxidants (periodate and the newly formed iodate) from the reaction mixture; the presence of oxidants also hinders determination of the degree of oxidation of dextran [23]. To remove the oxidative ions from the reaction mixture, sorption of IO_4^- and IO_3^- ions on AB-17 anionite equilibrated with 0.05 M acetate buffer (pH 5.5) was used. Because the affinity of the used anion-exchanger to IO_3^- and IO_4^- is significantly higher than that to acetate ions [24], the oxidized polysaccharide was almost completely isolated from the oxidants. It is also important that acetate ions do not influence determination of the degree of oxidation of the polysaccharide and do not inactivate physiologically active substances including those of protein nature [11].

The newly formed aldehyde groups are able to form Schiff bases with the primary amino groups of physiologically active substances, in particular, proteins.

On treatment of the product of protein modification with sodium borohydride, azomethine moieties are transformed to alkylamino groups, which are more stable in acidic medium, and the excess aldehyde groups of the polymer are reduced to hydroxyl groups (Fig. 1b). We synthesized BBI–DAD conjugates with the degree of oxidation of DAD of 5, 10, and 20%, the BBI/DAD ratio being 1 : 8 (w/w). The resulting BBI–DAD conjugates were isolated from the unreacted inhibitor and desalted by gel filtration. Biogels P-60, P-20, and P-300, Sephadex G-50, Ultragel-54, Toyopearl HW 40, 50, 55, 60, and 75 were tried as the carriers. The best separation of the native BBI from BBI–DAD conjugates was attained on a Sephadex G-50 column equilibrated with 0.05 M NH_4HCO_3, pH 8.0 (Fig. 2). However, the use of a polysaccharide matrix decreases the yield of synthesized conjugate during chromatography because of the interaction of DAD with the sorbent. Analysis of chromatograms allowed evaluation of the effect of the degree of oxidation

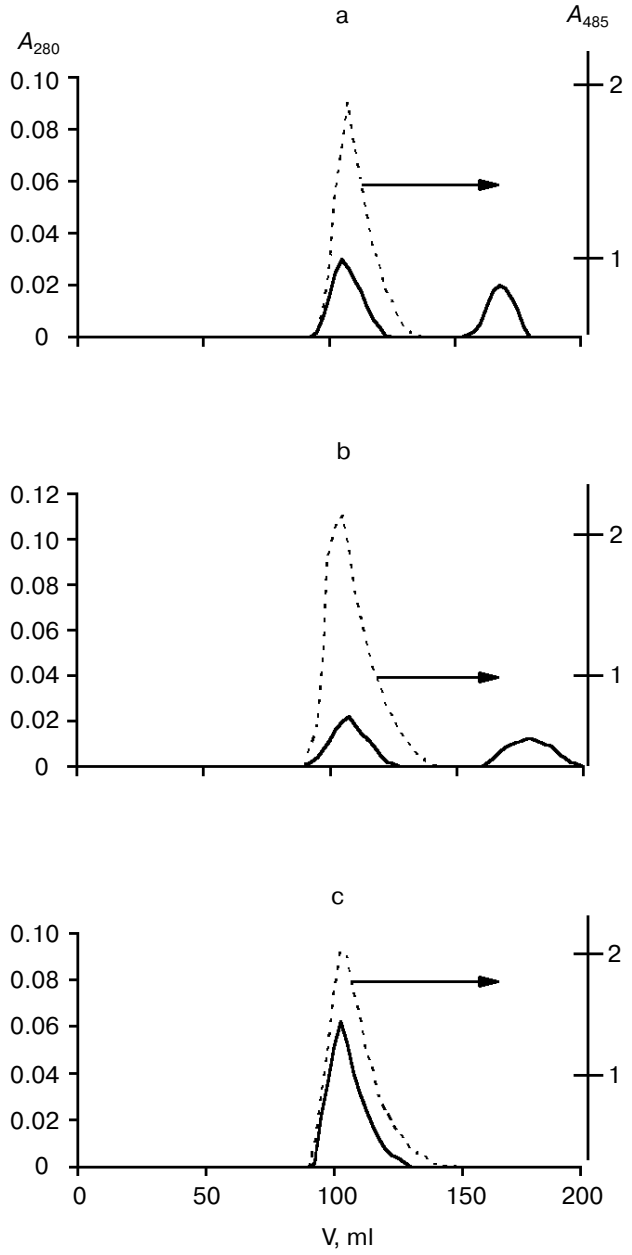

Fig. 2. Effect of the degree of oxidation of dextran on the binding of BBI (chromatography on a Sephadex G-50 column). Eluent, 0.05 M NH_4HCO_3, pH 8.0; column volume, 270 ml. The degree of oxidation of the dextran was: a) 5%; b) 10%; c) 20%.

of DAD on the inhibitor binding. We concluded that almost complete protein binding occurs at the 20% degree of oxidation of the dextran.

