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NOTE

Antipain Y, a new antipain analog that inhibits neurotransmitter release from rat dorsal root ganglion neurons

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Protease-activated receptors (PARs) are a unique class of G protein-coupled receptors activated by proteolytic cleavage of the receptor N terminus. The exposed new N terminus acts as a tethered ligand that interacts with extracellular loop 2 of its receptor, resulting in the initiation of intracellular signaling. To date, four PAR subtypes (PAR1-4) have been reported; these are expressed in a variety of organs and have important functions both physiologically and pathologically.[1,2] Activation of PARs is initiated by several serine proteases. PAR1 is activated by thrombin and PAR2 is activated by several serine proteases, including trypsin and mast cell tryptase. Recently, it was reported that PAR activation closely relates to sensory neuron-associated disorders, such as inflammation, pain and pruritus.[3–5] Consequently, inhibitors of PAR signaling, including protease inhibitors, antagonists and intracellular signaling inhibitor, are expected to be therapeutic candidates for these disorders and biological tools.

In the screening program for inhibitory compounds of PAR signaling from microbial metabolites, a new antipain analog, antipain Y (Figure 1a), was identified in the culture medium of *Streptomyces* sp. MJ218-CF4. In this study, we report the isolation, physicochemical properties, structural determination and biological activities of antipain Y.

MATERIALS AND METHODS

Determination of absolute configuration

Hydrolysis of antipain Y yielded the corresponding arginine, tyrosine and valine. Each antipain Y and standard amino acid (0.2 mg) was dissolved in 6 M HCl (50 μl), heated at 110 °C for 3 h, and then evaporated to dryness. Each hydrolysate was dissolved in water (50 μl) and treated with 1% Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in a solution of acetone (100 μl) and 7.5% aqueous $NaHCO_3$ (20 μl) at 40 °C for 1 h. After quenching with 3.5 μl of 6 M HCl, the resulting FDAA derivatives were analyzed by reversed-phase liquid chromatography-HR-electrospray ionization-MS. FDAA derivatives were eluted with a linear gradient between (a) CH_3CN containing 0.01% TFA and (b) 0.01% aqueous TFA from 20 to 50% over 25 min at a flow rate of 0.2 ml min^{-1} using ODS column (CAPCELL PAK C18MG 5 μm, 2.0 i.d. ×150 mm, Shiseido, Tokyo, Japan). Each peak in the accurate mass chromatograph was identified by comparing the retention time with those of the FDAA derivatives of corresponding authentic amino acids. Standards of amino acid gave the following retention times in minutes: 5.35 for D-Arg, 5.65 for L-Arg; 13.11 for L-Tyr, 14.60 for D-Tyr; 15.80 for L-Val and 19.34 for D-Val. On the other hand, the FDAA derivatives of the amino acids liberated from antipain Y showed peaks at 5.73, 13.13 and 15.84 min.

Biological assays

The activities of tryptase (Promega, Madison, WI, USA) and trypsin (bovine pancreas trypsin, Worthington Biochemical, Freehold, NJ, USA) were measured by the use of a fluorescent substrate. The assay buffer contained 50 mM tris-HCl (pH 8.0), 120 mM NaCl, 0.02% Triton X-100, 5.8 μM Boc-Phe-Ser-Arg-MCA (Peptide Institute, Osaka, Japan) as a substrate and 15 μg ml^{-1} tryptase or 1 μg ml^{-1} trypsin. The reaction was started by adding the substrate, and the reaction mixture was incubated for 30 min at room temperature, the reaction was terminated by adding 20% SDS. The fluorescence intensity of released 7-amino-4-methyl coumarin was measured by fluorescence spectrophotometer at 460 nm with excitation at 355 nm. The following biological assays were described previously.[6] In brief, a Ca^{2+} mobilization assay was performed using a Ca^{2+}-specific fluorescent indicator. Ca^{2+} loading buffer was prepared with Fluo-3AM (Dojindo Laboratories, Kumamoto, Japan) containing 0.01% pluronic F-127 (Invitrogen, Carlsbad, CA, USA) in the Ca^{2+} assay buffer (Hank's balanced salt solution, 17 mM HEPES pH 7.4, 0.1% bovine serum albumin (BSA), 1 mM probenecid). A human epithelial carcinoma cell line, A431 cells, was incubated with Ca^{2+} loading buffer for 60 min at 37 °C. The emitted 480 nm fluorescence induced by stimuli was measured by FDSS6000 fluorimeter (Hamamatsu Photonics, Shizuoka, Japan). The culture of primary dorsal root ganglion (DRG) neurons was prepared from newborn rats (SD strain). Dispersed single cells were obtained by mechanical dissociation

