VOL. 56 NO. 4, APR. 2003 THE JOURNAL OF ANTIBIOTICS pp. 358 — 363

YM-254890, a Novel Platelet Aggregation Inhibitor Produced by *Chromobacterium* sp. QS3666

MASATOSHI TANIGUCHI*, KOJI NAGAI†, NAKAKO ARAO†, TOMIHISA KAWASAKI, TETSU SAITO, YUMIKO MORITANI, JUN TAKASAKI, KAZUMI HAYASHI, SHIGEO FUJITA, KEN-ICHI SUZUKI† and SHIN-ICHI TSUKAMOTO

Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21, Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan
† Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 1-1-8 Azusawa, Itabashi-Ku, Tokyo 174-8511, Japan

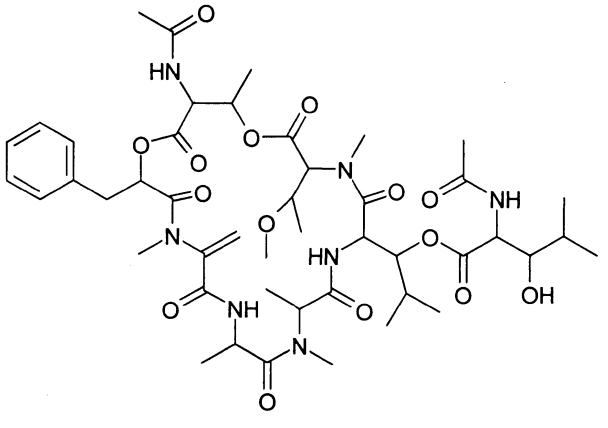
(Received for publication December 4, 2002)

A novel platelet aggregation inhibitor, YM-254890, was isolated from the culture broth of strain QS3666. This strain was isolated from a soil sample collected at Okutama, Tokyo, Japan, and was identified as *Chromobacterium* sp. by morphological and physiological criteria. YM-254890 was purified from the culture supernatant by solvent extraction, ODS and silica gel flash chromatography, followed by preparative HPLC. YM-254890 inhibited ADP-induced platelet aggregation in human platelet-rich plasma with an IC_{50} value below 0.6 μM by blocking the $P2Y_1$ receptor-signal transduction pathway.

Platelets play a crucial role in thrombus formation particularly in the arterial system[1]. When the vascular endothelium is ruptured, platelets adhere to subendothelial components such as collagen, and this induces platelet aggregation. The adherent platelets spread over the surface and are stimulated to release their dense granule contents including ADP, thromboxane A_2 and serotonin. These agonists then activate surrounding platelets, leading to thrombus formation. This process underlies various thromboembolic diseases such as unstable angina, myocardial infarction, stroke and peripheral arterial occlusive diseases. Antiplatelet drugs such as aspirin and ticlopidine inhibit platelet activation and may be useful in the treatment of these diseases[2]. However, since these drugs remain insufficient for the prevention of thrombosis, new types of antiplatelet agents are required[3].

In the course of screening for platelet aggregation inhibitors, YM-254890 (Fig. 1) was found from the culture broth of *Chromobacterium* sp. QS3666 . In this paper, we describe the taxonomy, fermentation, isolation and biological properties of YM-254890. The physico-chemical properties and structure elucidation of YM-254890 will be reported elsewhere.

Fig. 1. Structure of YM-254890.



* Corresponding author: taniguti@yamanouchi.co.jp

Material and Methods

Taxonomic Studies

The producing bacterial strain QS3666 was isolated from a soil sample collected at Okutama, Tokyo, Japan. Examination of growth on various media, and tests of physiological characteristics, were based on the methods of COWAN[4] and SNEATH[5]. The morphology of cells grown on meat extract agar at 28°C for 24 hours was studied with an optical (OPTIPHOT-2, Nikon) and an atomic force microscope (Nanoscope III, Digital Instruments). Isoprenoid quinones were extracted with $CHCl_3$-MeOH (2:1)[6] and analyzed by mass spectroscopy (LC/ESI-MS and EI-MS). The guanine plus cytosine (G+C) content of the DNA was determined by the method of MESBAH *et al.*[7]. 16S rRNA gene sequencing was performed with the MicroSeq 16S rRNA Gene Kit (Applied Biosystems) according to the manufacturer's instructions. All sequences were analyzed with the ABI PRISM 377 DNA sequencer (Applied Biosystems) and compared with the MicroSeq 16S rRNA sequence database.

