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ORIGINAL ARTICLE

Phlorofucofuroeckol-A, a Potent Inhibitor of Aldo-keto Reductase Family 1 Member B10, from the Edible Brown Alga *Eisenia bicyclis*

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Abstract Aldo-keto reductase family 1 member B10 (AKR1B10) belongs to a superfamily of NADPH-dependent aldo-keto reductases and is considered a biomarker of several cancers. Inhibition of recombinant human AKR1B10 (rhAKR1B10) was assayed using 31 seaweed extracts, among which, an *Eisenia bicyclis* extract was selected for further study. To identify the compounds in *E. bicyclis* responsible for inhibitory effects on rhAKR1B10, five compounds were isolated by bioactivity-guided fractionation and isolation. Among them, phlorofucofuroeckol-A (PFF-A), isolated from an ethyl acetate fraction, exhibited the greatest inhibition of rhAKR1B10. The inhibitory rate of PFF-A against rhAKR1B10 was 61.41% at 10 μM, with an IC_{50} of 6.22 μM. Enzyme kinetic analyses revealed non-competitive inhibition with a K_D of 2.76 μM. These results indicate that PFF-A from *E. bicyclis* may be a promising anticancer agent.

Keywords aldo-keto reductase · aldo-keto reductase family 1 member B10 · *Eisenia bicyclis* · phlorofucofuroeckol-A · phlorotannins

Introduction

Aldo-keto reductases (AKRs) are NAD(P)H-dependent oxidoreductases that convert ketone and aldehydes into alcohols. The resulting alcohols can be conjugated with sulfate or glucuronide for excretion (Penning, 2005). The AKR superfamily is composed of more than 140 proteins with different physiological roles (Barski et al., 2008). At present, the proteins of the AKR superfamily can be divided into 15 families according to their amino acid sequences (>40% sequence identity among the members of each family). The human genome project has identified 13 human AKRs in families AKR1, AKR6, and AKR7 (Jin and Penning, 2007). Among these AKRs, eight belong to the AKR1 family including an aldehyde reductase (the AKR1A subfamily), aldose reductases (the AKR1B subfamily), hydroxysteroid/dihydrodiol dehydrogenases (the AKR1C subfamily), and steroid 5β-reductase (the AKR1D subfamily).

The Human AKR1B subfamily is composed of two members, AKR1B1 and AKR1B10 (Ruiz et al., 2011). AKR1B1 is by far the best-studied AKR due to its potential role in mediating hyperglycemic injury and in the development of secondary diabetic complications (Oates, 2008). This enzyme also has broad substrate specificity for carbonyl compounds, which are of physiological significance, and plays an essential role in osmotic homeostasis, steroid and xenobiotic metabolisms, signal processing, and oxidative defense mechanisms (Sato et al., 1993). In comparison, AKR1B10 was first identified in a human hepatocarcinoma (Cao et al., 1998). This enzyme, which is also known as an aldose reductase-like 1 (ARL1) or small intestine aldose reductase (Hyndman and Flynn, 1998), shares 71% sequence identity and a similar tertiary structure with AKR1B1. However, unlike AKR1B1, the specific function of AKR1B10 in the metabolism of endogenous compounds has not been thoroughly elucidated. Nevertheless, a number of studies have indicated a correlation between AKR1B10 and several cancers. It has been suggested that AKR1B10 mediates tumorigenesis through retinoic acid depletion, cancer cell dedifferentiation, and chemoresistance. For

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example, a recent study reported that AKR1B10 does not reduce endogenous glucose, and, relative to AKR1B1, exhibits a low k_{cat} for pyridine-3-aldehyde (Crosas et al., 2003). However, AKR1B10 converts retinals into retinols with much higher catalytic efficiency than AKR1B1, indicating a substrate preference for retinals (Gallego et al., 2006). AKR1B10 also mediates chemoresistance by reducing daunorubicin, a carbonyl group-bearing anticancer drug used in the treatment of lung cancer (Martin et al., 2006). AKR1B10 is not expressed in most human tissues, but has been detected in the adrenal gland, small intestine, liver, and thymus (Cao et al., 1998). AKR1B10 is also overexpressed in colorectal, breast, and lung cancer, for which it is considered as a diagnostic marker (Díez-Dacal et al., 2011). AKR1B10 is highly overexpressed in non-small cell lung carcinoma, indicating a close association with smoking and smoking-related carcinogenesis (Penning, 2005). Therefore, AKR1B10 constitutes a primary target for the development of inhibitors with anticancer potential.

