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Covalent binding of rofecoxib, but not other COX-2 inhibitors, to allysine aldehyde in elastin of human aorta

Masataka Oitate, Takashi Hirota, Takahiro Murai, Shin-ichi Miura, and Toshihiko Ikeda

Drug Metabolism & Pharmacokinetics Research Laboratories (M.O., T.M., S.M., T.I.),

and Global Project Management Department (T.H.), Daiichi Sankyo Co., Ltd., Tokyo, Japan

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Running Title: Covalent binding of rofecoxib to allysine in human elastin

Corresponding Author:

Masataka Oitate

Drug Metabolism & Pharmacokinetics Research Laboratories

Daiichi Sankyo Co., Ltd.

1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

TEL: (81)3-3492-3131, FAX: (81)3-5436-8567

E-mail: oitate.masataka.i3@daiichisankyo.co.jp

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Abbreviations: COX-2, cyclooxygenase-2; CS-706, (2-(4-ethoxyphenyl)-4-methyl 1-(4-sulfamoylphenyl)-1H-pyrrole); HAOEC, human aortic endothelial cells; CV, cardiovascular; PGI_2, prostacyclin; TxA_2, thromboxane A_2; allysine, α-aminoadipic-δ-semialdehyde; LOX, lysyl oxidase; BAPN, β-aminopropionitrile; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; TCA, trichloroacetic acid; HBSS, Hanks' Balanced Salt Solution.

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Abstract

In rats, it has been reported that rofecoxib, a cyclooxygenase-2 (COX-2) inhibitor, reacts with the aldehyde group of allysine in elastin to give a condensation covalent adduct, thereby preventing the formation of cross-linkages in the elastin and causing degradation of the elastic fibers in aortas *in vivo*. Acid-, organic solvent- and proteolytic enzyme-treatments of human aortic homogenate after incubation with [^{14}C]rofecoxib demonstrated that most of the radioactivity is covalently bound to elastin. The *in vitro* covalent binding was inhibited in the presence of β-aminopropionitrile, D-penicillamine and hydralazine, which suggested that the aldehyde group of allysine in human elastin was relevant to the covalent binding. The *in vitro* covalent binding of [^{14}C]rofecoxib was significantly decreased by the addition of only non-radiolabeled rofecoxib, but not the other COX-2 inhibitors, celecoxib, valdecoxib, etoricoxib and CS-706 (2-(4-ethoxyphenyl)-4-methyl 1-(4-sulfamoylphenyl)-1H-pyrrole), a novel selective COX-2 inhibitor. All the above COX-2 inhibitors except for rofecoxib had no reactivity with the aldehyde group of benzaldehyde used as a model compound of allysine aldehyde under a physiological pH condition. On the other hand, no retention of the radioactivity of [^{14}C]rofecoxib was observed in human aortic endothelial cells (HAOEC) *in vitro*, suggesting that rofecoxib is not retained in aortic endothelial cells *in vivo*. These results suggest that rofecoxib, but not other COX-2 inhibitors, is capable of covalently binding to the aldehyde group of allysine in human elastin. This might be one of the

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main causes of cardiovascular events by rofecoxib in clinical situations.

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Introduction

In 2004, rofecoxib (VIOXX), a potent and highly selective cyclooxygenase-2 (COX-2) inhibitor, was withdrawn from the worldwide market based on the results of some clinical studies indicating its association with increased risk of cardiovascular (CV) events, such as heart attack and stroke (Merck, 2004). In the last few years, it has been reported that other selective COX-2 inhibitors, e.g. etoricoxib, valdecoxib (and its prodrug, parecoxib), and even non-selective non-steroidal anti-inflammatory drugs, e.g. naproxen, may also have a potential for increased CV risk (Aldington et al., 2005; Nussmeier et al., 2005; NIH, 2004). However, rofecoxib has a distinctly higher potential for CV risk, in comparison with these drugs (Graham et al., 2005; Fredy et al., 2005). Although some hypotheses regarding the mechanism have been proposed so far, such as prostacyclin (PGI_2)/thromboxane A_2 (TxA_2) imbalance in arteries (McAdam et al., 1999), most of these hypotheses have not been verified yet.

In our previous study using rat aortas (Oitate et al., 2007), it was suggested that rofecoxib itself reacts with the aldehyde group of allysine (α-aminoadipic-δ-semialdehyde) in elastin to give a condensation covalent adduct (Figure 1B). This was also strongly supported by an *in vitro* reaction using benzaldehyde, a model compound of allysine, in which rofecoxib reacted with the aldehyde group of benzaldehyde in a kind of condensation reaction under a physiological pH condition. Similar to collagen, elastin is a key extracellular matrix protein which provides CV tissues, e.g.

arteries and heart valves, with tensile strength and elasticity (Vrhovski and Weiss, 1998). The elastic property is due to the existence of covalent cross-linkages, such as desmosine and isodesmosine, and allysine is the most important precursor of physiologically essential cross-linkage formation in elastin and collagen, and is formed from the lysine residue by lysyl oxidase (LOX) (Figure 1A). It has been reported that the prevention of these cross-linkages in elastin or collagen causes serious lesions in the connective tissues in animals and humans (Yoshikawa et al., 2001; Junker et al., 1982; Light et al., 1986; Hashimoto et al., 1981; Herd and Orbison, 1966; Andrews et al., 1975). In fact, multiple oral administration of rofecoxib to rats caused a marked degradation of the elastic fibers in the aorta *in vivo*, conceivably by covalently binding to allysine aldehyde and by the inhibition of the normal cross-linking process of elastin (Oitate et al., 2007).

In the present study, to investigate whether the above observations seen in rats also occur in clinical situations, that is, whether rofecoxib can bind with allysine aldehyde in human elastin, we conducted *in vitro* binding studies using a human aorta sample. In addition, the reactivity of other COX-2 inhibitors, celecoxib (CELEBREX), valdecoxib (BEXTRA), etoricoxib (ARCOXIA), and CS-706 (2-(4-ethoxyphenyl)-4-methyl 1-(4-sulfamoylphenyl)-1H-pyrrole), a novel COX-2 inhibitor, with allysine aldehyde was estimated using benzaldehyde, and their inhibitory potencies in the cross-linking process of human elastin were evaluated.

