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Enantioselective immunorecognition of protein modification with optically active ibuprofen using polyclonal antibody

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Abstract

Formation of covalently bound protein adducts with 2-arylpropionic acids (2-APAs) has been proposed as a possible explanation for hypersensitivity and toxic responses to chiral carboxylic acid drugs. To identify the cellular proteins chemically modified with optically active (*S*)-ibuprofen, we generate polyclonal antibodies by immunizing rabbits with immunogen coupled to bovine serum albumin (BSA) via the spacer of 4-aminobutyric acid. The resulting antibodies largely cross-reacted with *N*-α-(*t*-butoxycarbonyl)-ε-(*S*)-ibuprofenyl lysine as well as with the conjuguated (*S*)-ibuprofen with glycine and taurine and unconjugated (*S*)-ibuprofen, enabling enantioselective detection of (*S*)-ibuprofen residues anchored on ovalbumin molecules, introduced by the reaction of the ibuprofen *p*-nitrophenyl ester. Furthermore, immunoblotting with an antibody allows the enantioselective detection of (*S*)-ibuprofen-introduced glutathione-*S*-transferase (GST). These results indicate that the developed method will be useful for monitoring the generation and localization of protein covalently bound with (*S*)-ibuprofen, which may be the cause of ibuprofen-induced toxicity.

Keywords: Immunorecognition; Protein modification; Ibuprofen; Polyclonal antibodies

1. Introduction

Many of the nonsteroidal anti-inflammatory drugs such as ibuprofen, naproxen, flurbiprofen, fenoprofen, and ketoprofen belong to the class of 2-arylpropionic acids (2-APAs) containing a carboxylic acid group at the C-2 chiral center of the propionic acid side chain, and their racemic forms can be resolved into (*R*)- and (*S*)-enantiomers. Nevertheless, the clinically used 2-APAs are marketed as racemates, with the notable exception of naproxen. It has become increasingly clear that the enantiomeric forms of drugs may differ in their potency, pharmacological actions, metabolism, tissue distribution, and toxicity. A unique feature of the metabolism of this class of compounds is conversion at a chiral center, generally referred to as chiral inversion [1]. This inversion is unidirectional; i.e. in mammalian organisms the pharmacologically inactive (*R*)-enantiomer is transformed to the active (*S*)-enantiomer, while the reverse reaction does not occur.

Another biotransformation pathway is conjugation with glucuronic acid to yield diastereomeric isomers of acyl glucuronides, which play a significant role in the metabolic disposition of most 2-APAs [2–4]. These acyl glucuronides are intrinsically reactive molecules both in vitro and in vivo, and are able to undergo a number of reactions including hydrolysis [2,5], intramolecular rearrangement [6–8], and covalent binding to proteins [9–11]. Each of these reactions can have pharmacological or toxicological implications; hydrolysis, catalyzed by hydroxide ions, β-glucuronidases, esterases, or serum albumin, leads to regeneration of the pharmacologically active drug. Rearrangement occurs by hydroxide ion-catalyzed acyl migration to yield the β-glucuronidase-resistant 2-, 3- and 4-*O* positional isomers. These isomers, unlike the acyl glucuronide itself, can exist transiently in the open-chain aldehyde of the sugar ring,

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and thereby react with amino groups of lysines on proteins to form imines that undergo rearrangement to yield the final adduct retaining the glucuronic acid moiety. Alternatively, the acyl glucuronide itself can directly interact with the thiol group of cystein, the phenolic hydroxyl group of tyrosine, and the ε-amino group of lysine on protein, resulting in the loss of the glucuronic acid moiety. Irrespective of the mechanism, it has been suggested that such covalent modification of native proteins by 2-APAs has caused allergic reactions and hepatotoxicity [2,12,13]. However, there have been relatively few studies that have examined the potentially stereoselective nature of irreversible binding of 2-APAs to protein via their acyl glucuronide. Additionally, the relative contributions of the transacylation and glycation mechanisms to total adduct formation and the generation of cytotoxic, genotoxic, or immune response is not known. If their toxicities are responsible for the damage sometimes observed when using 2-APAs, the identity and location of proteins modified with chiral carboxylic acid drugs will be significant in understanding the mechanism of toxicity.