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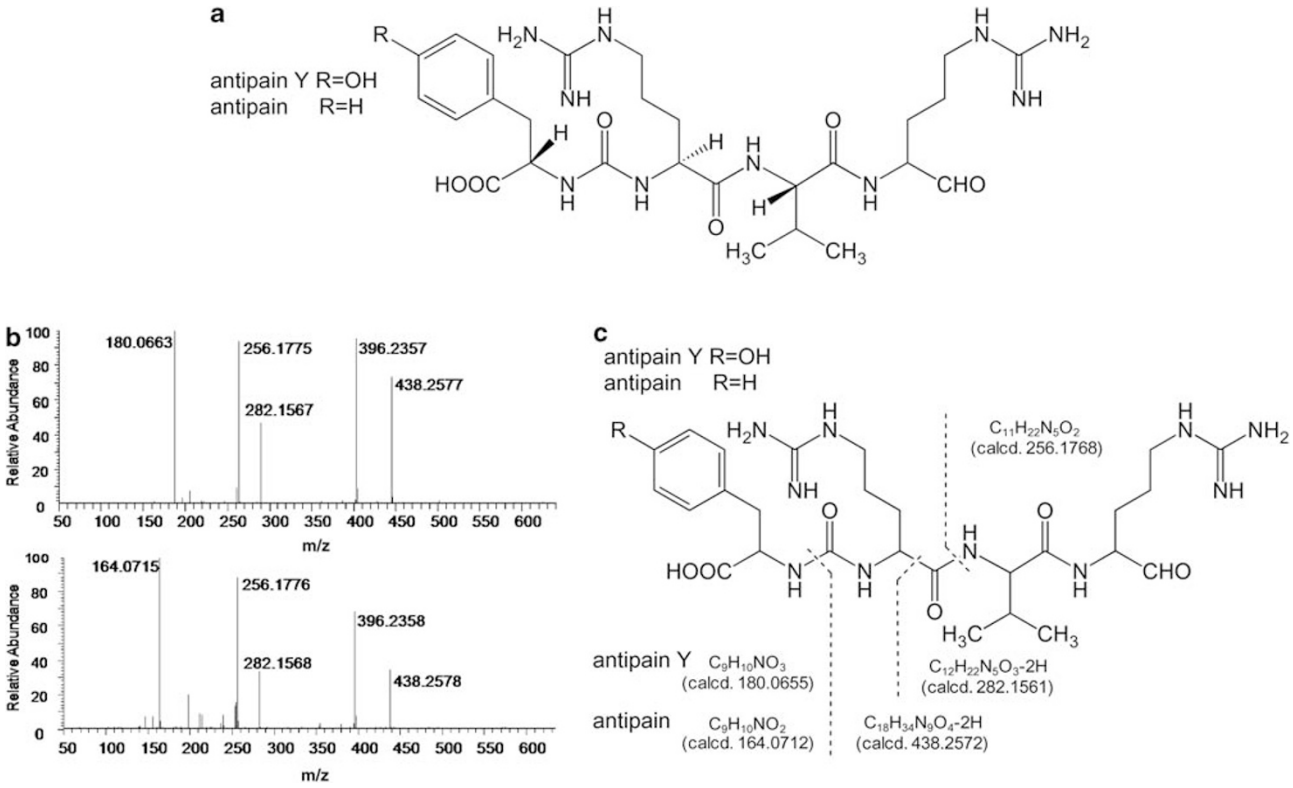

Figure 1 (**a**) Structure of antipain Y and antipain. (**b**) Negative ESI-MS/MS spectra of antipain Y at *m/z* 619.33 (upper) and antipain at *m/z* 603.34 (lower). (**c**) Assignments of the fragment ions of antipains.

in collagenase A solution and cultured for 3 days in HAM's F-12 medium containing 80 ng ml^{-1} nerve growth factor, 0.1 mM 5-fluorouracil, 7.5 mg ml^{-1} L-ascorbic acid and 10% fetal calf serum in collagen type I precoated 96-well plates. For the substance P (SP) release assay, DRG neurons were washed with the assay buffer (Hank's balanced salt solution, 17 mM HEPES, 0.1% BSA, pH 7.4, including 200 nM iloprost). SP release into the assay buffer, induced by trypsin for 30 min at 37 °C, was quantified using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA).