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Materials and Methods

Chemicals and Reagents

[^{14}C]Rofecoxib (17 mCi/mmol) and [^{14}C]celecoxib (13 mCi/mmol) were synthesized at GE Healthcare Bio-Sciences (Little Chalfont, Buckinghamshire, UK, radiochemical purities; >98%), and non-radiolabeled rofecoxib, celecoxib, valdecoxib, etoricoxib and CS-706 were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan) (Figure 2). β-Aminopropionitrile (BAPN), D-penicillamine, hydralazine, and benzaldehyde were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other reagents and solvents used were commercially available and were of extra pure, guaranteed or high performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS) grade.

Animals and Sample Collection

Male Sprague-Dawley rats (6 weeks of age) were obtained from Charles River Japan, Inc. (Yokohama, Japan), and were used after one week of acclimatization. The rats were housed in a temperature-controlled room with a 12-h light/dark cycle, and were allowed free access to water and a diet (FR-2, Funabashi Farm Co., Ltd., Chiba, Japan) throughout the study. The thoracic aortas were collected from the rats weighing 210 – 230 g after being euthanized by exsanguination under

diethyl ether anesthesia (N = 10).

Procurement of human aorta sample

A human abdominal aorta (Caucasian, female, 69 years of old) was obtained from the Human Animal Bridging Research Organization (HAB) in Chiba, Japan. The sample was legally procured from the National Disease Researcher Interchange (NDRI) in Philadelphia, USA, with permission to use the aorta for research purposes only, based on the international partnership between the NDRI and HAB.

Cell culture

Human aortic endothelial cells (HAOEC) were obtained from Cell Applications, Inc. (San Diego, CA, USA). The cells were cultured in Endothelial Cell Medium (HAOEC Total Kit, Cell Applications, Inc.) according to the supplier's instructions. For the uptake and efflux experiments, HAOEC were seeded at 9.0×10^4 cells/cm^2 in 24-well dishes (Type I collagen coated plate, Asahi Techno Glass, Tokyo, Japan), and used at 90% confluence.

Acid-, organic solvent- and proteolytic enzyme-treatments of human aorta

After removal of the adhering tissues, the human aortic sample was weighed, and

homogenized (5% w/v) in isotonic saline using a motor-driven homogenizer. The homogenate in final volume of 10 mL was incubated with [^{14}C]rofecoxib (100 μM) at 37°C for 2 h (N = 1). After the incubation, to the homogenates 30 mL of 0.9 M trichloroacetic acid (TCA) was added to precipitate the proteins. Then, the mixture was centrifuged at 2,000 g at room temperature for 10 min. The supernatant was discarded, and the precipitate was washed by resuspension and centrifugation successively with 0.6 M TCA, 80% (v/v) methanol and 100% methanol, until only background radioactivity was measured in each wash. According to a previous report (Oitate et al., 2006), the resulting precipitate was enzymatically treated successively with collagenase (25,000 units, from *Clostridium histolyticum*, Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.1 M Tris-HCl buffer (pH 8.0) at 37°C for 16 h, elastase (4,000 units, from porcine pancreas, Wako Pure Chemical Industries, Ltd.) in 0.1 M Tris-HCl buffer (pH 8.5) at 37°C for 16 h, and pronase (10,000 units, from *Streptomyces griseus*, Merck, Darmstadt, Germany) in 0.1 M sodium phosphate buffer (pH 7.5) at 37°C for 16 h and then the radioactivity recovered in each proteolytic fraction was measured.

***In vitro* covalent binding of [^{14}C]rofecoxib to human aorta homogenate and effect of protein modifiers**

Homogenate samples of the human aorta were prepared as described above. According

to a previous report (Oitate et al., 2007), the homogenates were each pretreated with 10 mM of protein modifiers, BAPN, D-penicillamine or hydralazine in potassium phosphate buffer (100 mM, pH 7.4) at 37°C for 0.5 h, and then incubated with [^{14}C]rofecoxib (100 μM) at 37°C for 2 h (N = 3). As a control, the homogenate was pretreated with buffer alone. For evaluating the non-specific binding, the same treatments were performed at 4°C. After the incubation, threefold volume of 0.9 M TCA was added to the incubation mixture to precipitate the proteins. Then, the mixture was centrifuged at 4°C for 10 min. The supernatant was removed for radiodetection-HPLC analysis, and the precipitate was washed by resuspension and centrifugation successively with 0.6 M TCA, 80% (v/v) methanol and 100% methanol, until only background radioactivity was measured in the centrifuged supernatants of each washing step. The resulting precipitates were air-dried and subjected to radioactivity measurement. The net amount of covalent-binding was calculated by subtracting the non-specific binding from the total. The LOX activity in the samples was measured as previously reported (Oitate et al., 2007).

***In vitro* covalent binding of [^{14}C]rofecoxib to rat and human aorta homogenates and effect of several COX-2 inhibitors**

After removal of the adhering tissues, the rat and human aortic samples were weighed, and homogenized (5% w/v) in isotonic saline using a motor-driven homogenizer. The homogenates

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were incubated with [^{14}C]rofecoxib (50 μM) in the presence or absence of 0.5 mM of non-radiolabeled COX-2 inhibitors, rofecoxib, celecoxib, valdecoxib, etoricoxib or CS-706 at 37°C for 2 h (N = 3). In a separate study, the homogenate was incubated with 25 μM [^{14}C]rofecoxib (only for humans), 100 μM [^{14}C]rofecoxib or 50 μM [^{14}C]celecoxib at 37°C for 2 h (N = 3). For evaluating the non-specific binding, the same treatments were performed at 4°C for 2 h (N = 3). After the incubation, the samples were washed and treated as described above, and the amount of net covalent binding was calculated. The LOX activity in the samples was also measured as described above.

Condensation reaction of COX-2 inhibitors with benzaldehyde as a model reaction

The experiment to examine the reaction of COX-2 inhibitors with benzaldehyde was performed according to a previous report (Oitate et al., 2007). Briefly, 100 μM of each compound (control; buffer only) was incubated with 1 mM of benzaldehyde in potassium phosphate buffer (pH 7.4) at 37°C up to 24 h. The reaction mixtures were frozen to stop the reaction, and stored at -80°C until analysis using LC/MS.