Immunoblotting of protein adducts of reactive metabolites has been successfully developed to identify target cellular proteins modified by reactive metabolites and to investigate the toxicological importance of protein modification in experimental animals and in humans pre-exposed to diclofenac [14,15], acetaminophen [16] and halotan [17]. In addition, the antibodies prepared for immunoblotting can be used for the development of enzyme-linked immunosorbent assays to monitor urinary mercapturic acid metabolites [18] and plasma protein adducts [19] derived from the reactive metabolite as biomarkers of human exposure to potential toxins. The objective of this study is to prepare polyclonal antibodies and to develop immnunoblotting used as a probe to detect proteins modified by optically active ibuprofen. These antibody-based techniques are expected to facilitate our investigation on the mechanism of 2-APAs-induced cytotoxicity.

2. Materials and methods

2.1. Materials

(*R*)- and (*S*)-Ibuprofen (**1a** and **1b**) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Amidated conjugates of (*R*)- and (*S*)-ibuprofen with glycine (**2a** and **2b**) and taurine (**3a** and **3b**), as well as *p*-nitorophenyl esters of (*R*)- and (*S*)-ibuprofen (**4a** and **4b**) were synthesized in our laboratory by a previously reported method [20] (Fig. 1). Endoproteinase Lys-C (lysylendopeptidase) was obtained from Wako (Osaka, Japan). α-Cyano-4-hydroxycinnamic acid (α-CHCA), bovine serum albumin (BSA), chicken egg albumin (OVA) and glutathione-*S*-transferase (GST) from *Schistosoma japonicum* expressed in *Escherichia coli* were purchased from Sigma Chemical Co. Freund's complete adjuvant was purchased from DIFCO (Detroit, MI, US).

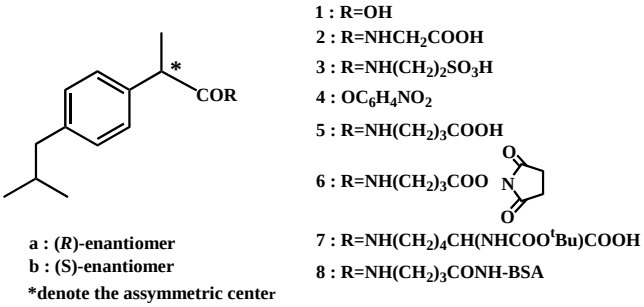

Fig. 1. Structures of ibuprofen, its related compounds, and the immunogen.

β-Galactosidase (EC 3.2.1.23) from *E. coli* (600–950 units per mg protein) was obtained from Boehringer Manheim Co. (Tokyo, Japan). Horseradish peroxidase conjugated anti-rabbit IgG antibody (Fc specific) and ENVISION kit were purchased from Chemicon International Inc. (Temecula, CA, USA) and Dako Cytomation Co. Ltd. (Glostrup, Denmark), respectively. 96-well EIA/RIA plates (No. 3590) were purchased from Coster Co. (Cambridge, MA, USA). ULTRACENT-10 microconcentrator was obtained from TOSOH Co. (Tokyo, Japan). The filtrate has a nominal molecular weight cut off of 10,000 and was used without preconditioning. EIA/RIA plates coated with goat anti-rabbit IgG antibody was kindly donated by Eiken Chemical Co. Ltd. (Tokyo, Japan). Zip-TiP C18 was from Millipore Waters (Milford, MA, USA). Nitrocellulose membrane (0.45 μm) was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All other reagents were of analytical reagent grade and all solvents were purified by distillation prior to use. Water from a Millipore water filtration system (Milli QUV Plus) was used for the aqueous solutions described below.

2.2. Buffers

The buffers used for this work were as follows: buffer A, 50 mM NaH_2PO_4–Na_2HPO_4 (pH 7.3); buffer B, buffer A containing gelatin (0.1% (w/v)), NaCl (0.9% (w/v)) and NaN_3 (0.1% (w/v)); buffer C, buffer A containing NaCl (0.9% (w/v)); buffer D, buffer C containing Tween 20 (0.05% (v/v)); buffer E, 1.5 mM KH_2PO_4–8.1 mM Na_2HPO_4 (pH 7.4) containing NaCl (0.8% (w/v)) and KCl (0.02% (w/v)).

2.3. Apparatus

Melting points were measured on an electric microhot-stage and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JNM-GS270 at 270 MHz using tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplets). The absorbance for ELISA was measured using a MPR A4i microplate reader (Tosoh, Tokyo, Japan). SDS-PAGE and immunoblotting were performed with NA-1013 mini gel slab electrophoresis equipment and NA-1510B double cassette

mini transfer instrument (Nihon Eido, Tokyo, Japan), respectively.