The strain MJ218-CF4 was isolated from a soil sample collected at Suginami-ku, Tokyo, Japan. Strain MJ218-CF4 formed well-branched vegetative mycelia and formed aerial hyphae, which bore spiral spore chains. The aerial hyphae of the strain were purplish white. The vegetative mycelia were pale yellow to pale yellowish brown. These characteristics were observed on yeast extract-malt extract agar (ISP medium no. 2) and inorganic salts-starch agar (ISP medium no. 4). The type of diaminopimelic acid isomers in whole-cell hydrolysates of the strain MJ218-CF4 was determined to be the LL-form by the method of Staneck and Roberts.[7] The 16S ribosomal RNA gene sequence (1481 bp, positions 28–1524, *Escherichia coli* numbering system[8]) of the strain showed high identity with those of genus *Streptomyces* such as *Streptomyces mauvecolor* NBRC 13854T (1456/1456 bp, 100%) and *S. violascens* NBRC 12920T (1466/1474 bp, 99%). These morphological characteristics and genetic analysis of the strain MJ218-CF4 suggested that the strain belongs to genus *Streptomyces*. Therefore, the strain was designated as *Streptomyces* sp. MJ218-CF4.

Antipain Y was produced by inoculation of *Streptomyces* sp. MJ218-CF4 into 110 ml of a culture medium (pH 7.4) composed of glycerol (2%), dextrin (2%), Bacto Soytone (Difco, Detroit, MI, USA, 1%), yeast extract (0.3%), $(NH_4)_2SO_4$ (0.2%), and $CaCO_3$ (0.2%) in each of 24 500-ml Erlenmeyer flasks, followed by cultivation at 27 °C for 5 days on a rotary shaker (220 r.p.m.).

Antipain Y was purified as follows. The filtrated broth (2.6 l) was applied to batch adsorption of charcoal (52 g) and eluted with 90% aqueous MeOH (pH 2.0). The eluate was concentrated, and the concentrate (8.0 g) was chromatographed on a column of Diaion HP-20 (36×145 mm, Mitsubishi Chemical, Tokyo, Japan) and eluted with 50% aqueous MeOH. The resulting active residue (0.6 g) was further chromatographed on a column of CM Sephadex C-25 (22×350 mm, GE Healthcare, Chalfont St Giles, UK) and eluted with 0.1 M ammonium formate. The active fractions were concentrated, and the residue (0.29 g) was chromatographed on a column of charcoal (8×60 mm) and eluted with 80% aqueous MeOH in 0.05 M HCl. The active fractions were concentrated, and the residues were lyophilized to give 13.1 mg of antipain Y as a white powder.

The physicochemical properties of antipain Y are as follows: m.p. 194–198 °C (hydrochloride); $[\alpha]_D^{22}$ −7.1° (*c* 0.27 H_2O); UV λ_{max} (H_2O) 275 nm (ε 1274); IR spectrum ν_{max} (KBr) cm^{-1} 3361, 3170, 2962, 1731, 1652, 1562, 1398, 1245, 1174, 1112, 1002; and positive color reaction with 2,4-dinitrophenylhydrazine reagent. These data suggested the presence of amide and aldehyde moieties in the molecule.

We show the ^{1}H and ^{13}C NMR spectra of antipain Y compared with those of antipain in the Supplementary data. The ^{1}H NMR spectrum of antipain Y was similar to that of antipain (Peptide Institute), except for the aromatic region (δ_H 6.89 (2H) and 7.17 (2H) in antipain Y; δ_H 7.29–7.40 (5H) in antipain) corresponding to *p*-hydroxyphenyl and phenyl group, respectively. The structure analysis of antipain suggested that antipain maintains equilibrium mixture of two hydrate forms, four cyclic carbinolamine forms and two unhydrated forms owing to the C-terminal aldehyde derivative of arginine (arginal),[9–11]