Uptake and efflux experiments with HAOEC

Immediately before the uptake experiment, the culture medium was removed and the cells

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were washed three times with 1 mL of Hanks' Balanced Salt Solution (HBSS, pH 7.4, 37°C, Invitrogen Corp., Carlsbad, USA). Uptake was initiated by adding 1 mL of Endothelial Cell Medium containing 10 µM of [^{14}C]rofecoxib or [^{14}C]celecoxib (final concentration of DMSO: 0.1%), and the cells were incubated at 37°C up to 360 min. After the incubation, the cells were washed three times with 1 mL of ice-cold HBSS to stop the uptake and to remove any extracellular [^{14}C]compounds. For the quantification of the compounds taken up, the cells were dissolved in 0.5 mL of HBSS containing Triton X (final 0.1%) by sonication.

For the efflux experiment, each [^{14}C]compound (10 µM) was incubated with the cells for 120 min at 37°C and then the cells were washed in the same manner as described above. After that, the cells were incubated at 37°C with 1 mL of Endothelial Cell Medium containing 20 µM of each non-radiolabeled compound to start the efflux. At the designated time, the cells were washed and dissolved as described above.

Both the uptake and efflux experiments were done in triplicate. The cellular protein content was measured using a *DC* Protein Assay Kit (Bio-Rad Laboratories, Inc., California, USA) in duplicate.

Radiodetection-HPLC analyses

After the incubation of [^{14}C]rofecoxib with aortic homogenates, the supernatants were

analyzed by radiodetection-HPLC as previously reported (Oitate et al., 2007). The chromatographic system included a LC-10A system (Shimadzu Corp., Kyoto, Japan) equipped with an Xterra MS C18 column (4.6 × 150 mm, 5 μm, Waters Corp., MA, USA) heated to 40°C. The mobile phase, consisting of distilled water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), was delivered at a flow rate of 1 mL/min, starting at 20% solvent B in solvent A and increasing the proportion of solvent B to 60% linearly for 20 min, and holding for 10 min at 60% solvent B. The effluent was monitored using a SPD-10A UV detector (Shimadzu Corp., 280 nm), and a Radiomatic 525TR radiochemical detector (PerkinElmer Life and Analytical Sciences, Boston, USA) with a 3 mL/min flow rate for the scintillation cocktail ULTIMA-FLO (PerkinElmer Life and Analytical Sciences).

LC/MS analyses

LC/MS was performed on a Waters Q-Tof Ultima mass spectrometer (Waters Corp.) operated in the positive and negative ion ESI modes for the analysis of the incubation mixture of each COX-2 inhibitor with benzaldehyde. The LC system used was a Waters Alliance 2695 separation module coupled with a 2996 photodiode array detector, scanned from 200 to 340 nm for 1 second. Chromatographic separations were carried out on an XTerra MS C18 column (2.1 × 150 mm, 5 μm, Waters Corp.) maintained at 30°C in a column oven. Solvent A (distilled water

containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) were delivered at a flow rate of 0.2 mL/min with the following linear gradient programs of solvent B (30 min): 30% to 90% for celecoxib and CS-706, 5% to 45% for etoricoxib, 20% to 80% for valdecoxib, 10% to 70% for rofecoxib.

Radioactivity measurements

Samples were solubilized with a tissue solubilizer NCS-II (GE Healthcare Bio-Sciences, 1-2 mL) under constant shaking at ca. 55°C. After solubilization, these samples were mixed with 10 mL of scintillator HIONIC-FLUOR (PerkinElmer Life and Analytical Sciences) and were subjected to radioactivity measurement by liquid scintillation counter, model 2300TR (PerkinElmer Life and Analytical Sciences). The radioactivity value of each proteolytic fraction of the aorta was converted to a percentage of the total covalent-binding radioactivity, which is not extractable by washing with TCA and methanol. The radioactivity in the aorta was calculated as an equivalent (Eq) value of [^{14}C]compound, and expressed as a concentration per g aorta. For the cell experiments, the intracellular concentrations were expressed as the amount per mg protein of cells (nmol/mg protein).

Data analyses

Statistical analyses were performed by analysis of variance (ANOVA) followed by Dunnett's test using SAS System Release ver. 9.1 (SAS Institute Inc., NC, USA). Differences were considered to be significant when $p < 0.05$.

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Results

Acid-, organic solvent- and proteolytic enzyme-treatments of human aorta

After incubation with [^{14}C]rofecoxib, the human abdominal aortic homogenate was treated by successive extraction and washing with 0.6 M TCA, 80% methanol and 100% methanol and then the resultant precipitated fraction was enzymatically treated successively with collagenase, elastase and pronase (N = 1). As shown in Table 1, the largest amount of the radioactivity from [^{14}C]rofecoxib was recovered in the elastolytic fraction (51.9% of the total covalent binding). The second largest amount of radioactivity was recovered in the collagenolytic fraction (40.6% of the total). The radioactivity recovered in the fraction after pronase treatment was 4.4%, and the radioactivity in the residue after the treatments with the proteolytic enzymes was 3.0%.

Effect of protein modifiers on *in vitro* covalent binding of [^{14}C]rofecoxib to human aorta homogenate

Homogenate of human aorta, which had been pretreated with 10 mM of protein modifiers was incubated with [^{14}C]rofecoxib (100 μM) under a physiological pH condition (pH 7.4), and the amount of covalent binding to the proteins was measured (Figure 3). The radioactivity from [^{14}C]rofecoxib bound covalently to the aorta without any protein modifiers was 1.10 ± 0.06 μg Eq/g

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(control). Pretreatment of the aortic homogenate with the protein modifiers BAPN, D-penicillamine or hydralazine, decreased the amount of covalent binding to 56%, 52% and 31% of the control, respectively. Radiodetection-HPLC analysis of the reaction mixture supernatant after the incubation demonstrated that the major component is the unchanged rofecoxib (> 95%, data not shown). In a separate experiment, BAPN and hydralazine significantly decreased the LOX activity to 4.1% and 0% of the control, respectively, whereas rofecoxib did not decrease it at all. The LOX activity in the presence of D-penicillamine could not be measured because the fluorescence in the assay mixture was too strong, perhaps due to the production of some unknown compound(s) with fluorescence.