Platelet Aggregation

Platelet-rich plasma was prepared by centrifugation of citrate-anticoagulated blood (3.8% sodium citrate : blood = 1 : 9) from healthy human volunteers who had not been on any medication for two weeks preceding the experiments. Platelet aggregation in human platelet-rich plasma was measured using an aggregometer (Hema Tracer 212, MC Medical) by recording the increase in light transmission through a stirred suspension maintained at 37°C for 7 minutes. Platelet aggregation in platelet-rich plasma ($3\times10^5/\mu l$) was induced by ADP (2, 5 and 20 μM). Inhibition was calculated by dividing the maximum rate of decrease in absorbance of a mixture containing test sample by the maximum rate in the buffer control. The IC_{50} values were calculated from each inhibition curve.

Intracellular Calcium Mobilization

$C_{6\text{-}15}$ cells[8], rat glioma cell line, transfected with human $P2Y_1$ receptor cDNA were seeded into 96 well black clear tissue culture plates 24 hours before assay. The cells were loaded for 1 hour at 37°C with 4 μM Fluo-3, AM (Molecular Probes) in Dulbecco's Modified Eagle Medium containing 10% FBS and 2.5 mM probenecid (Sigma). After washing with Hanks Balanced Salt Solution (GIBCO BRL) and 20 mM HEPES containing 2.5 mM probenecid, transient changes in intracellular calcium concentration caused by 2MeSADP (2-methylthioadenosine 5′-diphosphate) were monitored using the FLIPR system (Molecular Devices). Test sample was added 5 minutes before addition of 2MeSADP. Data were obtained by the maximum fluorescence counts after addition of the agonist.

Table 1. Morphological characteristics of strain QS3666.

Gram stain	Negative
Cell shape	Rod
Cell size	0.7-0.8 X 1.7-2.2 μm
Spore formation	Negative
Color of colony	Pale yellowish brown
Motility	Positive
Flagella	Peritrichous

Results and Discussion

Taxonomy of the Producing Strain

The morphological characteristics of strain QS3666 are summarized in Table 1. QS3666 is a motile Gram-negative rod ($0.7\sim0.8\times1.7\sim2.2\,\mu$m) with peritrichous flagellae. Colonies on meat extract agar are circular and pale yellowish brown with smooth or rugose surface.

Physiological characteristics are listed in Tables 2 and 3. It is a facultative anaerobe and the oxidation-fermentation test revealed it to be of fermentative type. It grows between 15 and 32°C with optimal growth from 20 to 28°C, and has a pH range of 5.0 to 9.0. It does not produce violet pigment and gives positive results for oxidase, catalase, nitrate reduction, and gelatin liquefaction. Voges-Proskauer reaction, indole production, lysine decarboxylase, ornithine decarboxylase, esculin hydrolysis, and deoxyribonuclease are negative. Acid is formed from D-glucose and D-fructose. The G+C content of its DNA is 66.1 mol%. The predominant isoprenoid quinone type is Q-8.

BERGEY's Manual of Systematic Bacteriology (Vol. 1) and BERGEY's Manual of Determinative Bacteriology (9th edition)[9] indicate that QS3666 belongs to the genus *Chromobacterium*. A comparison of the physiological characteristics of strain QS3666 and *Chromobaterium violaceum* is shown in Table 4. Strain QS3666 differs from *C. violaceum* in its ability to grow at 37°C as well as in producing acid from trehalose and mannose. QS3666 and *C. violaceum* are 98.11% similar in their 16S rRNA

Table 2. Physiological characteristics of strain QS3666.