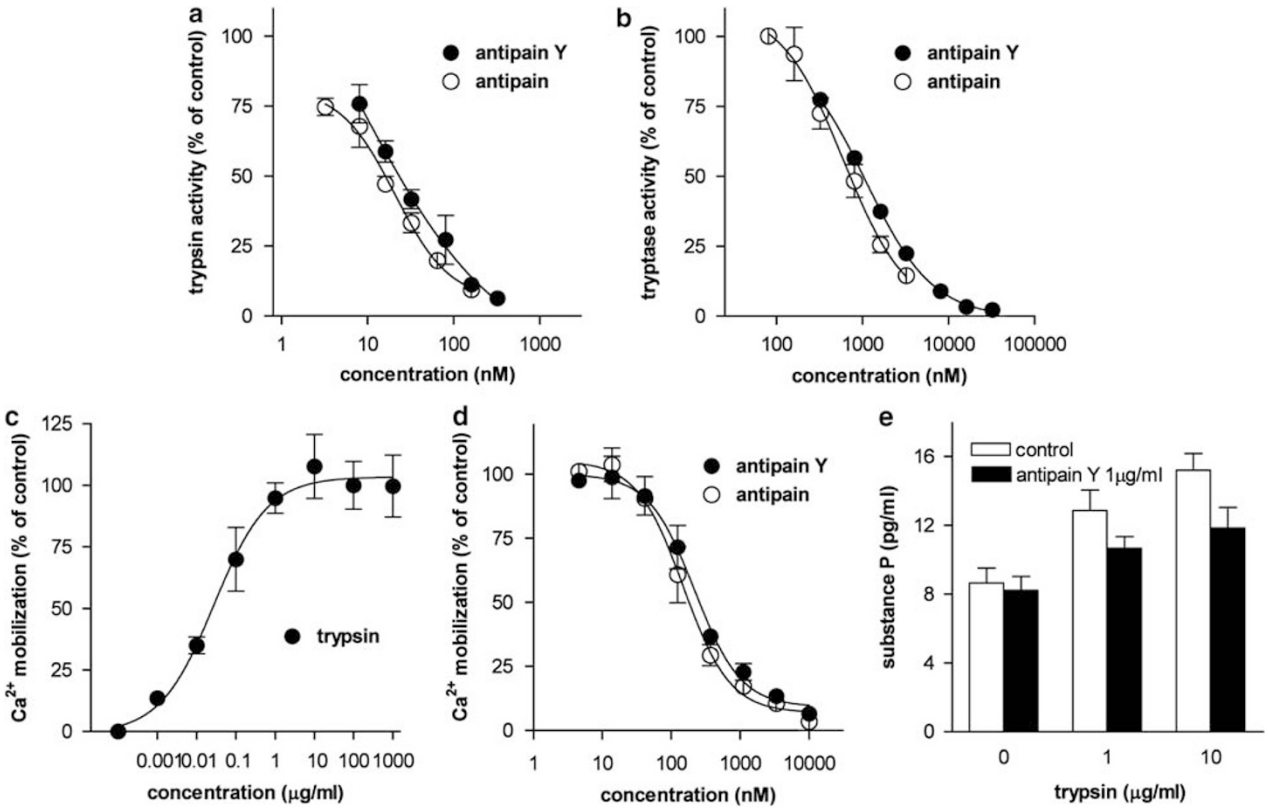

Figure 2 Biological activity of antipain Y. Inhibitory activities of antipains against trypsin (**a**) and tryptase (**b**) activity. (**c**) Effects of trypsin on Ca^{2+} mobilization in A431 cells. (**d**) Effects of antipains on trypsin-induced Ca^{2+} mobilization in A431 cells. (**e**) Effect of antipain Y on substance P release from dorsal root ganglion neurons induced by trypsin.