Determination of composition and antiproteinase activity of the BBI–DAD conjugates. The synthesized BBI–DAD conjugates were characterized by their composition, i.e., their protein and polysaccharide contents, and by the retention of the inhibiting properties of the BBI. As shown in the table, on increasing the degree of oxidation of dextran, the BBI content in the conjugate increases.

At the maximal degree of oxidation of DAD (20%), the BBI–DAD conjugate contained 13% protein. The BBI/DAD molar ratio in this conjugate is 1 : 1.

Retention of the activity of BBI within the conjugate was studied by determination of the inhibition constants (K_i) of trypsin and α-chymotrypsin by the preparations.

Earlier we demonstrated that BBI belongs to a group of slowly interacting inhibitors tightly binding to proteinases [20, 25]. The values of K_i for the systems BBI–serine proteinase are in the range 10^{-9}-10^{-10} M [9, 20, 24, 25].

The titration curve of α-chymotrypsin with the BBI–DAD conjugate with the 20% degree of oxidation of DAD is presented in Fig. 3. Analogous data were obtained for the BBI–DAD conjugates with 5 and 10% oxidation of DAD (the titration curves are not presented here). The values of K_i of α-chymotrypsin by three BBI–DAD preparations are given in the table. Comparison of these values with the K_i of the native BBI demonstrates that modification of the inhibitor by dextran activated with sodium periodate does not result in any significant change in the thermodynamic parameters of the association reaction with α-chymotrypsin. It should be noted that the possibility of direct chemical modification of the chymotrypsin-binding site of BBI (Leu_{43}-Ser_{44}) during synthesis is excluded. It is possible that a slight increase in K_i of chymotrypsin by the BBI–DAD conjugates as compared to the native inhibitor can be explained by some steric hindrances around this site or by structural changes occurring in the BBI molecule as a result of the synthesis. High sensitivity of the antichymotrypsin site to the structural changes was mentioned earlier [27]. Since the Lys_{16} residue is a constituent of the trypsin-binding site of BBI, there is a theoretical possibility of modification of the NH_2 group of this site during the synthesis. However, the

Composition and antiproteinase activity of the BBI–DAD conjugates

Preparation	Degree of oxidation of dextran, %	Protein in the conjugate, %	K_i, nM	
			trypsin	α-chymotrypsin
BBI–DAD	5	8.3	0.3 ± 0.06	24 ± 5
BBI–DAD	10	11	0.27 ± 0.05	17 ± 2
BBI–DAD	20	13	0.22 ± 0.04	27 ± 5
BBI			0.1 ± 0.02	6.4 ± 0.6
			[25]	[26]

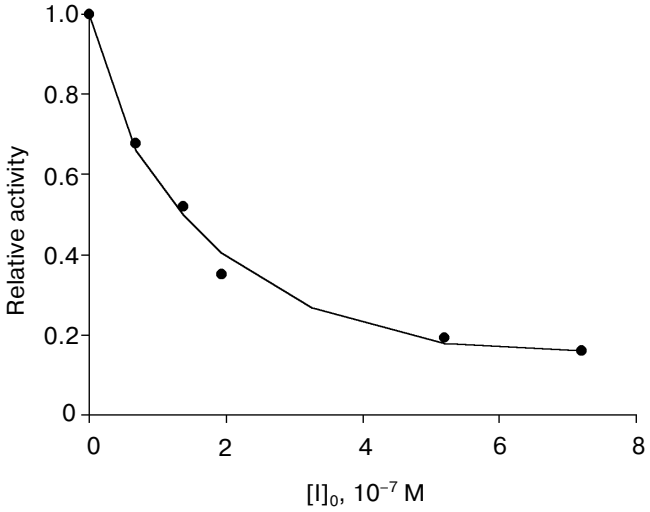

Fig. 3. Inhibition of α-chymotrypsin by BBI–DAD conjugate (degree of oxidation of DAD is 20%): $[E]_o$ = 40 nM; $[Bz\text{-}Tyr\text{-}EE]_o = 2 \cdot 10^{-4}$ M; 0.05 M Tris-HCl, pH 8.0; 0.02 M $CaCl_2$.