Nitrate reduction	Positive	Tween 80 esterase	Positive
Denitrification	Positive	Starch hydrolysis	Negative
Methyl red test	Negative	Gelatin liquefaction	Positive
Voges-Proskauer test	Negative	Esculin hydrolysis	Negative
Production of		Lysine decarboxylase	Negative
Indole	Negative	Ornithine decarboxylase	Negative
H_2S	Negative	Arginine dihydrolase	Positive
Utilization of		Range of growth	
Citrate	Positive	Temperature	15-32°C
$NaNO_3$	Negative	(Optimum)	20-28°C
$(NH_4)_2SO_4$	Positive	pH	5.0-9.0
Violet pigment	Negative	(Optimum)	5.0-6.0
β-Galactosidase	Negative	Facultative anaerobes	Positive
Urease	Negative	O-F test	Fermentative
Oxidase	Positive	Growth in 6% NaCl	Negative
Catalase	Positive	Mol % G + C of DNA	66.1
DNase	Negative	Quinone type	Q-8

Table 3. Utilization of carbon sources and formation of acid by strain QS3666.

	Acid formation	Utilization
L-Arabinose	-	-
D-Xylose	-	-
D-Glucose	+	+
D-Mannose	-	+
D-Fructose	+	-
Sucrose	-	-
Inositol	-	-
Rhamnose	N.T.	-
Raffinose	N.T.	-
D-Mannitol	-	-
D-Galactose	-	-
Maltose	-	+
Toleharose	-	-
Lactose	-	-
D-Sorbitol	-	-
Salicin	N.T.	-
Melibiose	N.T.	-
Glycerol	-	+
Starch	-	+
Xanthine	N.T.	-
Chitin	N.T.	-

sequences. In the light of these results, QS3666 was classified and designated as *Chromobacterium* sp. QS3666. Strain QS3666 has been deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan, with accession no. FERM P-18672.

Fermentation

A slant culture of QS3666 grown on Bennett's agar was used to inoculate a 500-ml Erlenmeyer flask containing 100ml of a seed medium consisting of glucose 1%, potato starch 2%, Polypeptone (Nihon Pharmaceutical) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4% (pH 7.0). After incubation at 28°C for three days on a rotary shaker at 220 rpm, the seed culture was inoculated into each of twenty five 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of glycerol 2%, glucose 0.5%, Polypepton 0.5%, meat extract 0.5%, yeast extract 0.1% and NaCl 0.1% (pH 6.5). Fermentation was carried out at 28°C for three days on a rotary shaker at 220 rpm.

Isolation

The procedure used to purify YM-254890 is outlined in Fig. 2. The fermentation broth (2.5 liters) was filtered, and

Table 4. Comparison of strain QS3666 with *Chromobacterium violaceum*.

	QS3666	*C. violaceum*[a]
Growth at 4°C	-	-
Growth at 37°C	-	+
Arginine dihydrolase	+	d(50)
Acid from:		
Glucose	+	+
Fructose	+	+
Trehalose	-	+
Mannose	-	[+](80)

[a] Data for *C. violaceum* are derived from BERGEY's Manual of Systematic Bacteriology (Vol. 1).

Symbols: +, all strains positive; [+], positive in 80% or more strains; d, positive in 31-79% of strains; [-], positive in 30% or fewer strains; -, negative in all strains. Numbers in parentheses indicate the % of strains giving a positive reaction.

Fig. 2. Purification procedure of YM-254890.