Effect of several COX-2 inhibitors on i*n vitro* covalent binding of [^{14}C]rofecoxib to rat and human aorta homogenates

Homogenate of rat aorta was incubated with [^{14}C]rofecoxib (50 μM) under a physiological pH condition (pH 7.4) in the presence or absence of non-radiolabeled COX-2 inhibitors (0.5 mM), and the amount of the radioactivity that bound covalently to the proteins was measured (Table 2). The covalently bound aortic radioactivity from [^{14}C]rofecoxib in the control was 1.66 ± 0.07 μg Eq/g. The amount was significantly decreased in the presence only of non-radiolabeled rofecoxib to 53% of the control, but not of the other COX-2 inhibitors at all. On the other hand, the binding amount

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of 50 μM [^{14}C]celecoxib (0.19 ± 0.17 μg Eq/g) was significantly lower than that of [^{14}C]rofecoxib (control).

Human aortic homogenate was treated in the same way as described above, and the effect of non-labeled COX-2 inhibitors (0.5 mM) on the covalent binding of [^{14}C]rofecoxib (50 μM) was investigated (Table 2). In the control, the covalent binding of [^{14}C]rofecoxib was 0.46 ± 0.02 μg Eq/g. The addition of non-radiolabeled rofecoxib to the aortic homogenate significantly decreased it to 22% of the control, while the other COX-2 inhibitors had no such effect at all. The binding of 50 μM [^{14}C]celecoxib (0.09 ± 0.09 μg Eq/g) was significantly lower than that of [^{14}C]rofecoxib (control). The binding of 25 μM and 100 μM [^{14}C]rofecoxib were 0.24 ± 0.01 and 0.88 ± 0.05 μg Eq/g, respectively.

Radiodetection-HPLC analysis of the reaction mixtures of rat and human samples demonstrated that the major component in the reaction mixtures was unchanged rofecoxib (>95%, data not shown). All the COX-2 inhibitors tested had no significant inhibitory effect on the LOX activity in rat and human aortas.

Condensation reaction of COX-2 inhibitors with benzaldehyde as a model reaction

COX-2 inhibitors (100 μM) were incubated with benzaldehyde (1 mM) in phosphate buffer under a physiological pH condition (pH 7.4) and the reaction mixtures were analyzed by

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LC/MS (Figure 4). In the case of rofecoxib, two peaks P_A and P_B, which had been identified by LC/MS and LC-NMR analyses as the covalent adducts of rofecoxib with benzaldehyde and diastereomers, were detected, as previously reported (Oitate et al., 2007). On the other hand, in the cases of other COX-2 inhibitors, no reaction products which were considered to be adducts with benzaldehyde were detected at all in either the positive and negative ion analytical modes. No dehydrated form of each estimated adduct was observed in any of the compounds tested.

Uptake and efflux of radioactivity with HAOEC

In the incubation of 10 μM [^{14}C]rofecoxib or [^{14}C]celecoxib with HAOEC, the uptake of radioactivity by the cells was observed (Figure 5). The radioactive concentration of [^{14}C]celecoxib at 120 min was about 3-fold higher than that of [^{14}C]rofecoxib (1.97 vs. 0.64 nmol/mg protein). After an uptake of 120 min, the efflux of both compounds was very rapid, independent of their initial intracellular concentrations. In each case, the intracellular concentration was hardly detected at only 15 min after the start of efflux.

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Discussion

After incubation with [^{14}C]rofecoxib, human abdominal aortic homogenate was washed and extracted using TCA/methanol and the residue was successively enzymatically treated with collagenase, elastase and pronase and then the radioactivity recovered in each fraction was measured. As shown in Table 1, the largest amount of the radioactivity from [^{14}C]rofecoxib that was covalently bound to the aorta was recovered in the elastolytic fraction, suggesting that rofecoxib is covalently bound to elastin in the human aorta. This result was consistent with that obtained in rat aorta (Oitate et al., 2006).

To investigate the reactivity of rofecoxib with the allysine aldehyde in human elastin compared to that in rat elastin (Oitate et al., 2007), *in vitro* binding studies using human aortic homogenate were conducted in the presence of some protein modifiers (Figure 3). Pretreatment of BAPN, a specific inhibitor of LOX (Tang et al., 1983), significantly decreased the covalent binding of [^{14}C]rofecoxib, probably via the prevention of allysine production from lysine. The covalent binding of [^{14}C]rofecoxib was also significantly decreased by pretreatment with D-penicillamine and hydralazine, which have been reported to react with protein aldehydes in connective tissues and to form thiazolidine or hydrazone analogue (hydralazine can also inhibit LOX activity) (Deshmukh and Nimni, 1969; Pinnell et al., 1968; Howard-Lock et al., 1986; Gallop and Paz, 1975; Numata et al., 1981). These results strongly suggested that the aldehydic functional group in human elastin, i.e.

the aldehyde group of allysine, is relevant to the covalent binding with rofecoxib, as it is in rat elastin (Oitate et al., 2007). In addition, the covalent binding of [^{14}C]rofecoxib to human aorta was concentration-proportional in the range between 25 to 100 μM (Table 2), suggesting that the binding capacity is fairly high.

In the enzymatic fractionation of the human aorta (Table 1), the rank order of recovery of radioactivity was similar to that in rat aortas after oral administration of [^{14}C]rofecoxib *in vivo* (Oitate et al., 2006). However, it differed in one way: in the rat aorta almost all of the radioactivity (>90%) was recovered in the elastolytic fraction with a small percentage in the collagenolytic fraction, whereas in the human aorta about 41% of the total binding radioactivity was recovered in the collagenolytic fraction as well as in the elastolytic fraction. This difference might come from dissimilarities in the parts of the aortas used (human: abdominal, rat: thoracic). It has been reported that the content of elastin in the aortic wall decreases progressively, while in contrast, the content of collagen increases, as the aorta is traversed longitudinally from the arch to abdominal (Grant RA, 1967). In collagen, allysine is also formed from the lysine residue by LOX and contributes importantly to the elasticity of CV tissues, as well as elastin (Eyre DR et al., 1984). Pinnell and Martin (1968) have reported that, in the case of elastin, 5-16 lysyl residues per 10^3 amino acids are converted to allysine residues, compared with one lysyl residue per 10^3 amino acids in collagen. From the above results in the human abdominal aorta, it was suggested that rofecoxib is capable of

covalently binding to the allysine aldehyde in elastin and partially binding to that in collagen.