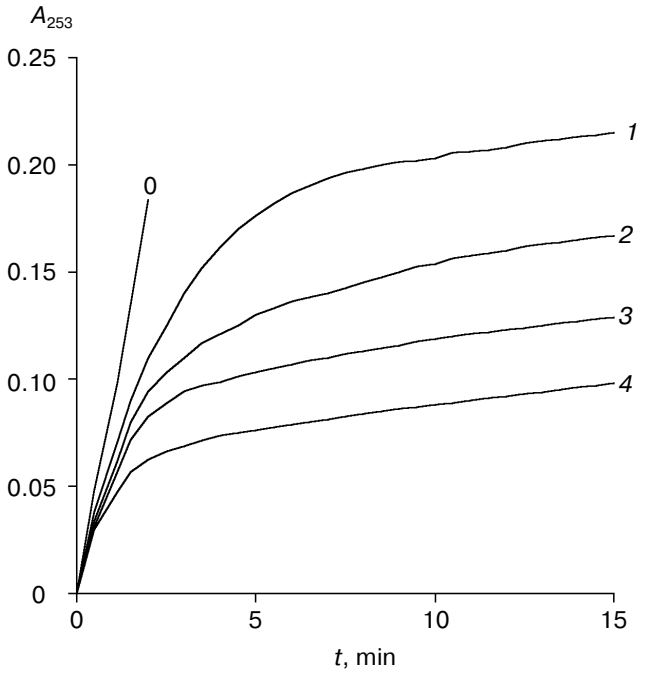

Fig. 4. Kinetics of Bz-Arg-EE hydrolysis catalyzed by trypsin in the presence of the BBI–DAD conjugate (20% degree of oxidation of DAD): $[E]_o$ = 57 nM; $[I]_o$ = 0 (*0*), 0.8 (*1*), 1.3 (*2*), 2.4 (*3*), 3.2 (*4*) μM; $[S]_o = 3 \cdot 10^{-4}$ M; 0.05 M Tris-HCl, pH 8.0; 0.02 M $CaCl_2$.

results of titration of the BBI–DAD conjugates with trypsin demonstrate that for trypsin the activity of these conjugates is close to 100% and consequently, such modification is insignificant.

Rather low inhibition constants, as in the case of trypsin (10^{-10} M), cannot be determined by the method described above. The relationship $1 < [E]_o/K_i < 10$ is one of the conditions for obtaining a smooth titration curve necessary for calculation of K_i using Eq. (1). Because we had no substrates that allow measuring trypsin concentrations in the range 10^{-9}-10^{-10} M, in this case we used a method specially developed for slowly reacting inhibitors [17]. A set of kinetic curves of accumulation with time of the product of BAEE hydrolysis catalyzed by trypsin in the presence of various concentrations of the BBI–DAD conjugate at 20% degree of oxidation of DAD is presented in Fig. 4. Analogous data were obtained for the conjugates of DAD with degrees of oxidation of 5 and 10%. The length and slope of the initial portion of each curve decreases with increase in the inhibitor concentration.

A specific feature of the inhibition process by slowly interacting inhibitors is that the curves of accumulation of the product of hydrolysis have asymptotes at rather high initial concentrations of the inhibitor. The slopes of these asymptotes are the equilibrium rates; they indicate that decrease in the enzymatic reaction rate is not caused by inactivation of the enzyme, consumption of the substrate, or inhibition by the product.

The values of K_i given in the table indicate that binding of the inhibitor with the polymeric carrier does not cause any significant decrease in affinity of the inhibitor molecule at either active site.

It should be noted that K_i were determined for different lots of BBI–DAD conjugates, i.e., conjugates with equal composition but obtained in different syntheses. The standard deviations of the K_i measurement account for this fact.

Since conjugates of dextran with proteins are known to circulate in the bloodstream for a long time [28-30], it can be suggested that the conjugate synthesized by us will also be a more prolonged preparation than native BBI. The clearance rate of dextrans used as neutral extravasating carriers depends on their molecular mass. Dextrans with molecular masses from 40 to 70 kD remain in the bloodstream from 6 to 24 h after intravenous administration [2]. It was also demonstrated earlier by the example of conjugates of antitumor drugs with dextrans [31] that they are more effective than the free drugs, although dextran does not have affinity to tumor cells and does not possess its own activity. This effect can be due to the fact that a neoplasm is more permeable for macromolecules than a normal tissue because of neovascularization and increase in intercapillary pores. It was also found that the conjugates of dextran (molecular mass 70 kD) with antitumor agents remain at the site of administration for a long time, are accumulated in lymph, penetrate into tumor cells via endocytosis, and act on cells resistant to the drugs [32]. Thus, our results suggest that BBI bound to DAD will be a more effective chemotherapeutic agent than native BBI.