2.4. MALDI-TOFMS analysis

Analysis by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOFMS) in the positive-ion detection mode was carried out with a Voyager RP (PerSeptive Biosystems, Framingham, MA, USA) equipped with a pulsed nitrogen laser (337 nm). The 0.5 ml aliquot of the samples in acetonitrile and water (1:1) containing 0.1% (v/v) trifluoroacetic acid was mixed with 0.5 ml of α-CHCA (10 mg/ml) in the same solvent, and 1 μl of the mixture was applied to the stainless steel sample plate and allowed to dry at room temperature. The solution was subjected to MALDI-TOFMS analysis. The measured *m/z* values were the average mass for the fragment ions. Peptides were initially analyzed in the linear mode with external calibration using the protonated ion $[M + H]^+$ of angiotensin I (1296.7), ACTH (clip 18–39) (2465.2), ACTH (clip 7–38) (3657.9) and insulin (5730.6). The data were obtained from 256 laser shots using the following parameters: 25.0 kV accelerating voltage, 14.0 kV grid voltage, and 50.0 V guide wire voltage.

2.5. Synthesis of N-((S)-Ibuprofenyl)-4-aminobutyric acid (5b)

To a solution of **4b** (680 mg) in pyridine (3 ml) was added a solution of 4-aminobutyric acid (600 mg) in 10% NaOH (1.5 ml) under stirring. After being stirred at room temperature overnight, the reaction mixture was diluted with H_2O and acidified with 5% HCl. The resulting mixture was extracted with ethyl acetate, washed with H_2O, and the organic layer was then dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on silica gel (15 g) using toluene-acetone (1:1 (v/v)) as an eluent. Recrystallization of the eluate from acetone-hexane gave **5b** (210 mg) as colorless crystalline product. mp 67–71 °C. ^{1}H NMR ($CDCl_3$) δ: 0.89 (6H, d, $J = 6$ Hz, –CH(CH_3)$_2$), 1.54 (3H, d, $J = 7.5$ Hz, –CH(C_6H_4)CH_3), 1.78 (2H, m, –$CH_2CH_2CH_2$COOH), 1.82 (1H, m, –CH_2CH(CH_3)$_2$), 2.33 (2H, t, $J = 7$ Hz, –$CH_2CH_2CH_2$COOH), 2.45 (2H, d, $J = 7.5$ Hz, –CH_2CH(CH_3)$_2$), 3.29 (2H, q, $J = 7$ Hz, –NH$CH_2CH_2CH_2$COOH), 3.61 (1H, q, $J = 7.5$ Hz, –CH_3(C_6H_4)CHCONH–), 5.98 (1H, t, $J = 7$ Hz, –NH–), 7.11 and 7.18 (each 2H, d, $J = 7.8$ Hz, aromatic).

2.6. Synthesis of N-((S)-Ibuprofenyl)-4-aminobutyric acid succinimidyl ester (6b)

To a solution of **5b** (200 mg) in dioxane (2 ml) was added *N*-hydroxysuccinimide (400 mg) and the mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with ethyl acetate, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on silica gel (10 g) using toluene-acetone (5:1 (v/v)) as an eluent. Recrystallization of the eluate from acetone-hexane gave **6b** (75 mg) as colorless needles. mp 92–94 °C. ^{1}H NMR ($CDC1_3$) δ: 0.84 (6H, d, $J = 6$ Hz, –CH(CH_3)$_2$), 1.48 (3H, d, $J = 7.5$ Hz, –CH(C_6H_4)CH_3), 1.86 (2H, m), 1.88 (1H, m, –CH_2CH(CH_3)$_2$), 2.44 (2H, d, $J = 7$ Hz, –CH_2CH(CH_3)$_2$), 2.55 (2H, t, $J = 7$ Hz, –CH_2COOH), 2.80 (4H, 4, succinimide), 3.27 (2H, m, –NHCH_2CH_2–), 3.53 (1H, q, $J = 7.5$ Hz, –CH(C_6H_4)CH_3), 6.01 (1H, m, –NH), 7.10 and 7.19 (each 2H, d, $J = 7.8$ Hz, aromatic).