After the incubation of [^{14}C]rofecoxib with rat aortic homogenate *in vitro* in the presence or absence of non-radiolabeled rofecoxib, celecoxib, valdecoxib, etoricoxib and CS-706, the amount of covalent binding was measured (Table 2). The binding of [^{14}C]celecoxib was significantly lower than that of [^{14}C]rofecoxib, supporting our previous result, that is, there was no retention of radioactivity in the aorta after oral administration of [^{14}C]celecoxib to rats *in vivo*, whereas the radioactivity from [^{14}C]rofecoxib was retained by and accumulated in the rat aorta (Oitate et al., 2006). The covalent binding of [^{14}C]rofecoxib to rat aortic homogenate *in vitro* was significantly decreased by the addition of only rofecoxib, while the other COX-2 inhibitors had no such effect. As well as the above results in rats, only the addition of non-radiolabeled rofecoxib to human aortic homogenate could significantly reduce the covalent binding of [^{14}C]rofecoxib, and the binding of [^{14}C]celecoxib was much less than that of [^{14}C]rofecoxib (Table 2). In the cases of both rats and humans, rofecoxib did not affect the LOX activity at all. Therefore, the decrease of [^{14}C]rofecoxib binding by non-radiolabeled rofecoxib was thought to be due to a competitive inhibition of allysine aldehyde. This suggested that other COX-2 inhibitors except for rofecoxib have no reactivity with the allysine aldehyde in human elastin the same as in rats, and collagen.

To estimate the reactivity of COX-2 inhibitors with the allysine aldehyde in human elastin and collagen with certainty, they were incubated with benzaldehyde, a model compound of allysine,

under a physiological pH condition, as previously reported (Oitate et al., 2007). In the case of rofecoxib (as a positive control), the two peaks P_A and P_B were detected as reported previously (Figure 4). By LC/MS and LC-NMR analyses, they had been identified as the covalent adducts of rofecoxib with benzaldehyde and diastereomers (Oitate et al., 2007). On the other hand, in the cases of other COX-2 inhibitors, no reaction products which were considered to be adducts with benzaldehyde were detected at all by LC/MS analyses operated in both the positive and negative ion modes. The C5 position in rofecoxib was considered to be sufficiently nucleophilic to react with aldehyde (Oitate et al., 2007). The other COX-2 inhibitors tested structurally do not contain a carbon atom carrying hydrogen α or allylic to the carbonyl group (Figure 2), and therefore it is reasonable to conclude that these compounds did not react with benzaldehyde.

In clinical situations, the C_{max} of rofecoxib at a general therapeutic dose (25 mg) was reported to be ~ 1 μM (Davies NM et al., 2003). On the other hand, the *in vitro* binding of [^{14}C]rofecoxib to the human aorta was linear, at least in the concentration range of 25 – 100 μM (Table 2), and the binding capacity was high, as mentioned above. Considering that rofecoxib binds to allysine in a covalent manner, rofecoxib has often been chronically dosed in clinical situations, and since both elastin and collagen have very slow turnover rates (Petersen et al., 2002; Maroudas A et al., 1992), rofecoxib would be increasingly accumulated in the human aorta depending on the dose-frequency. From the above results, it is presumed that in clinical situations

rofecoxib, but not other COX-2 inhibitors, might prevent the cross-linking process of elastin and collagen, leading to the degradation of elastin and collagen, the dysfunction of arteries, and finally CV events.

It has been generally accepted that CV events caused by selective COX-2 inhibitors might be partially due to an imbalance of the concentration ratio of two prostanoids with major CV actions: PGI_2, a vasodilator and inhibitor of platelet aggregation, and TxA_2, a vasoconstrictor and promoter of platelet aggregation. That is, selective COX-2 inhibitors diminish the production of PGI_2 in the endothelium, but not TxA_2 in the platelets, so that the relative concentration of TxA_2 increases around the affected area, which might increase the CV risks (McAdam et al., 1999). Both [^{14}C]rofecoxib and [^{14}C]celecoxib were taken up by HAOEC, and the intracellular concentration of [^{14}C]celecoxib at 120 min was about 3-fold higher than that of [^{14}C]rofecoxib (Figure 5). On the other hand, the efflux of both compounds was very rapid without any retention of radioactivity in the cells, suggesting that both compounds might not be retained by and accumulated in human aortic endothelial cells *in vivo*. When the concentrations in the plasma are high immediately after the administrations, it is thought that the concentrations in the endothelial cells and platelets are also high, and that all selective COX-2 inhibitors, including rofecoxib and celecoxib, would theoretically cause a PGI_2/T_XA_2 imbalance. However, it is quite difficult to postulate that rofecoxib has an especially strong effect in causing an imbalance, compared with other compounds.

In conclusion, it was suggested that rofecoxib, but not the other COX-2 inhibitors, can covalently bind to the allysine aldehyde in human elastin as well as that in rats (Figure 1B) and that it can bind partially in human collagen. This would lead to the prevention of the normal-cross linking processes and dysfunction of the arteries. This might be one of the main causes of CV events by rofecoxib in clinical situations. In this view, other COX-2 inhibitors could be less toxic in terms of CV risks than rofecoxib.

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Footnotes

Reprint request to:

Masataka Oitate

Drug Metabolism & Pharmacokinetics Research Laboratories

Daiichi Sankyo Co., Ltd.

1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

TEL: (81)3-3492-3131, FAX: (81)3-5436-8567

E-mail: oitate.masataka.i3@daiichisankyo.co.jp

Legends for figures

Figure 1. Schematic diagram illustrating the structure and route of the formation of elastin cross-linkages under normal conditions (A) and a proposed reaction mechanism for the covalent binding of rofecoxib with elastin (B).

Figure 2. Chemical structures of rofecoxib, celecoxib, valdecoxib, etoricoxib and CS-706. Asterisks indicate the positions of the radiolabels.

