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Protein Binding Characteristics of 2′-Benzoyloxycinnamaldehyde

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ABSTRACT The protein binding characteristic of 2′-Benzoyloxycinnamaldehyde (BCA) was investigated, which has demonstrated a potent antitumor effect against several human solid tumor cell lines and in human tumor xenograft nude mice. Protein binding of BCA in human serum was 86±0.91% and the predominant binding protein of BCA was fatty-acid-free human serum albumin (HSA) (81±0.91%). The binding of BCA to HSA was outlined by one class, and Ka and n of BCA were 1.65×10^5 M^{-1} and 0.374, respectively. Displacement studies with fluorescence probes suggested that BCA mainly binds to site I on HSA, and BCA-induced enhancement in site II binding. The limited drug–drug interaction experiments suggested that BCA influences both site I and site II drug–HSA bindings via different mechanisms; a competitive displacement and a probable allosteric conformational change in HSA, respectively.

KEYWORDS Cinnamaldehyde, Protein binding, Displacement

INTRODUCTION

Cinnamomum cassia Blume (Lauraceae) has been used as carminative and antispasmodic agents (Lee, 2002) in traditional oriental medicines. Recently, it was reported that the extract of *C. cassia* exhibited cytotoxicity against the human tumor cells (Ka et al., 2003; Kwon et al., 1998) and antimutagenetic activity (Ohta, 1993), and potentiated the cell-inactivating effect of cis-diamminedichloroplatinum (II) in human NHIK 3025 cells (Dornish et al., 1988, 1989). The inhibitory effect on farnesyl protein transferase activity in vitro was also reported, which relates to immune cell activation as well as carcinogenesis (Koh et al., 1998).

Cinnamaldehydes (CAs) such as 2′-hydroxycinnamaldehyde and *o*-methoxycinnamaldehyde (Choi et al., 2001), the major constituents of volatile oil of *C. cassia*, showed a strong antitumor effect, while other components of *C. cassia*, such as cinnamic acid, cinnamate, and cinnamoyl alcohol, did not exhibit the antitumor effect. It was believed that the key functional group of the CA-related compounds in the antitumor activity is the propenal group (Kwon et al., 1998). CA-related compounds were synthesized from various cinnamic acids, and the antitumor activity of those synthetic CA analogues was studied in order to develop new anticancer drugs (Kwon et al., 1998).

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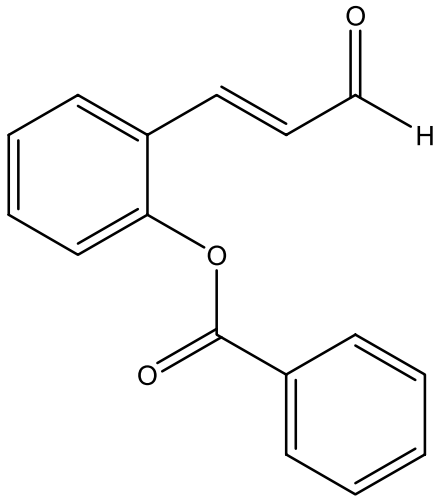

FIGURE 1 **Structure of 2′-Benzoyloxycinnamaldehyde (BCA, MW 252.3).**

2′-Benzoyloxycinnamaldehyde (BCA, Fig. 1), prepared from 2′-hydroxycinnamaldehyde and benzoyl chloride, strongly inhibited in vitro growth of 29 kinds of human cancer cells and in vivo growth of SW-620 human tumor in xenograft nude mice (Lee et al., 1999). BCA also showed antiproliferative effect in a proliferative vitreoretinopathy model in the rabbit (Lee et al., 2002). The majority of human malignancies have deregulation of cyclin-dependent kinases (Cdks), leading to uncontrolled cellular proliferation. To date, it is believed that the mechanism of action of these CAs relates to the inhibitory effect on Cdks (Jeong et al., 2003), which play a key role in regulating cell cycle progression in eukaryotic cells and, consequently, leads to G_2/M phase arrest (Seong et al., 2003).

As is well known, protein binding is a critical factor affecting the volume of distribution, and both hepatic and renal clearance of a compound. In this paper, the protein-binding characteristic of BCA, a new antitumor agent, was investigated.