Previously reported inhibitors of AKR1B10 include synthetic aldose reductase inhibitors (tolrestat, epalrestat, and ranirestat), flavonoids, anti-inflammatory agents, plant phenolics, bile acids, and fibrates (Takemura et al., 2011). Among these, tolrestat is the most potent inhibitor of AKR1B10. However, tolrestat failed the Phase III trial in the U.S. due to its toxicity and never received FDA approval. Therefore, there is currently great interest in identifying AKR1B10 inhibitors in natural products. Several triterpenoids derived from plants, including oleanolic acid, are potent inhibitors of AKR1B10 (Takemura et al., 2011). We previously investigated the inhibitory effects of polyphenols from *Rhus verniciflua* (Song et al., 2010), local plant extracts (Pan et al., 2009), and dicaffeoylquinic acids from *Artemisia dubia* (Lee et al., 2010) on AKR1B10. However, in the search for AKR1B10 inhibitors, the most recent attention has been focused on terrestrial plants. Relatively few studies have been directed toward marine plants, despite the tremendous diversity of novel natural compounds, including carotenoids, phenols, and sulfur compounds, that can be isolated from marine plants (Cardozo et al., 2007).

In the current study, we attempted to isolate and identify novel inhibitors of AKR1B10 from marine seaweeds collected from the eastern and southern coasts of Korea using recombinant human AKR1B10 (rhAKR1B10). The activity-guided isolation of potential inhibitors was performed using an extract of *Eisenia bicyclis*, a common perennial brown alga belonging to the Laminariaceae family. In addition, enzymatic kinetic analyses were performed with a potent inhibitor, phlorofucofuroeckol-A (PFF-A), to determine the type of enzymatic inhibition and dissociation constant (K_D) of the inhibitor and AKR1B10 complex.

Materials and Methods

Samples. Thirty-one seaweed species (Table 1) were collected between December 2005 and June 2006 from the eastern and southern coastal waters of Korea. Identification of the seaweeds was performed by Dr. Hyung-Seop Kim (Gangneung-Wonju National University, Gangneung, Korea). Voucher specimens were deposited at the Herbarium of Korea Institute of Science and Technology, Gangneung Institute (Gangneung, Korea). The seaweeds were rinsed with tap water and dried in the shade. The dried samples were cut into small pieces and extracted three times with 95% ethanol. Each extract was concentrated by vacuum evaporation at 40°C and stored at −20°C until use.

Preparation of soluble fractions and isolation of phlorotannins from *E. bicyclis*. The serial solvent fractionation and isolation of bioactive compounds from *E. bicyclis* was performed as described by Kim et al. (2011a) with slight modifications. Briefly, serial solvent fractions (*n*-hexane, methylene chloride, ethyl acetate, *n*-butanol, and water) were obtained from 30 g of crude extract. The ethyl acetate fraction (5 g) was sequentially separated on a silica gel column with a chloroform:methanol:water solvent system, a Sephadex LH-20 column with methanol, and preparative high performance liquid chromatography column with a solvent system consisting of acetonitrile and water containing 0.1% trifluoroacetic acid. Finally, five phlorotannin compounds (eckol; 6,6'-bieckol; 8,8'-bieckol; dieckol; and PFF-A) were purified, and their chemical structures were confirmed by several spectroscopic methods (Kim et al., 2011a).