Figure 3. Effect of protein modifiers on the covalent binding of [^{14}C]rofecoxib to human aorta homogenate (Mean ± SD, N = 3). [^{14}C]Rofecoxib (100 μM) was incubated with human aortic homogenate (5% w/v) with or without the pretreatment of each of the protein modifiers (10 mM) and the amount of covalent binding was measured as described in *Materials and Methods*. ***, $p <$ 0.001, significantly different from the control by ANOVA followed by Dunnett's test.

Figure 4. Reconstructed ion chromatograms of *m/z* 488 (A), 421 (B), 465 (C), 463 (D) and 421 (E), corresponding to the estimated protonated molecules of the adducts formed from each COX-2 inhibitor with benzaldehyde. Each COX-2 inhibitor (100 μM) was incubated with benzaldehyde (1

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mM) in potassium phosphate buffer (pH 7.4) at 37°C for 24 h. Positive ion mode results are representatively shown. Nominal mass: celecoxib (381), valdecoxib (314), etoricoxib (358), CS-706 (356), rofecoxib (314), benzaldehyde (106). Peaks shown as asterisks were confirmed not to be peaks derived from the adducts with benzaldehyde.

Figure 5. Uptake and efflux of [^{14}C]rofecoxib (circle) and [^{14}C]celecoxib (triangle) in HAOEC (N = 3, Mean ± SD). For the uptake experiment (closed symbols), each compound (10 μM) was incubated with HAOEC at 37°C up to 360 min. For the efflux experiment (open symbols), each compound (10 μM) was incubated with HAOEC for 120 min and then the cells were washed and incubated at 37°C with 20 μM of each non-labeled compound.

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Table 1. Enzymatic fractionation of covalently bound radioactivity in human aortic homogenate (5% w/v) after incubation with 100 μM of [^{14}C]rofecoxib (N = 1). Covalently bound radioactivity in the aorta was fractionated by treatment with collagenase, elastase and pronase in series as described in *Materials and Methods*. In each step after the enzyme treatment, the removable radioactivity from the tissue was measured.

	Fraction			
	Collagenase	Elastase	Pronase	Residue
Radioactivity recovery (% of total covalent binding)	40.6	51.9	4.4	3.0

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Table 2. Effect of several COX-2 inhibitors on the covalent binding of [^{14}C]rofecoxib to aorta homogenate of rat and human (Mean ± SD, N = 3). [^{14}C]Rofecoxib (50 μM) was incubated with aortic homogenate (5% w/v) in the presence or absence of each of the COX-2 inhibitors (0.5 mM) at 37°C for 2 h. In a separate study, the homogenate was incubated with 25 μM [^{14}C]rofecoxib (only for humans), 100 μM [^{14}C]rofecoxib or 50 μM [^{14}C]celecoxib at 37°C for 2 h (N = 3). The amount of covalent binding was measured as described in *Materials and Methods*. **, $p < 0.01$; ***, $p < 0.001$, significantly different from the control by ANOVA followed by Dunnett's test. ND, no data.

Substrate	Inhibitor	Covalent binding (μg Eq/g aorta)	
		Rat	Human
[^{14}C]Rofecoxib (50 μM)	- (control)	1.66 ± 0.07	0.46 ± 0.02
	Rofecoxib	0.88 ± 0.47 **	0.10 ± 0.05 ***
	Celecoxib	1.90 ± 0.15	0.50 ± 0.01
	Valdecoxib	1.66 ± 0.11	0.53 ± 0.06
	Etoricoxib	1.74 ± 0.17	0.49 ± 0.03
	CS-706	1.77 ± 0.06	0.51 ± 0.02
[^{14}C]Rofecoxib (25 μM)	-	ND	0.24 ± 0.01 ***
[^{14}C]Rofecoxib (100 μM)	-	3.75 ± 0.12 ***	0.88 ± 0.05 ***
[^{14}C]Celecoxib (50 μM)	-	0.19 ± 0.17 ***	0.09 ± 0.09 ***