2.7. Synthesis of N-α-(t-butoxycarbonyl)-ε-(S)-ibuprofenyl lysine ((S)-Ibuprofenyl-N^a-BOC-lysine) (7b)

To a solution of **4b** (160 mg) in pyridine (2 ml) was added a solution of *N*-α-(*t*-butoxycarbonyl)lysine (143 mg) in 10% NaOH (1.0 ml) under stirring. After being stirred at room temperature overnight, the reaction mixture was diluted with H_2O and acidified with 5% HCl. The resulting mixture was extracted with ethyl acetate, washed with H_2O, and the organic layer was then dried over anhydrous Na_2SO_4. After evaporation of the solvent in vacuo, the crude product was purified by column chromatography on silica gel (15 g) using toluene-acetone (5:1 (v/v)) as an eluent to give **7b** (200 mg) as colorless granules. mp 65–68 °C. ^{1}H NMR ($CDC1_3$) δ: 0.9 (6H, d, $J = 6$ Hz, –CH(CH_3)$_2$), 1.3 (4H, m), 1.45 (9H, s, –C(CH_3)$_3$), 1.5 (3H, d, $J = 7.5$ Hz, –CH(C_6H_4)CH_3), 1.69 (1H, m), 1.84 (2H, m), 2.45 (2H, d, $J = 7.5$ Hz), 3.18 (2H, m), 3.54 (1H, q, $J = 7.5$ Hz, –CH(CH_3)$_2$), 4.2 (1H, m), 5.3 (1 H, m), 7.12 and 7.18 (each 1H, d, $J = 7.8$ Hz, aromatic).

2.8. Preparation of anti (S)-ibuprofen antiserum

Hapten–BSA conjugate was prepared by the reaction of **6b** with BSA (~60 mol eq. to BSA). The hapten/BSA molar ratio was determined to be 34 by titration of the residual free amino groups on BSA using trinitrobenzensulfonic acid. The hapten–BSA conjugate (**8**, 1 mg) was dissolved in sterile saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). The emulsion was injected into domestic female albino rabbits (1.5–2 kg body weights) subcutaneously at multiple sites over the back. This procedure was repeated 1 week intervals at first month and then once every month. The antisera prepared from blood by centrifugation at 3000 rpm for 10 min was stored at 4 °C with NaN_3 (0.1% (w/v)).

2.9. Preparation of β-galactosidase-labeled N-((S)-ibuprofenyl)-4-aminobutyric acid conjugate

The enzyme-labeled antigen was prepared by reacting **6b** with 1 mg of β-galactosidase in 50 mM sodium phosphate buffer (pH 8.0)–dioxane (1:1 (v/v), 400 μl) at 4 °C

overnight. The molar ratio of the activated ester to enzyme in this reaction was adjusted to 10. Removal of unreacted substances by dialysis afforded the desirable enzyme-labeled (*S*)-ibuprofen, which were stored at 4 °C until use.

2.10. Characterization of antibodies

A suitably diluted antiserum diluted with buffer B (100 μl) were distributed in each well of the second antibody coated microplate. After incubation at 37 °C for 1 h, the solutions were aspirated off and the wells were washed three times with buffer D. The β-galactosidase-labeled (*S*)-ibuprofen (50 ng) in buffer B (100 μl) and a 50% methanolic solution (50 μl) of ibuprofen related compound were then added, mixed and incubated at 37 °C for 2 h. After washing in the same manner, bound enzymic activity on the plate was colorimetrically measured using a substrate solution (100 μl) containing *o*-nitrophenyl-β-D-galactopyranoside (0.06% (w/v)), $MgCl_2$ (0.2% (w/v)), and 2-mercaptoethanol (0.7% (v/v)). After incubation at 37 °C for 1 h, the enzyme reaction was terminated by the addition of 0.1 M NaOH (100 μl). The absorbance at 415 nm was measured using a MRP A4i microplate reader.