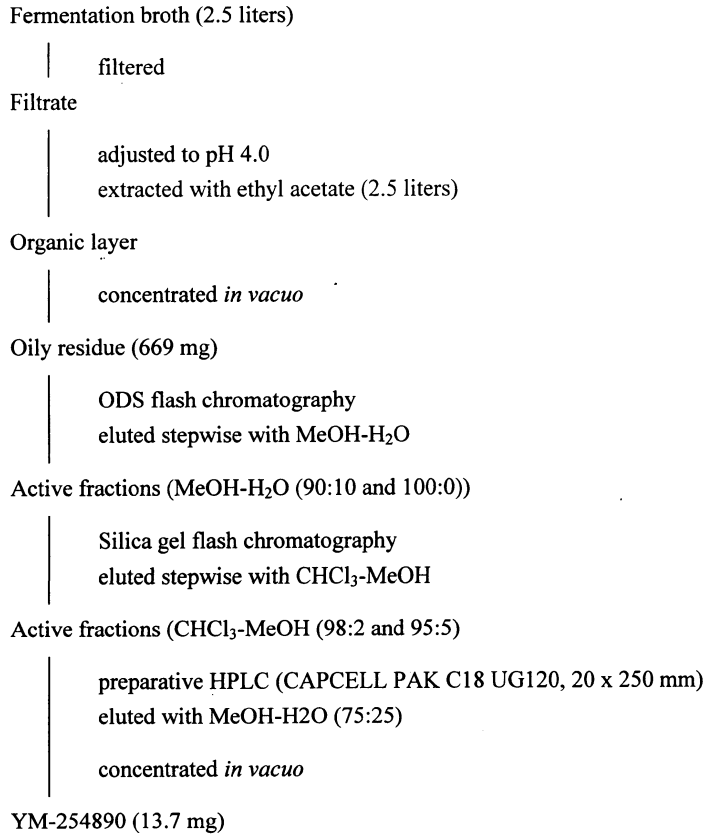


Fig. 3. Effect of YM-254890 on ADP-induced platelet aggregation in human platelet-rich plasma.

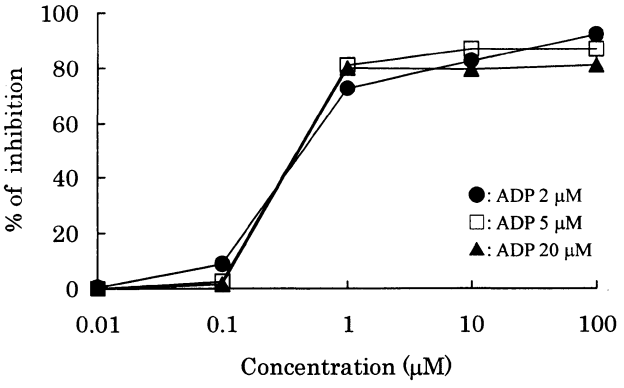
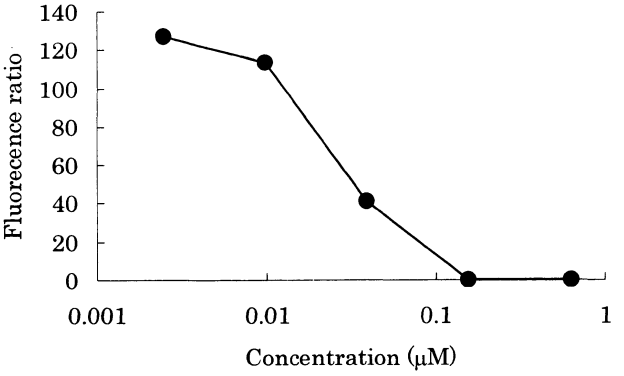


Fig. 4. Effect of YM-254890 on 2MeSADP-induced intracellular calcium mobilization in $P2Y_1$-$C_{6\text{-}15}$ cells.



the filtrate adjusted to pH 4.0 with 1 N HCl and extracted with 2.5 liters of EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown extract (669 mg). This oily extract was subjected to ODS flash chromatography (YMC-GEL ODS-A 120~130/70, 40×55 mm, YMC) and eluted with a step gradient from 40% to 100% MeOH. The fractions eluted with 90% and 100% MeOH were combined and concentrated to dryness *in vacuo* (278 mg). The yellow residue was subjected to Silica gel flash chromatography (Kieselgel 60 0.040~0.063 mm, 40×45 mm, MERCK), with elution by a step gradient of $CHCl_3$ - MeOH solvent system. The active fractions eluted with $CHCl_3$ - MeOH (98 : 2 and 95 : 5) were evaporated to dryness (30 mg). Finally the residue was purified by preparative HPLC (CAPCELL PAK C18 UG120, 20×250 mm, Shiseido) with MeOH/H_2O (75 : 25) at a flow rate of 8 ml/minute to yield 13.7 mg of YM-254890 as a white powder.