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Figure 1

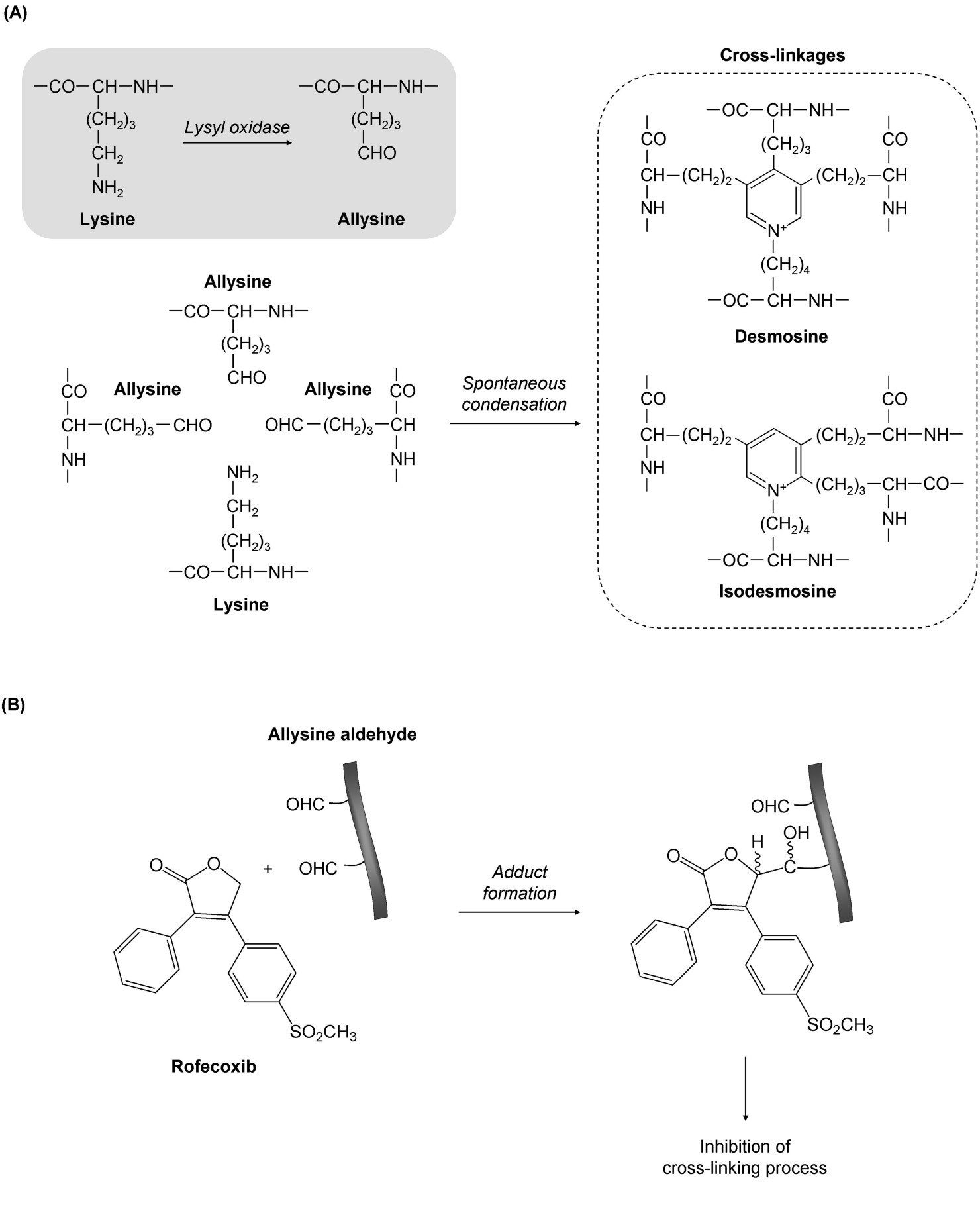

Figure 2

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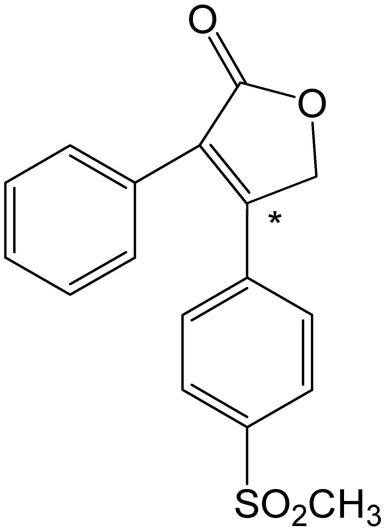
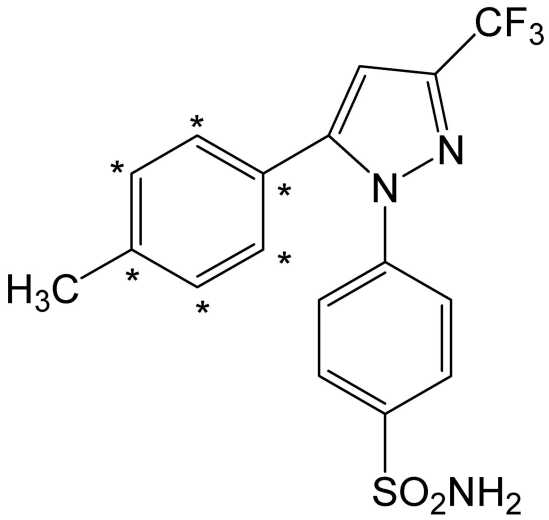


Rofecoxib



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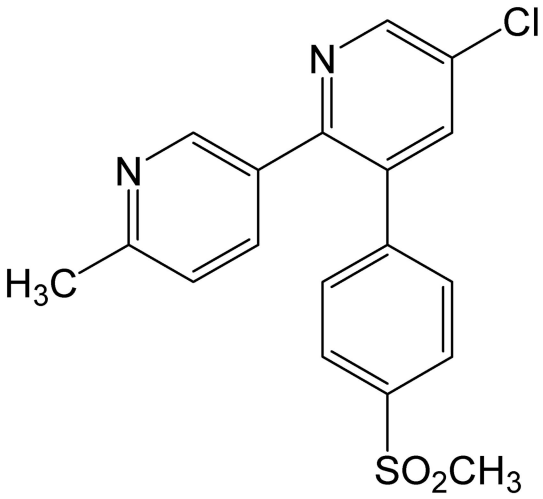
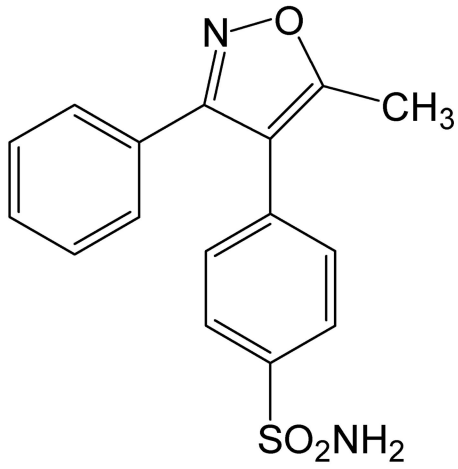
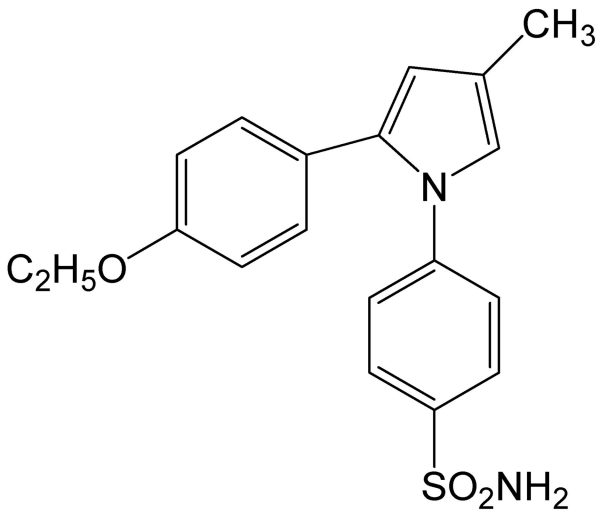