2.11. Detection of ibuprofen residues on OVA introduced by the reaction of (R)- and/or (S)-ibuprofen p-nitrophenyl ester

A solution of OVA diluted with buffer A was distributed in each well of the 96-well EIA/RIA plates, which were left overnight at room temperature. After washings three times with buffer C, **4a** and/or **4b** in 50% methanol were added to the wells. After incubation at 37 °C for 24 h, the solutions were aspirated off and the wells were washed three times with buffer C. The wells were blocked with 5% skimmed milk in buffer C (300 μl) at 37 °C for 2 h, and washed three times with buffer C. A 1:1000 (v/v) diluted antiserum (100 μl) diluted with buffer B were added to the wells, which were incubated at 37 °C for 1 h. After washings three times with buffer D, a solution (100 μl) of 1:1000 diluted HRP-labeled anti-rabbit IgG antibody diluted with buffer B was added, and incubated at 37 °C for 1 h. After washings as already described, a citrate-phosphate buffer (pH 5.0) containing 0.04% *o*-phenylenediamine 2HCl and 0.018% H_2O_2 was distributed to the wells and the plates were incubated at room temperature for 15 min. The enzyme reaction was terminated by the addition of 1 M H_2SO_4 (100 μl), and the absorbance at 492 nm was measured using MPR A4i microplate reader.

2.12. Formation of (R)- and (S)-ibuprofen–GST adducts and structural elucidation

A solution of **4a** or **4b** (each 75 μg) in methanol (7.5 μl) was added to a solution of GST (250 μg) in 50 mM potassium phosphate buffer (pH 8.5, 250 μl). The resulting mixture was incubated at 37 °C for 96 h, and the reaction was stopped by addition of acetic acid (50 μl). The mixture was submitted to centrifugal filtration to remove any low molecular weight substances, followed by centrifugations in buffer A. A potion of the mixture containing modified and unmodified GST was then subjected to reductive S-alkylation and proteolytic digestion with endoproteinase Lys-C, as follows. The mixture (17.6 μg) was reduced with dithiothreitol (3.6 μg) in 100 μl of 0.1 M Tris–HCl buffer (pH 8.0) containing 8 M urea and 2 mM EDTA for 30 min at 50 °C, and carboxymethylated with sodium iodoacetate (4.8 μg) at room temperature in the dark for 1 h under flushing with a stream of N_2. A solution of endoproteinase Lys-C (0.16 μg) in H_2O (10 μl) was added to the reaction mixture, and the resulting mixture was incubated at 37 °C for 4 h. The reaction was terminated by addition of acetic acid (100 μl). After desalting the mixture with Zip-Tip C18, 1 μl of the mixture was submitted to peptide mapping by MALDI-TOFMS.

2.13. Immunoblotting

For SDS-PAGE, the samples were diluted to 1:1 with 125 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, and 0.02% pyronin Y and heated at 100 °C for 5 min. Duplicate gels were routinely run to assess immunochemical specificity. Gels were stained with coomassie brilliant blue. Proteins resolved by polyacrylamide gels were transferred to nitrocellulose membrane at 55 V for 1 h in 25 mM Tris–192 mM glycine buffer (pH 8.3). After transferring, the blots were blocked by shaking overnight at 4 °C in buffer E containing 5% skimmed milk. The blotted membranes were incubated with a 1:1000 diluted anti-(*S*)-ibuprofen antiserum (S_1) for 3 h at room temperature with shaking. The blots were rinsed for three 5 min washes in buffer E containing 0.05% Tween 20, and then for two 5 min washes in buffer E. The immunoblots were incubated for 1 h with 1:100 diluted ENVISION kit diluted with buffer E. After washing as above, the blotted protein bands were visualized with 3,3′-diaminobenzidine for 5 min.

3. Results and discussion

It has been reported that, following administration of racemic ibuprofen to humans, about 80% of the drug is excreted as (*S*)-ibuprofen glucuronide [21]. This is much higher than the ratio of diastereomeric glucuronides obtained with human liver preparations [22]. This difference reflects the chiral inversion of (*R*)- to (*S*)-ibuprofen, which increases the amount of (*S*)-ibuprofen and decreases the amount of (*R*)-ibuprofen that can undergo glucuronidation [23]. These findings led to identification of the cellular proteins chemically modified with (*S*)-ibuprofen as a step in elucidating the mechanism of ibuprofen-induced cytotoxicity.

Western blotting and immunostaining techniques have been used as powerful tools to identify protein adducts by

detecting the hapten moiety of protein adducts formed during the exposure of cellular proteins to electrophilic species (haptens). In an attempt to identify target proteins modified with (*S*)-ibuprofen through its carboxyl group, we took advantage of immunoblotting as a detection tool by using a polyclonal antibody. For this purpose, the antibody should be able to discriminate both the asymmetric center and the isobutylphenyl moiety of (*S*)-ibuprofen–protein adducts, where the drug is coupled via the ϵ-amino group of lysine residue on the proteins. Several studies show that the use of carrier proteins such as BSA and keyhole limpet hemocyanin improved stereorecognition of chiral molecule by antibodies when the asymmetric center is far away from the coupling site. If the chemical group involved in the link between the hapten and the carrier is bound to the asymmetric center, it is desirable to increase the distance between the asymmetric center and the carrier protein in order to increase the probability of raising antibodies with chiral recognition [24,25].