AKR1B10 inhibition assay. rhAKR1B10 was prepared from *Escherichia coli* BL21 (DE3) harboring the pET23b-AKR1B10 plasmid as described by Song et al. (2010). The rhAKR1B10 inhibition was assessed with a BioTek Power Wave XS spectrophotometer (BioTek Instruments, USA) by measuring the decrease in absorption of NADPH at 340 nm over a 5-min period with DL-glyceraldehyde as the substrate. The reaction mixture (1 mL) consisted of 1 μM rhAKR1B10, 0.1 M sodium phosphate buffer (pH 6.2), and 0.3 mM NADPH with or without 10 mM substrate and inhibitor. All samples were dissolved in DMSO to a concentration of 20 mg/mL for the extracts and fractions and 20 mM for the test compounds. The reaction mixtures were diluted to a final concentration of 10 μg/mL for the extracts and fractions and 10 μM for the test compounds. IC_{50} values (i.e., the concentration of each compound at which 50% inhibition of rhAKR1B10 was observed) were calculated from the least-squares regression line of the logarithmic concentrations plotted against the residual activity. Epalrestat was used as a positive control of AKR1B10 inhibition.

Enzyme kinetic assay of PFF-A. To determine the type of inhibition exerted by PFF-A against rhAKR1B10, a kinetic study was performed using DL-glyceraldehyde at various concentrations as the substrate. The reaction mixture consisted of 0.1 M potassium phosphate (pH 7.0), 0.16 mM NADPH, 2 μM rhAKR1B10, DL-glyceraldehyde at various concentrations, and PFF-A in a total volume of 200 μL. The inhibitory reaction with rhAKR1B10 was initiated by addition of the substrate; the subsequent decrease in NADPH absorption was measured spectrophotometrically at 340 nm. Dixon plots for the inhibition of AKR1B10 by PFF-A were obtained in the presence of DL-glyceraldehyde at concentrations from 0.04 to 0.2 μM. PFF-A was tested at three different

Table 1 Inhibitory effects of ethanol extracts from various seaweeds collected in South Korea on rhAKR1B10

Seaweed species	Family name	Inhibition % (at 10 μg/mL)[a]	IC_{50} (μg/mL)[b]
Green algae			
Codium fragile	Codiaceae	48.28±1.89	-
Ulva armoricana	Ulvaceae	27.83±2.40	-
Enteromorpha intestinalis	Ulvaceae	26.94±0.97	-
Enteromorpha linza	Ulvaceae	29.47±1.57	-
Phyllospadix iwatensis	Zosteraceae	42.51±3.16	-
Brown algae			
Undaria pinnatifida	Alariaceae	26.59±1.72	-
Cutleria cylindrica	Cutleriaceae	37.69±1.76	-
Desmarestia viridis	Desmarestiales	54.51±0.56	13.89
Desmarestia ligulata	Desmarestiales	44.81±2.21	-
Dictyopteris pacifica	Dictyotaceae	58.81±2.71	10.96
Dictyota coriaceum	Dictyotaceae	34.57±2.91	-
Pardina arborescens	Dictyotaceae	22.62±1.57	-
Dictyopteris divaricata	Dictyotaceae	25.23±2.24	-
Ecklonia cava	Laminariaceae	32.94±1.88	-
Laminaria japonica	Laminariaceae	24.83±1.96	-
Costaria costata	Laminariaceae	20.22±2.99	-
Eisenia bicyclis	Laminariaceae	66.19±3.27	8.11
Sargassum thunbergii	Sargassaceae	20.88±2.09	-
Sargassum horneri	Sargassaceae	20.70±3.27	-
Sargassum confusum	Sargassaceae	25.60±3.22	-
Sargassum sagamianum	Sargassaceae	88.53±0.97	4.70
Scytosiphon lomentaria	Scytosiphonaceae	62.44±1.03	5.79
Red algae			
Bonnemaisonia hamifera	Bonnemaisoniaceae	28.78±2.03	-
Delesseria serrulata	Ceramiaceae	20.06±1.82	-
Carpopeltis cornea	Halymeniaceae	52.00±1.36	9.28
Grateloupia filicina	Halymeniaceae	24.08±5.85	-
Gracilaria textorii	Phacelocarpaceae	14.29±1.24	-
Gracilaria verrucosa	Phacelocarpaceae	17.03±4.54	-
Polysiphonia morrowii	Rhodomelaceae	26.09±3.66	-
Laurencia nipponica	Rhodomelaceae	33.09±1.28	-
Symphyocladia latiuscula	Rhodomelaceae	25.33±3.67	-

[a] rhAKR1B10 inhibition (%)=(1-Δabsorbance of the sample for 1 min/Δabsorbance of the control for 1 min)×100, where the control refers to the reaction mixture in the absence of sample.
[b] IC_{50} values were calculated for extracts showing >50% inhibition of rhAKR1B10 activity at 10 μg/mL.

concentrations (3, 5, and 7 μM). As a positive control, epalrestat was used at four different concentrations (0.5, 1, 7, and 10 μM) in the presence DL-glyceraldehyde. The inhibition constant (K_i) was determined by interpreting the Dixon plots, in which the value of the x-axis implies -K_i.