REFERENCES

1. Virnik, A. D., Khomyakova, K. P., and Skokova, I. F. (1975) *Uspekhi Khim.*, **7**, 1280-1307.
2. Molteni, L. (1979) in *Drug Carriers in Biology and Medicine* (Gregoriadis, G., ed.) Academic Press, London-N. Y.-San Francisco, pp. 108-125.
3. Vercauteren, R., Schacht, E., and Duncan, R. (1992) *J. Bioact. Biocomp. Polym.*, **7**, 346-357.
4. Tikhonova, T. V., Gladysheva, I. P., and Larionova, N. I. (1995) *FEBS Lett.*, **362**, 225-228.
5. Larionova, N. I., Balabushevich, N. G., and Zhatikov, P. A. (1998) *Biochemistry (Moscow)*, **63**, 1078-1082.
6. Kennedy, A. R. (1993) in *Protease Inhibitors as Cancer Chemopreventive Agents* (Troll, V., and Kennedy, A. R., eds.) Plenum Publishing Corporation, N. Y., pp. 9-64.
7. Shonbaum, J. R., Zerner, B., and Bender, M. (1961) *J. Biol. Chem.*, **236**, 2930-2935.
8. Chase, T., and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.*, **29**, 508-514.
9. Larionova, N. I., Gladysheva, I. P., Tikhonova, T. V., and Kazanskaya, N. F. (1993) *Biochemistry (Moscow)*, **58**, 1046-1052.
10. Gladysheva, I. P., Balabushevich, N. G., Moroz, N. A., and Larionova, N. I. (2000) *Biochemistry (Moscow)*, **65**, 198-203.
11. Lindenbaum, G. M., Mirgorodskaya, O. A., and Moskvichev, B. V. (1977) *Khim. Farm. Zh.*, **11**, 80-83.
12. Torchilin, V. P., Reizer, I. M., Smirnov, V. N., and Chazov, E. I. (1976) *Bioorg. Khim.*, **2**, 1252-1258.
13. Dubois, M., Gilse, K. A., Hamilton, S. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.*, **28**, 350-356.
14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
15. Kakade, M. L., Simons, N., and Liener, J. E. (1969) *Cereal. Chem.*, **46**, 518-526.
16. Hummel, C. (1959) *J. Biochem. Phys.*, **37**, 1393-1399.
17. Bieth, J. (1974) in *Bayer-Symposium V Proteinase Inhibitors* (Fritz, H., Tschesche, H., Green, L. J., and Truscheit, E., eds.) Springer-Verlag, Berlin, pp. 463-469.
18. Morrison, J. F., and Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.*, **61**, 201-301.
19. Larionova, N. I., Gladysheva, I. P., Topchieva, I. N., and Kazanskaya, N. F. (1993) *Biochemistry (Moscow)*, **58**, 1220-1225.
20. Larionova, N. I., Gladysheva, I. P., and Gladyshev, D. P. (1997) *FEBS Lett.*, **404**, 245-248.
21. Koziner, V. B., and Fedorov, N. A. (1974) *Mechanism of Action of Polyglukin* [in Russian], Moskwa Publishers, Moscow.
22. Mirgorodskaya, O. A., and Poletaeva, L. V. (1985) *Khim. Farm. Zh.*, **5**, 594-599.
23. Pakshver, A. B. (ed.) (1957) *Handbook for Analytical Control in Fabrication of Artificial and Synthetic Fibers* [in Russian], Gizlegprom Publishers, Moscow, p. 42.
24. Tremillion, B. (1967) in *Separation on the Ion-Exchange Resins* [Russian translation], Leningrad, p. 36.
25. Gladysheva, I. P. (1994) Candidate's dissertation [in Russian], Moscow State University.
26. Gladysheva, I. P., Larionova, N. I., Gladyshev, D. P., Tikhonova, T. V., and Kazanskaya, N. F. (1994) *Biochemistry (Moscow)*, **59**, 371-374.
27. Werner, M. H., and Wemmer, D. E. (1992) *Biochemistry*, **31**, 999-1010.
28. Wileman, T. E., Foster, R. L., and Elliott, P. N. C. (1986) *J. Pharm. Pharmacol.*, **38**, 264-271.
29. Melton, R. G., Wiblin, C. N., Baskerville, A., Foster, R. L., and Sherwood, R. F. (1987) *Biochem. Pharmacol.*, **36**, 113-121.
30. Mikolajczyk, S. D., Meyer, D. L., Fagnani, R., Hagan, M. S., Law, K. L., and Starling, J. J. (1996) *Bioconjug. Chem.*, **7**, 150-158.
31. Bernshtein, A., Hurwitz, E., Maron, R., Arnon, R., Sela, M., and Wilchek, M. (1978) *J. Natl. Cancer. Inst.*, **60**, 379-384.
32. Onishi, H., and Nagai, T. (1986) *Chem. Pharm. Bull.*, **34**, 2561-2567.