Biological Properties

The antiplatelet activities of YM-254890 are shown in Fig. 3. YM-254890 inhibited platelet aggregation induced by ADP (2, 5 and 20 μM) in human platelet-rich plasma with IC_{50} values of 0.37, 0.39 and 0.51 μM. It is well established that ADP mediates platelet aggregation *via* two G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$[10]. The $P2Y_1$ receptor is responsible for the mobilization of intracellular calcium stores *via* the Gq pathway, while the $P2Y_{12}$ receptor is coupled to Gi and adenylyl cyclase inhibition. We therefore examined the effect of YM-254890 on the $P2Y_1$ and $P2Y_{12}$ signal transduction pathways using $C_{6\text{-}15}$ cells stably expressing the human $P2Y_1$ or $P2Y_{12}$ receptors. Stimulation of $P2Y_1$-$C_{6\text{-}15}$ cells by 2MeSADP leads to increases in intracellular calcium mobilization. In this assay, YM-254890 inhibited the increase in $[Ca^{2+}]_i$ with an IC_{50} value of 0.031 μM (Fig. 4). In contrast, 2MeSADP-induced inhibition of forskolin-stimulated adenylyl cyclase activity in $P2Y_{12}$-$C_{6\text{-}15}$ cells was unaffected by YM-254890 at 40 μM (data not shown). These results suggest that YM-254890 inhibits platelet aggregation induced by ADP by blocking the $P2Y_1$ signal transduction pathway. Further pharmacological studies and investigation of the mechanism of action of this compound are now underway.

References

1) PACKHAM, M. A.: Role of platelets in thrombosis and hemostasis. Can. J. Physiol. Pharmacol. 72: 278~284, 1994
2) SCHRÖR, K.: Antiplatelet drugs-a comparative review. Drugs 50: 7~28, 1995
3) TOPOL, E. J.; T. V. BYZOVA & E. F. PLOW: Platelet GPIIb-IIIa blockers. Lancet 353: 227~231, 1999
4) COWAN, S. T. (*Ed.*): Manual for the identification of medical bacteria. Cambridge University Press, 1974
5) SNEATH, P. H. A.: Genus *Chromobacterium* Bergonzini 1881, 153. *In* BERGEY's Manual of Systematic Bacteriology. Vol. 1., *Eds.*, N. R. KRIEG & J. G. HOLT, pp. 580~582, Williams & Wilkins Co., Baltimore, 1984
6) COLLINS, M. D.; T. PIROUZ, M. GOODFELLOW & D. E. MINNIKIN: Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100: 221~230, 1977
7) MESBAH, M.; U. PREMACHANDRAN & W. B. WHITMAN: Precise measurement of G+C content of deoxy-

ribonucleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. 39: 159~167, 1989

8) TAKASAKI, J.; M. KAMOHARA, T. SAITO, M. MATSUMOTO, S. MATSUMOTO, T. OHISHI, T. SOGA, H. MATSUSHIME & K. FURUICHI: Molecular cloning of the platelet $P2T_{AC}$ ADP receptor: pharmacological comparison with another ADP receptor, the $P2Y_1$ receptor. Mol. Pharmacol. 60: 432~439, 2001

9) BERGEY's Manual of Determinative Bacteriology 9th Edition, *Ed.*, J. G. HOLT *et al.*, Williams & Wilkins Co., Baltimore, USA, 1994

10) GACHET, C.: ADP receptors of platelets and their inhibition. Thromb. Haemost. 86: 222~232, 2001