Determination of K_D for PFF-A with rhAKR1B10. The interaction between rhAKR1B10 and PFF-A was tested using a ForteBio Octet Red System (ForteBio Inc., USA). Purified rhAKR1B10 was biotinylated by reacting with EZ-Link NHS-LC-LC-biotin (Pierce Thermo, USA) in a two-fold molar excess for 30 min at 4°C. Biotinylated rhAKR1B10 was coated onto super Streptavidin Biosensor tips (ForteBio Inc.) for 60 min at 4°C in 0.1 M potassium phosphate buffer (pH 7.0). The tips were then washed in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1% DMSO (v/v). The binding experiments were carried out in triplicate with PFF-A at concentrations from 1 to 50 μM (Abdiche et al., 2008). The binding constants were determined by global fits of the binding curves using Octet Red software (Data Analysis v6.2, ForteBio Inc.).

Results and Discussion

Screening for rhAKR1B10 inhibitory activity in seaweed extracts. Ethanol extracts were prepared from 31 seaweeds obtained from the southern coastal areas of Korea and tested for

Table 2 Inhibitory effects of solvent-soluble fractions from an ethanol extract of *E. bicyclis* on rhAKR1B10

Fraction	Concentration (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Ethanol extract	10	66.19±3.27	8.11±0.35
Hexane fraction	10	30.34±2.94	>10
Methylene chloride fraction	10	50.55±3.52	>10
Ethyl acetate fraction	10	77.63±2.94	5.35±0.61
Butanol fraction	10	78.09±2.48	5.73±0.18
Water fraction	10	28.49±4.43	>10

Table 3 Inhibitory effects of phlorotannins isolated from an ethyl acetate fraction of *E. bicyclis* on rhAKR1B10

Compound	Concentration (μM)	Inhibition (%)	IC_{50} (μM)
Epalrestat[a]	10	87.09±0.85	1.14±0.05
Eckol	10	12.64±2.26	>10
Phlorofucofuroeckol-A	10	61.41±1.34	6.22±1.23
Dieckol	10	27.34±6.35	>10
6,6-Dieckol	10	9.86±3.80	>10
8,8-Dieckol	10	48.55±8.78	>10

[a] Eplarestat was used as a positive control at the same concentration.

their inhibitory effects on rhAKR1B10 *in vitro* (Table 1). Among the extracts, six (from *Dictyopteris pacifica*, *Desmarestia viridis*, *Sargassum sagamianum*, *Scytosiphon lomentaria*, *Carpopeltis cornea*, and *E. bicyclis*) exhibited >50% inhibition of rhAKR1B10 activity at a concentration of 10 μg/mL. The IC_{50} values of these extracts are shown in Table 1. *S. sagamianum* was the most potent AKR1B10 inhibitor, with an IC_{50} value of 4.70 μg/mL. The examined species can be grouped into 15 families and 3 classes. All brown algae (class Phaeophyceae) showed potent inhibitory activities (>50%) with the exception of *C. cornea* (red alga, class Rhodophyceae). However, it cannot be said that brown algae is a better source of AKR1B10 inhibitors over the other two seaweed groups after examining only 31 samples. Nevertheless, to the best of our knowledge, this study is the first to screen for potent AKR1B10 inhibitors from marine seaweeds.