and detailed ^{1}H NMR study of antipain revealed that even the most major form accounts for only 20% of the total in aqueous solution.[12] Therefore, further detailed structural analysis of antipain Y was conducted by HR-electrospray ionization-MS/MS and acid hydrolysis. The molecular formula for antipain Y was determined to be $C_{27}H_{44}N_{10}O_7$ by HR-electrospray ionization-MS (positive ion mode; calculated, 621.3469 $(M+H)^+$; found, 621.3467 (as $C_{27}H_{45}N_{10}O_7$)), which revealed the addition of an oxygen atom to the formula for antipain ($C_{27}H_{44}N_{10}O_6$). As shown in Figure 1b, the precursor ions at *m/z* 619.33 and *m/z* 603.34 in the negative electrospray ionization-MS/MS spectra for antipain Y and antipain, respectively, revealed a similar fragment pattern, except for fragments at 180.0663 ($C_9H_{10}NO_3$) and 164.0715 ($C_9H_{10}NO_2$), which resulted from the difference of only one constituent amino acid, namely tyrosine in antipain Y and phenylalanine in antipain (Figure 1c). The absolute configuration of antipain Y was determined using acid hydrolysis and Marfey's method.[13] The detailed analysis condition for absolute configuration of antipain Y was described in Materials and methods section. The FDAA derivatives of the amino acids liberated from antipain Y were in accord with the retention times of L-Arg, L-Tyr and L-Val, respectively.

The inhibitory activity of antipain Y against trypsin and tryptase was evaluated by *in vitro* enzyme assay. Antipain Y inhibited trypsin and tryptase in a dose-dependent manner with IC_{50} values of 22 and 1000 nM (Figures 2a and b), respectively. The inhibitory potency of antipain Y was similar to that of antipain (IC_{50} values of 16 and 710 nM, respectively). The effect of antipain Y on the following signaling pathways associated with PARs was furthermore examined by cell-based assays using a PAR-expressing cell line. PAR1 and 2 are reported to couple to the $G\alpha_q$ type of G protein, and the activation of these receptors results in the increase of intracellular Ca^{2+} concentration.[2,14] We confirmed that A431 cells express PAR1 and PAR2 by PCR analysis (data not shown). Functional signaling downstream of PARs was detected by measuring the effect of trypsin on Ca^{2+} mobilization. As shown in Figure 2c, trypsin dose-dependently increased intracellular Ca^{2+} in A431 cells. The Ca^{2+} mobilization induced by 1 μg ml^{-1} trypsin was inhibited by antipain Y and antipain in a dose-dependent manner (IC_{50} values 260 and 180 nM, respectively) (Figure 2d). We also confirmed that the synthetically prepared tethered ligand peptides of PAR1 and PAR2 increased intracellular Ca^{2+} in A431 cells, and that antipain Y had no effect on Ca^{2+} mobilization induced by peptides (data not shown). These findings show that antipains affect PAR signaling at the cellular level.

Protease-activated receptors are abundantly expressed in DRG neurons containing the excitatory neuropeptide SP and calcitonin gene-related peptide (CGRP), a nociceptive neurotransmitter in the peripheral nervous system. Activation of PAR2 stimulates the release of SP and CGRP from afferent nerves.[15–17] Therefore, we investigated the inhibitory effect of antipain Y on SP release from DRG neurons. As shown in Figure 2e, treatment of DRG neurons with trypsin increased the release of SP, and antipain Y (1 μg ml^{-1}) inhibited its release. It has been reported that PARs closely relate to inflammation, pain and pruritus.[3–5] For instance, injections of proteases are known to cause hyperalgesia through PAR2 in response to both thermal and mechanical stimuli, and injection of trypsin evokes scratching behavior in mice.[5] PAR2 also sensitizes sensory neurons by the transient receptor potential vanilloid 1 (TRPV1)–protein kinase C pathway.[18] Although we have not examined whether antipain Y

inhibits the signaling pathway underlying sensitization mediated by TRPV1, it is likely that antipain Y and its related inhibitors are potential therapeutic compounds for sensory neuron-related disorders by inhibition of PAR activation.

In summary, a new antipain analog, antipain Y, was isolated from the fermentation medium of *Streptomyces* sp. MJ218-CF4, and its structure was elucidated to be a new type of antipain with a tyrosine in place of phenylalanine. Antipain Y inhibits trypsin and tryptase activity and dose-dependently suppresses the increase in intracellular calcium concentration induced by trypsin in PAR-expressing cells. Furthermore, antipain Y inhibits the trypsin-induced release of the excitatory neuropeptide SP from primary-cultured rat DRG neurons. The results of this study show that a new antipain analog, antipain Y, inhibits PAR signaling by acting as a protease inhibitor and thereby inhibits the release of excitatory neuropeptide.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)