MATERIALS AND METHODS

Materials

BCA was synthesized and donated by Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). Warfarin, diazepam, penylbutazone, dansylamide, dansylsarcosine, fatty-acid-free human serum albumin (HSA), human-γ-globulin (IgG), and human α_1-acid glycoprotein (AAG) were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Human serum was prepared from whole blood that was drawn from healthy volunteers. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Solvents for HPLC were filtered through a 0.45 μm filter and thoroughly degassed in an ultrasonic bath before use. Sorenson's buffer was purchased from Invitrogen Corp. (Carlsbad, CA, USA) and was used as received.

Methods

Protein binding of BCA was measured using an ultrafiltration method. A micropartition system MPS-3 equipped with YMT ultrafiltration membrane (exclusion limit 10,000 Da) was used. Protein binding of BCA (50 μM) was determined in human serum, HSA (4 g/100 mL), AAG (75 mg/100 mL), and IgG (1 g/100 mL) solutions. BCA–protein solutions were incubated for 30 min at 37°C with gentle shaking (EYELA LTI 601-SD, Tokyo Rikakikai, Tokyo, Japan). An aliquot of 100 μL was taken to determine the total concentration of BCA before ultrafiltration. An aliquot of 1 mL was further transferred into the ultrafiltration kit and centrifuged at 3000 rpm for 30 min. BCA in the ultrafiltrates was analyzed by the HPLC system, and its concentration was calculated by a calibration curve. The bound fraction of BCA was calculated from the total concentration and the filtrate concentration before and after ultrafiltration.

In order to obtain the binding parameters to HSA, BCA–HSA binding measurement was conducted at 2.5 to 100 μM of BCA in 40 μM HSA solution. The number of binding sites on the protein molecules (n) and the association constant of the drug to protein (Ka) were estimated from the Scatchard plot (Eq. 1):

$$\frac{r}{D_F} = nKa - rKa \qquad (1)$$

where r is the number of moles of bound drug per mole of protein and D_F is the free drug concentration.

The binding site of BCA on HSA was determined using two binding-site-specific fluorescent probes, dansylamide (site I or subdomain IIA) and dansylsarcosine (site II or subdomain IIIA). The fluorescence intensity of HSA solutions (20 μM) containing 2 μM probe was measured before and after the addition of BCA using a fluorescence spectrophotometer (excitation: 380 nm; emission: 470 nm) and varying BCA concentrations in the range of 2.5 to 80 μM. Effects of

incubation temperature (4, 23, and 37°C) and protein concentration (1 to 6% HSA) on BCA binding to HSA were investigated at 50 μM of BCA concentration.

Drug–drug interactions of BCA were investigated against warfarin, phenylbutazone, and diazepam. BCA (50 μM) was added to the HSA solution (4%), and subsequently, interacting drugs were added to BCA–HSA solutions in the concentration range of 50 to 5000 μM. The BCA–HSA drug solutions were incubated for 30 min at 37°C with gentle shaking, and the bound fraction of BCA was calculated from the total concentration and the filtrate concentration before and after ultrafiltration.

Analysis

BCA was analyzed by an HPLC system equipped with a Model 305 pump (Gilson, VILLIERS LE BEL, France), Gilson 805 nanometric module, Gilson 234 auto injector, Waters™ 486 turnable ultraviolet (UV)/visible absorbance detector (Waters, Milford, MA, USA), and a luminescence spectrometer SLM AB2 (SLM-Aminco, Urbana, IL, USA). A Waters Spherisorb Silica column (4.6 × 250 mm ID, particle size 5 μm; Waters, Millford, MA, USA) was used. BCA was well separated with a mobile phase of 70% methanol and 30% water ($R_T = 11.23$ min) and detected at 254 nm. The calibration curve was constructed in the concentration range of 1 to 50 μM, and intra- and interday variation was less than 5%, and the accuracy was achieved within a standard deviation of 10%.

RESULTS AND DISCUSSION

Protein binding of BCA in human serum was 86±0.91%. The predominant binding protein of BCA in human serum was HSA (81±0.91%), compared to the binding of AAG (60±1.40%), and IgG (42±3.05%). The association constant (*Ka*) and the binding capacity (*n*) of BCA to HSA were estimated from the Scatchard plot. The data were fitted well with a linear curve (Fig. 2), indicating that the binding of BCA to HSA is outlined by one class. Ka and n of BCA were 1.65×10^5 M^{-1} and 0.374, respectively. The effects of incubation temperature and HSA concentration on BCA protein binding were examined. Protein binding of BCA was independent of incubation temperature (Fig. 3A), while BCA binding increased as HSA concentration increased, and reached a plateau above 4% HSA (Fig. 3B).