AKR1B10 inhibition assay using fractions and compounds from *E. bicyclis*. Relative to the inhibitory effects of *S. sagamianum*, the ethanol extract of *E. bicyclis* demonstrated only moderate inhibitory activity against rhAKR1B10 (IC_{50}=8.11 μg/mL). However, *E. bicyclis* was selected for further study, because it is edible and easy to obtain. Potent AKR1B10 inhibitors were isolated from the *E. bicyclis* extract using an activity-guided procedure. Solvent-soluble fractions were prepared from the ethanol extract, and their inhibitory activities were tested against rhAKR1B10 (Table 2). Only two fractions (ethyl acetate and butanol) exhibited higher activities than the ethanol extract at a concentration of 10 μg/mL. The IC_{50} values of those fractions were 5.35 and 5.73 μg/mL, respectively. The hydrophobic (hexane and methylene chloride) and aqueous fractions showed relatively low inhibitory activities, with IC_{50} values >10 μg/mL. In our previous study, ethyl acetate and butanol fractions were identified as rich sources of phlorotannins, exhibiting strong antioxidant activity (Kim et al., 2011a). Given these results, we hypothesized that phlorotannins are possible target compounds and that radical scavenging may be correlated to AKR1B10 inhibition. Thus, the ethyl acetate fraction in the current study was separated using multiple chromatographic columns to isolate five phlorotannins (Fig. 1). Among them, only PFF-A could be considered a potent inhibitor of AKR1B10 (Table 3). At a concentration of 10 μM, PFF-A inhibited 61.41% of rhAKR1B10 activity. whereas epalrestat, the positive control, showed 87.09% inhibition. The IC_{50} value of PFF-A was relatively high (6.22 μM) compared to that of epalrestat (1.14 μM), indicating that PFF-A is a moderate inhibitor of AKR1B10. AKR1B10 inhibition does not appear to be correlated with radical-scavenging activity, because all isolated phlorotannins exhibited strong radical scavenging activities (Kim et al., 2011b). This is supported by data showing no correlation between the inhibitory activity of local plant extracts on AKR1B10 and radical scavenging activity (Pan et al., 2009). PFF-A contains dibenzo-1,4-dioxin and dibenzofuran elements in its structure (Fukuyama et al., 1990) and is generally found in brown algae (Nagayama et al., 2002; Kang et al., 2003; Sugiura et al., 2007). To date, a variety of biological activities, including antioxidant (Shibata et al., 2007), antibacterial (Nagayama et al., 2002), hyaluronidase inhibitory (Shibata et al., 2002), antiviral (Ahn et al., 2004), and algicidal effects (Nagayama et al., 2003), have been attributed to PFF-A. Among them, hyaluronidase inhibitory activity is known to be involved in anticancer activity (Shibata et al., 2007). In addition, Kim et al. (2011b) reported recently that tumor necrosis factor-α (TNF-α) was significantly reduced by treatment of PFF-A in LPS-stimulated RAW 264.7 cells, suggesting association of PFF-A with anticancer effect. The one possible role of PFF-A in the anticancer effect may be a synergistic activity to anthracyclines such as daunorubicin and idarubicin by reducing drug resistance induced by AKR1B10 expression (Zhong et al., 2011). However, the inhibition of