In the preceding paper, we designed an (*S*)-ibuprofenyl-β-alanine–BSA conjugate as a preferential immunogen, resulting in the production of specific antibodies that recognize the asymmetric center of (*S*)-ibuprofen and its amino acid conjugates. Contrary to this, a report on the production of an antiserum against (*S*)-ibuprofen describes that the antiserum obtained by immunization of rabbits with immunogen coupled via the spacer 4-aminobutyric acid with BSA, expressing 50% of cross-reactivity with (*R*)-ibuprofen [26]. The differences in chiral recognition are supposed to be attributed to chiral inversion during the hapten synthesis, since the haptenic derivative is prepared by the activation of carobxy group of (*S*)-ibuprofen by using thionyl chloride. To ascertain the above remarks, we decided to re-examine the amidated (*S*)-ibuprofen with 4-aminobutyric acid (**5b**) as a haptenic derivative. This compound, prepared by the activated ester method via *p*-nitrophenyl ester (**4b**), was covalently coupled with BSA, resulting in the (*S*)-ibuprofen–BSA conjugate as an immunogen. The immunogen was then subcutaneously injected into two rabbits with Fruend's complete adjuvant. We checked the antibody at each stage of the immunization regimen with a competitive ELISA system using an enzyme-labeled hapten, β-galactosidase-labeled (*S*)-ibuprofen 4-aminobutyric acid conjugate, as a probe. The antisera obtained at 3 and 6 months after the initial immunization were shown by ELISA to have optimal dilutions (20,000 for S_1 antiserum and 1000 for S_2 antiserum (v/v)) and feasible dose–response curves of (*S*)-ibuprofen-N^{α}-BOC-lysine in the range of 20 ng–5 μg per assay (Fig. 2). We conducted an inhibition test by the addition of optically active ibuprofen and its amino acid conjugates to compete with an enzyme-labeled antigen for binding to the antibody, and the cross-reactivities were determined by the 50% displacement method, taking the reactivity with (*S*)-ibuprofen-N^{α}-BOC-lysine as 100%. Table 1 shows that both antisera displayed significantly high reactivity to (*S*)-ibuprofen and its amino acid conjugates.

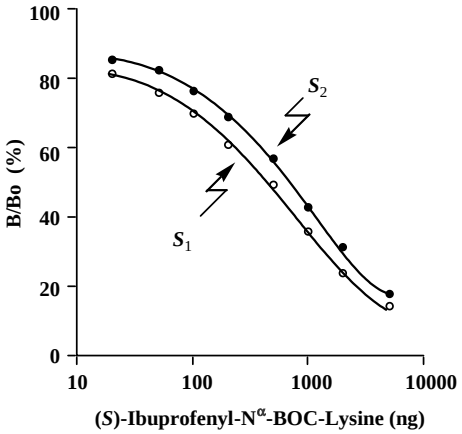

Fig. 2. Typical dose–response curves for (*S*)-ibuprofenyl-N^{α}-BOC-lysine in the competitive ELISA.

Hence, in immunoblotting the developed antisera may be useful for detection of (*S*)-ibuprofen anchored on proteins.

Additional cross-reactivity experiments on (*R*)-antipodes were carried out to gather detailed information about antigenic recognition with respect to the chiral center. These experiments demonstrated that there was—in addition to the specific antibody population for (*S*)-ibuprofen-another antibody population, which is expressed by a cross-reaction of less than 63%. That the cross-reactivity of (*R*)-antipodes is in the high range leads us to suggest that the antigenic recognition of the additional populations contained the isobutylphenyl moiety of (*R*)-ibuprofen. This result is caused by the use of a polyclonal antibody, hence the use of a monoclonal antibody should overcome this problem.