In order to identify the location of binding sites on HSA, site marker displacement experiments were carried out using fluorescent probes that specifically bind to known sites on HSA. The fluorescence intensities of dansylamide (a site I marker) and dansylsarcosine (a site II marker) were monitored before and after addition of BCA in HSA solution. BCA reduced the fluorescence of the site I binding marker by 56%, while BCA increased the fluorescence of the site II binding marker by 32% at a BCA concentration of 80 μM (the molar concentration ratio of BCA to HSA, approximately 4:1, Fig. 4). The fluorescent marker displacement experiments implied that BCA binds primarily to site I on HSA and influences the binding of ligands at site II.

The protein-binding interaction of BCA was examined against the site I marker (warfarin and phenylbutazone) and site II marker (diazepam). As the concentrations of warfarin and phenylbutazone increased to 500 μM, BCA–HSA binding decreased to 52% and 45%, respectively (Fig. 5). The displacement of BCA from HSA with the addition of warfarin and phenylbutazone suggested that BCA interacts with HSA site I binding drugs.

Interestingly, BCA–HSA binding increased to 87.5% at the concentration of diazepam 500 μM (Fig. 5). It was known that small molecule binding sites I and II on HSA are distinct, but not independent, and

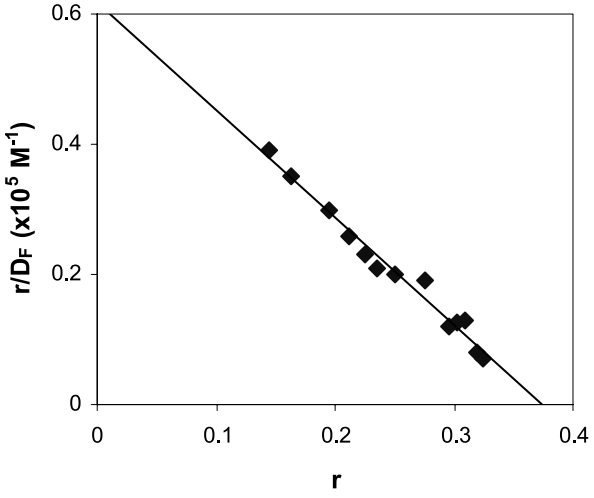

FIGURE 2 **BCA Binding to HSA According to Scatchard Plot. r is the Number of Moles of Bound Drug Per Mole of Protein, and D_F is the Free Drug Concentration.**

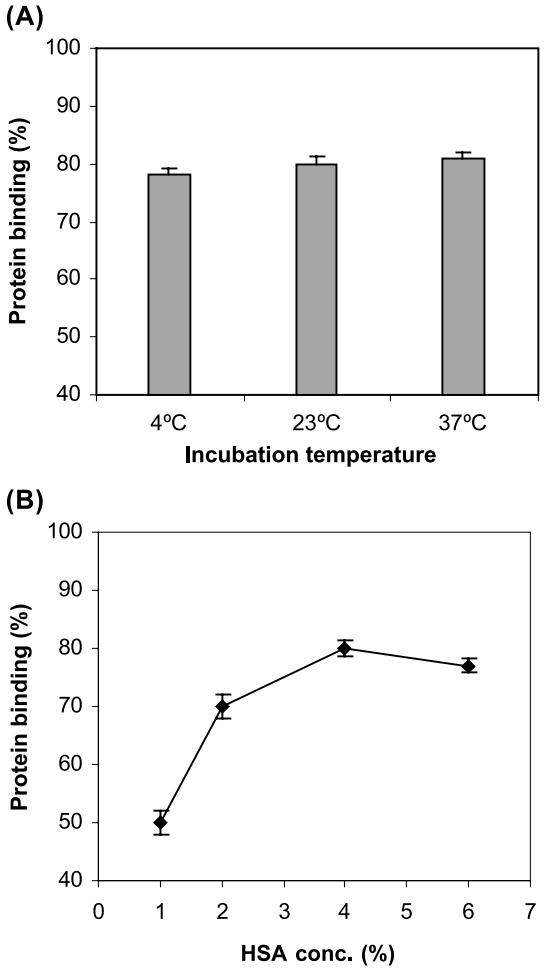

FIGURE 3 **Dependence of BCA Binding to HSA on (A) Incubation Temperature and (B) Protein Concentration. Results Represent the Mean ± SD of Three Determinations.**