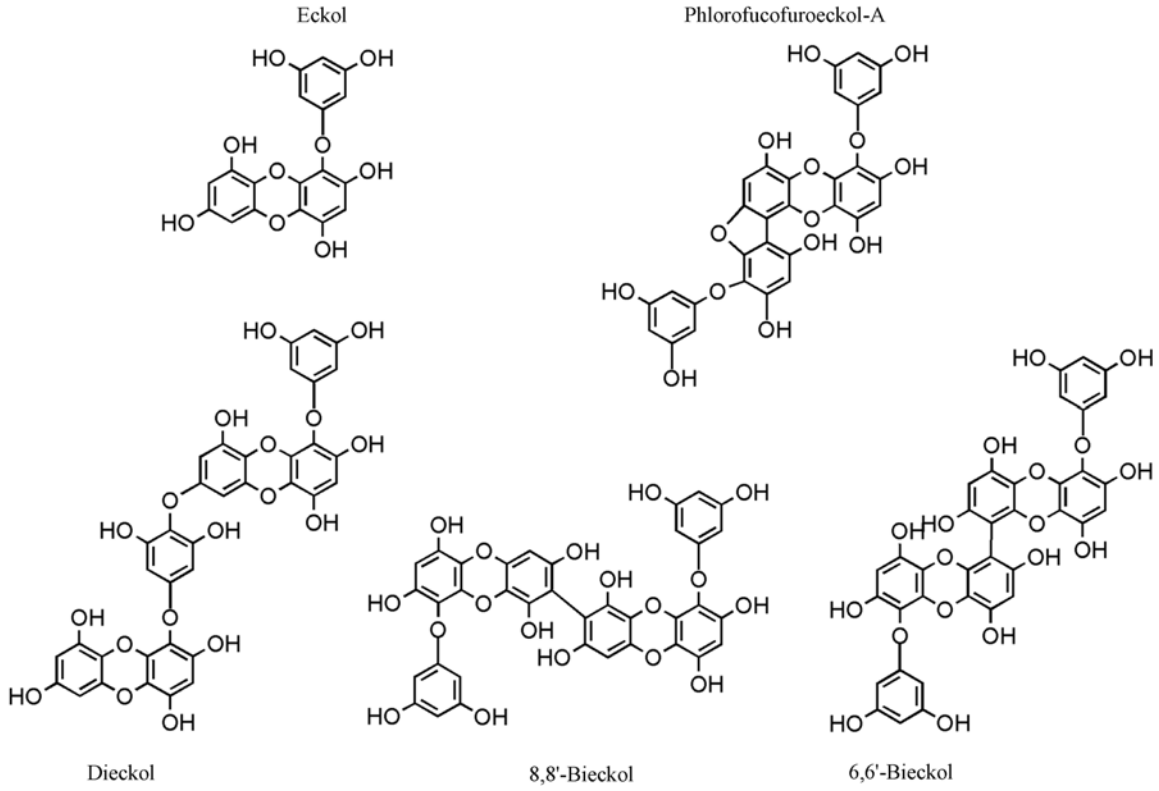

Fig. 1 Chemical structures of the phlorotannins isolated from an ethyl acetate fraction of *E. bicyclis*.

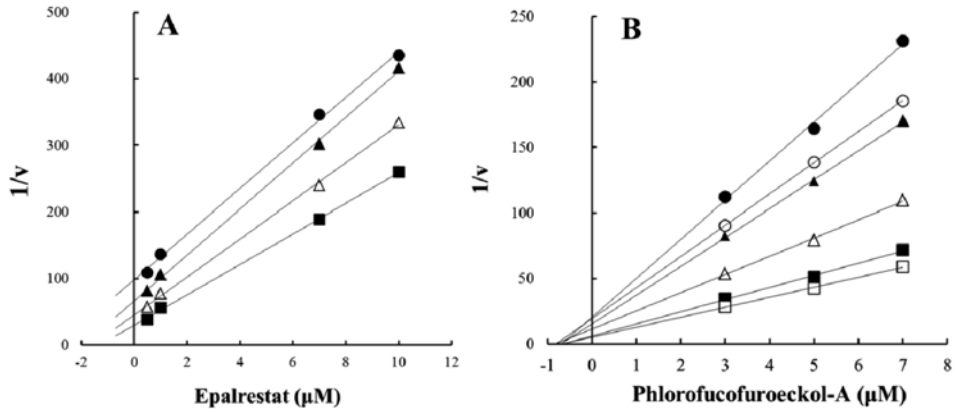

Fig. 2 Dixon plots for the inhibition of rhAKR1B10 by (A) epalrestat and (B) PFF-A. Symbols indicate the DL-glyceraldehyde concentration: (●) 0.2 μM, (▲) 0.13 μM, (△) 0.08 μM, and (■) 0.04 μM for epalrestat and (●) 0.2 μM, (○) 0.16 μM, (▲) 0.10 μM, (△) 0.08 μM, and (■) 0.04 μM for PFF-A.

AKR1B10 or any other enzyme in the AKR superfamily with PFF-A has not been reported.