Etoricoxib



Valdecoxib



CS-706

Figure 3

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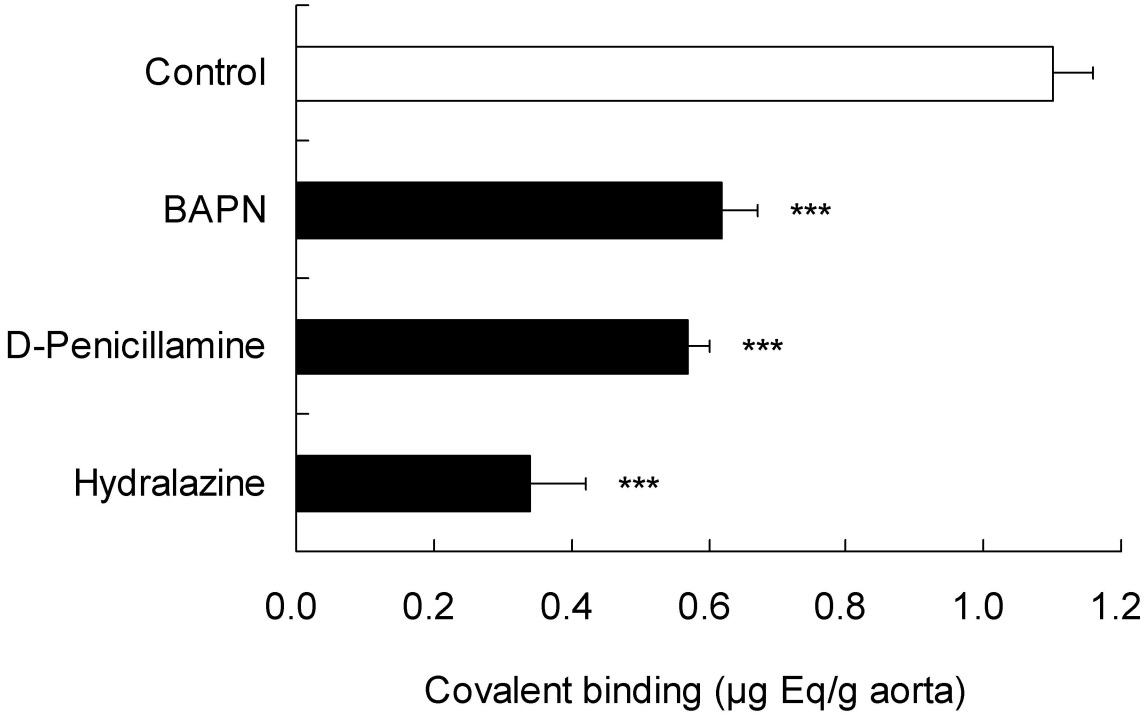

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Figure 4

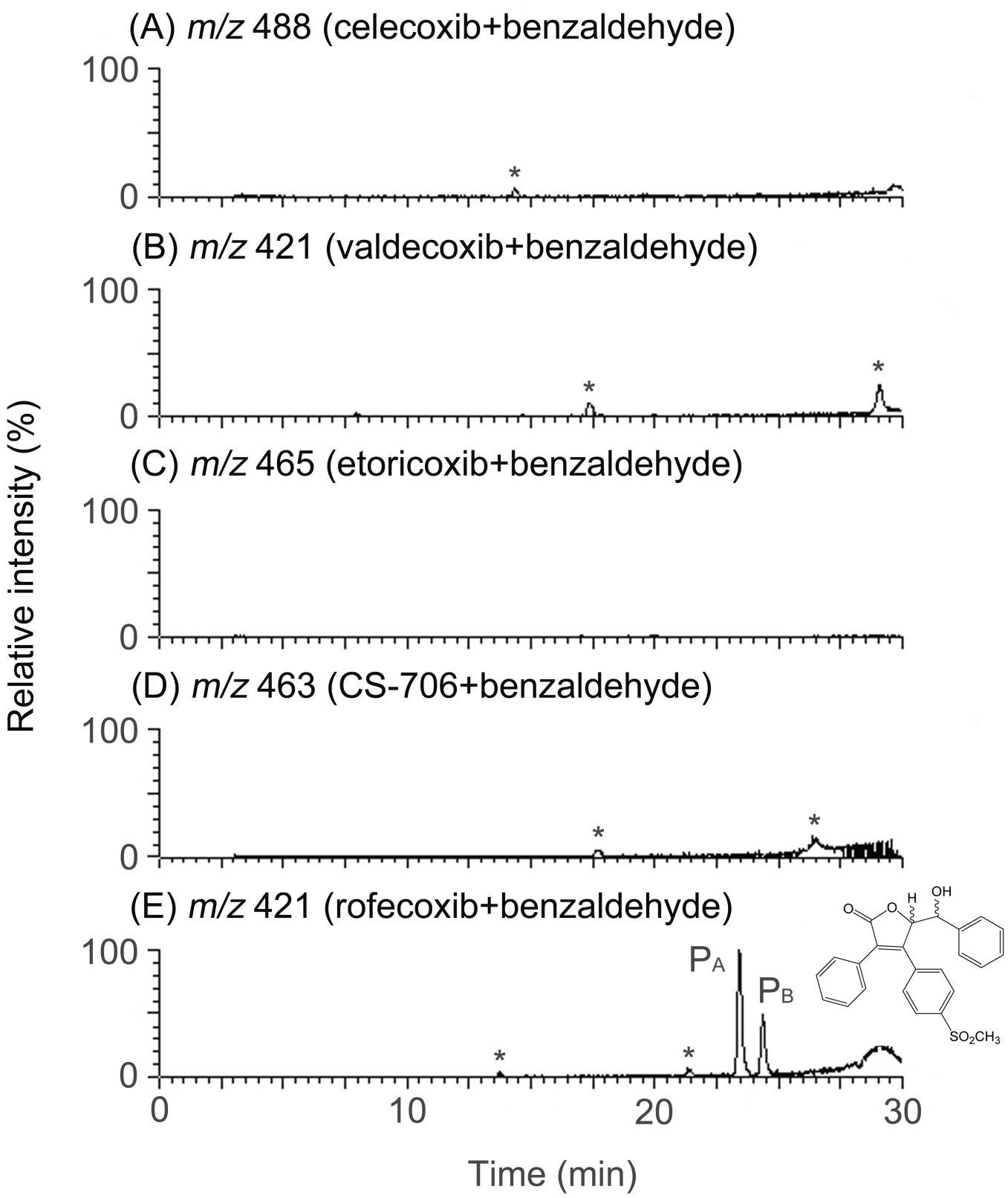

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Figure 5