We evaluated the antibody for its utility in the enantioselelctive detection of (*S*)-ibuprofen residues using the (*R*)- and/or (*S*)-ibuprofen–OVA conjugate as model modified proteins. In the experiment, various amounts of OVA placed on the microtiter plate (0.5–5 mg of OVA per well) were incubated with a fixed amount of (*R*)- and/or

Table 1
Percent cross-reaction of anti-(*S*)-ibuprofen antibody as determined by competitive ELISA

Compound	Cross-reactivity (%)[a]	
	S_1[b]	S_2[b]
(*S*)-Ibuprfenyl-N^{α}-BOC-lysine	100	100
(*S*)-Ibuprofen	89	69
(*S*)-Ibuprofenyl glycine	158	96
(*S*)-Ibuprofenyl taurine	121	67
(*S*)-Ibuprofenyl 4-aminobutyric acid	219	135
(*R*)-Ibuprofen	52	40
(*R*)-Ibuprofenyl glycine	63	47
(*R*)-Ibuprofenyl taurine	57	15
Titer	1:20000	1:1000
Midpoint (ng)	430	680

[a] Calculated by the 50% displacement method.
[b] Reference of individual rabbit.

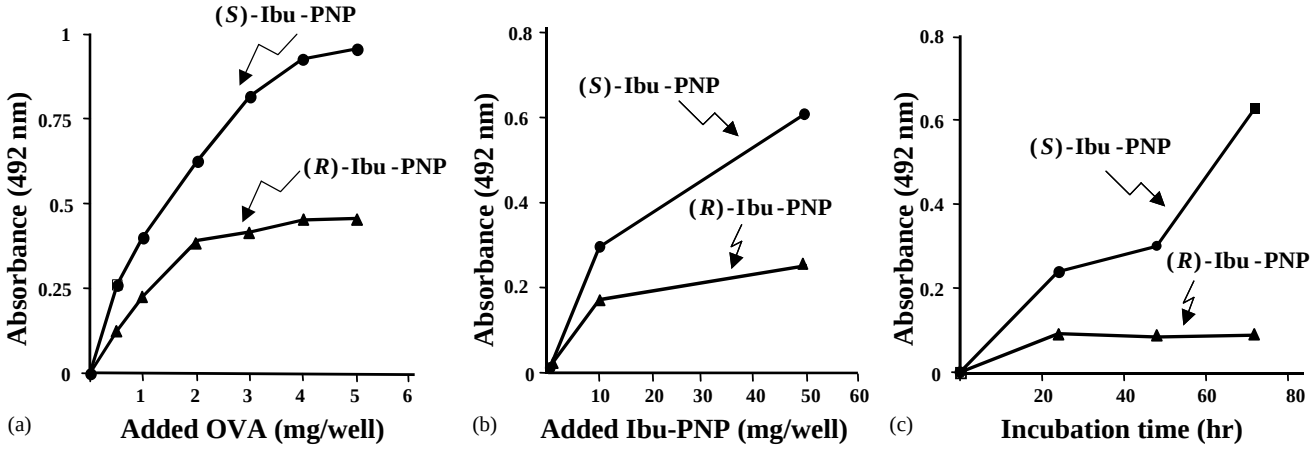

Fig. 3. Chiral discrimination of enantiomeric ibuprofen residue anchored on OVA molecule introduced by the reaction of Ibu–PNP as functions of amount of Ibu–PNP (b), amount of OVA loaded on microwell (a), and incubation time (c).

(*S*)-ibuprofen–*p*-nitrophenyl ester (Ibu–PNP) at 37 °C for 24 h. After removing unreacted materials with washings, the ibuprofen residues anchored on the formed adducts were determined by a sandwich-type ELISA using 1:1000 diluted S_1 antiserum and the HRP-labeled anti-rabbit IgG antibody. As expected, we observed increased signal intensities with increasing amounts of OVA placing the microwells and the difference was significantly in favor of (*S*)-Ibu–PNP (Fig. 3a). The extent of the intensity depended not only on the amount of the added (*S*)-Ibu–PNP, but also the incubation time (Fig. 3b and 3c). From these findings with results showing stereoselective binding against optically active (*S*)-ibuprofen anchored on OVA, it seems likely that the antibody may be useful for enantioselective detection of (*S*)-ibuprofen–protein adducts by immunoblottings.