Mode of inhibition for PFF-A against rhAKR1B10. The kinetics of rhAKR1B10 inhibition were measured in the presence of 3, 5, and 7 μM PFF-A using DL-glyceraldehyde as the substrate at concentrations ranging from 0.04 to 0.2 μM. As shown in Fig. 2(B), PFF-A appeared to act as a non-competitive inhibitor of rhAKR1B10 with respect to DL-glyceraldehyde. This result indicates that PFF-A binds to the enzyme or enzyme-substrate complex at locations other than the catalytic site. In contrast, Dixon plots for epalrestat (Fig. 2(A)) revealed non-competitive inhibition of rhAKR1B10. The K_i values for rhAKR1B10 inhibition were 0.73 μM for PFF-A and 0.58 μM for epalrestat in the presence of DL-glyceraldehyde. Generally, lower K_i values indicate tighter binding between the inhibitor and enzyme with the consequent enhancement of inhibition. Eplarestat and zopolrestat have been reported to have K_i values of 11 μM in the mixed non-competitive mode (Balendiran et al., 2009) and 116 μM in the non-competitive mode

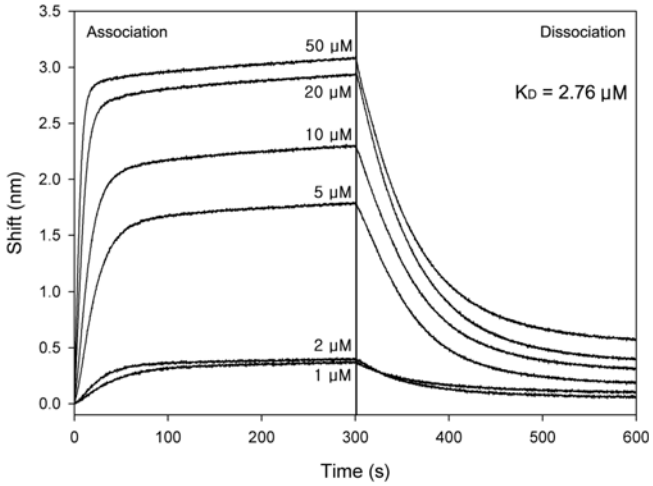

Fig. 3 Binding assay between PFF-A and biotinylated rhAKR1B10 by biolayer interferometry using the ForteBio Octet system. The association and dissociation curves of PFF-A with the enzyme were constructed from 1 to 50 μM PFF-A without substrate.

(Shen et al., 2010), respectively, for AKR1B10 with DL-glyceraldehyde. Other potent inhibitors examined in those reports possessed K_i values ranging from 13 to 250 μM in the mixed non-competitive and non-competitive inhibition modes. The K_i values of chromene-3-carboxamide derivatives against AKR1B10, determined with pyridine-3-aldehyde as the substrate, ranged from 2.7 to 24 nM in the competitive inhibition mode (Endo et al., 2010). Based on these reports, the potency of PFF-A inhibition on rhAKR1B10 is comparable to that of other well-known inhibitors. To confirm the inhibitory activity of PFF-A against rhAKR1B10, the K_D was measured using an Octet platform. Because the K_D is equivalent to the inhibition constant (K_i) in the absence of substrate, K_D can be used to determine the binding affinity between rhAKR1B10 and PFF-A at various concentrations of PFF-A. The Octet platform is a relatively new technology that has been applied using biotinylated rhAKR1B10 and superstreptavidin biosensors as a means of quantitating the affinity between rhAKR1B10 and PFF-A (Abdiche et al., 2008). As shown in Fig. 3, the K_D was 2.76 iM, which is similar to the K_i value determined above.

In summary, ethanol extracts prepared from 31 seaweeds were examined for their inhibitory effects on rhAKR1B10, a potential cancer therapeutic target, among which an extract of *E. bicyclis* exhibited potent inhibitory activity. Activity-guided assay was used to isolate the potent inhibitor PFF-A from an ethyl acetate fraction. This phlorotannin compound inhibited AKR1B10 non-competitively with a relatively low K_i of 0.73 μM and a K_D of 2.76 μM. In addition, any cytotoxic effects of this compound on human lung carcinoma cell line A549 were not found up to 100 μM concentration of PFF-A in MTT assay, indicating no cytotoxic effect of PFF-A (data not shown). Therefore, PFF-A, the active component in the *E. bicyclis* extract, could be developed as a potential therapeutic agent for cancer by inhibiting AKR1B10.

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