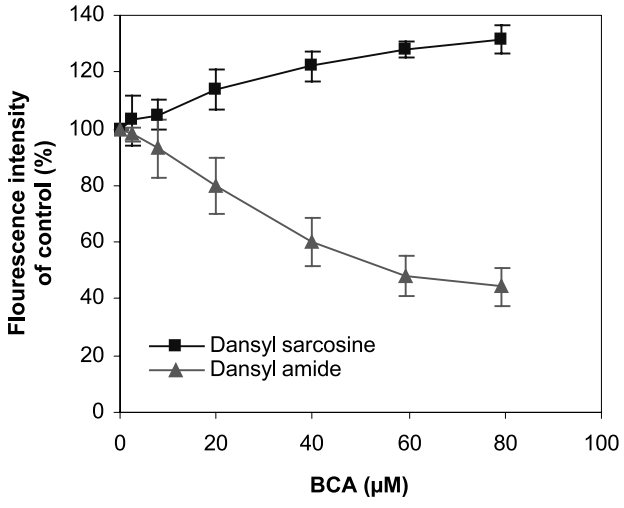

FIGURE 4 **Changes in the Fluorescence Intensities of Dansylamide and Dansylsarcosine: Dansylamide and Dansylsarcosine were Preincubated with HSA, and the Fluorescence Intensities were Measured Before and After Addition of BCA to the System. Results Represent the Mean ± SD of Three Determinations.**

an allosteric regulation exists between the binding sites (Ascoli et al., 1995; Sakai et al., 2001; Yamasaki et al., 1999). Bree et al. (1993) reported that tenoxicam, which mainly binds to site I on HSA, increased diazepam–HSA binding, and diazepam increased tenoxicam–HSA binding. It was reported that the neutral (N)–base (B) transition of albumin (Wanwimolruk & Birkett, 1982) and the addition of fatty acids (Wanwimolruk et al., 1983) and diazepam (Bree et al., 1993) increased HSA binding of site I markers. In contrast, tolmetin (a site I binder) and tenoxicam caused a significant increase in HSA binding of site II markers (Bree et al., 1989, 1993; Matsuyama et al., 1987). These binding enhancement effects toward site I or site II on HSA were attributed to an allosteric conformation change in the protein by the microenvironmental change. BCA enhanced the binding of a site II marker (dansylsarcosine) to HSA, and a site II marker (diazepam) enhanced BCA–HSA binding. The result of BCA with site II marker interaction experiments is similar to that of tenoxicam with diazepam (Bree et al., 1993).

Recently, Benet and Hoener (2002) reported that changes in plasma protein binding by drug–drug interactions would usually not influence the free drug concentration, which is more critical for therapeutic effects, and the clinical exposure such as AUC (area under curve) of a patient to a drug (Benet & Hoener, 2002). Therefore, despite the experimental result of BCA–drug protein-binding interaction, its clinical consequence could be marginal.

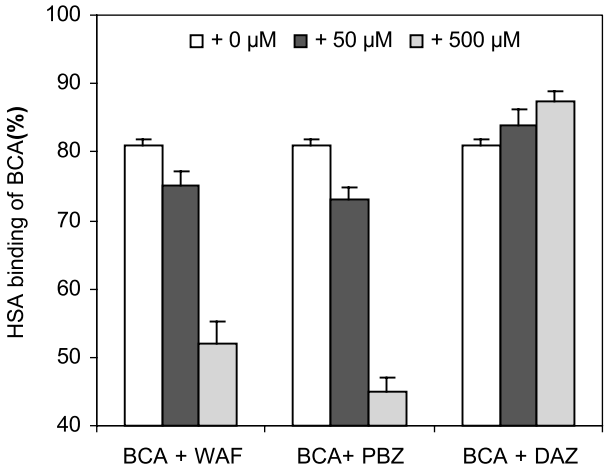

FIGURE 5 **Changes in BCA–HSA Binding in the Presence of Warfarin (WAF), Phenylbutazone (PBZ), and Diazepam (DAZ). Results Represent the Mean ± SD of Three Determinations.**

CONCLUSION

Protein binding of BCA in human serum was 86±0.91%. The major binding protein in human serum was HSA, and the binding to HSA was described as one-class binding. Displacement studies with fluorescence probes suggested that BCA mainly binds to site I on HSA, and BCA induced enhancement in site II binding. The limited drug–drug binding interaction experiments suggested that BCA influences both site I and site II drug–HSA bindings via different mechanisms: a competitive displacement and a probable allosteric conformational change in HSA, respectively.

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