In an effort to develop immunostaining for enantioselective detection of (*S*)-ibuprofen–protein adducts using antiserum S_l, we incubated GST as a model protein with (*R*)- and (*S*)-Ibu–PNP as model reactive species. A MALDI-TOFMS analysis of the protein modification was carried out by mass spectrometric peptide mapping of the peptide fragment mixtures obtained by proteolytic digestion

Table 2
Calculated and observed peptide fragments of covalent adducts formed from (*R*)- and (*S*)-ibuprofen and glutathione-*S*-transferase

Amino acid residue		Sequence	Ion	*m/z*		Binding site
From	To			Calculated	Observed	
(*R*)-Ibuprofen–GST adduct						
1	9	MSPILGYWK	$[M+H]^+$	1095.36	1090.08	
1	9	SPILGYWK	$[M+H]^+$	1194.45	1196.39	K-9
1	9	SPILGYWK	$[M+K]^+$	1232.54	1234.15	K-9
181	191	KRIEAIPQIDK	$[M+H]^+$	1311.57	1314.01	
120	131	VDFLSKLPEMLK	$[M+H]^+$	1436.77[a]	1433.53[a]	
65	78	LTQSMAIIRYIADK	$[M+H]^+$	1678.06[a]	1680.83[a]	
28	40	YEEHLYERDEGDK	$[M+H]^+$	1683.74	1684.04	
181	197	KRIEAIPQIDKYLKSSK	$[M+H]^+$	2018.42	2014.76	
45	64	KFELGLEFPNLPYYIDGDVK	$[M+H]^+$	2358.71	2359.38	
(*S*)-Ibuprofen–GST adduct						
65	78	LTQSMAIIRYIADK	$[M+H]^+$	1623.96	1624.15	
65	78	LTQSMAIIRYIADK	$[M+H]^+$	1662.06	1664.36	
12	27	GLVQPTRLLLEYLEEK	$[M+H]^+$	1902.25	1900.64	
198	218	YIAWPLQGWQATFGGGDHPPK	$[M+H]^+$	2327.62	2324.78	
45	64	KFELGLEFPNLPYYIDGDVK	$[M+H]^+$	2358.71	2358.78	
176	194	LVCFKKRIEAIPQIDKYLK	$[M+H]^+$	2364.91	2366.70	
88	113	ERAEISMLEGAVLDIRYGVSRIAYSK	$[M+H]^+$	2944.40[a]	2945.87[a]	
88	113	ERAEISMLEGAVLDIRYGVSRIAYSK	$[M+H]^+$	2966.50	2970.87	
88	113	ERAEISMLEGAVLDIRYGVSRIAYSK	$[M+H]^+$	3133.66	3134.83	K-113
12	40	GLVQPTRLLLEYLEEKYEEHLYERDEGD	$[M+H]^+$	3566.97	3564.14	
12	40	GLVQPTRLLLEYLEEKYEEHLYERDEGD	$[M+H]^+$	3605.07	3603.04	
46	87	FELGLEFPNLPYYIDGDVKLTQSMAIIRY	$[M+H]^+$	4869.76	4874.26	

[a] Methionine oxidized ion.

Fig. 4. Immunoblots of GST (1), (*R*)-ibuprofen–GST (2), and (*S*)-ibuprofen–GST (3) adducts. Proteins were separated by SDS-PAGE and immunoblotted with S_1 antiserum.

with endopeptidase Lys-C after reduction with dithiothreitol and alkylation with iodoacetic acid of the reaction mixture. As a result, we confirmed the covalent (*R*)- and (*S*)-ibuprofen–GST adducts bound through lysine-9 and lysine-113 of GST (Table 2). Accordingly, the resulting samples were separated by SDS-PAGE, followed by blotting to a nitrocellulose membrane. The blotted membranes were incubated with the S_1 antiserum and incubated succession with the ENVISION kit, followed by staining with 3,3′-diaminobenzidine. As shown in Fig. 4, the immunoblot results exhibited a strongly stained band in lane 3 loaded with (*S*)-ibuprofen–GST adducts, although no significantly stained bands were observed in lanes 1 and 2 loaded with GST and (*R*)-ibuprofen–GST adducts. This indicates that binding to (*S*)-ibuprofen–GST attributed to enantioselective immunorecognition of the modification of lysine residue on the protein by (*S*)-ibuprofen.

In summary, we successfully raised polyclonal antibodies to detect (*S*)-ibuprofen–protein adducts. The antibodies are able to detect (*S*)-ibuprofen-modified cellular proteins with no cross-reactivity toward native cellular proteins, providing a powerful tool for the investigation of the mechanism of cytotoxicity induced by ibuprofen.

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