STUDIES ON WF-5239, A NEW POTENT PLATELET AGGREGATION INHIBITOR

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WF-5239 was produced by a fungal strain identified as *Aspergillus fumigatus* Fresenius. The substance was purified by solvent extraction followed by chromatography on silica gel and then crystallized ($C_9H_9NO_2$, mp 142~145°C). The chemical structure was determined from its physico-chemical properties as *N*-[2-*cis*(4-hydroxyphenyl)ethenyl]formamide.

WF-5239 has inhibitory activity against rabbit platelet aggregation induced by arachidonic acid and collagen, with IC_{50} values of 1.25 and 5.0 μg/ml, respectively. Arachidonic acid induced mortality in mice was reduced by a single intraperitoneal dose of WF-5239 (30 mg/kg). These biological properties have been compared with those of aspirin.

In the course of screening for potential antithrombotic drugs, we have tested a wide range of microbial products for effects on platelet aggregation. The role of the platelet is believed to be very important in the process of thrombosis. The antithrombotic properties of platelet inhibitory drugs have been reviewed recently by RODEN *et al.*[1] Aspirin, for example, prevents peripheral ischemia in patients with thrombocytosis and this effect is considered to be associated with inhibition of platelet aggregation[2,3]. Although many synthetic antithrombotic drugs including nonsteroidal anti-inflammatory drugs have been evaluated in animal models of thrombosis, no microbial products have been reported as potential antithrombotic drugs. Therefore, we undertook a screening program directed towards the isolation and evaluation of new compounds with inhibitory activity against platelet aggregation from microbial products. As a result, we discovered a new substance initially designated WF-5239 from fungal culture broth, which has an inhibitory activity against rabbit platelet aggregation. In this paper we describe characterization of the producing strain, fermentation and isolation procedures, and physico-chemical and biological properties of WF-5239 compound.

Methods

Fermentation

The growth of *Aspergillus fumigatus* No. 5239 on mature slant culture was used to inoculate five 500-ml flasks containing 100 ml of each of sterile seed medium containing soluble starch 1%, gluten meal 1%, dried yeast 0.5% and corn steep liquor 0.5%. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) for 3 days at 30°C. The content of the flasks was used to inoculate 20 liters of fermentation medium in a 30-liter volume of jar fermentor. The composition of the medium is as follows: soluble starch 3%, peanut meal 0.5% and Na_2CO_3 0.06%. Fermentation was allowed to proceed for 3 days at a temperature of 30°C, air flow of 20 liters per minute and with agitation of 250 rpm.

Detection of WF-5239

The active compound present in the fermentation broth or in preparations obtained during the purification process was detected by its inhibitory activity against rabbit platelet aggregation induced by

arachidonic acid.

Platelet Aggregation

Blood was collected from the central ear arteries of male Japanese White rabbit (2.5~3.0 kg body weight). The blood was prevented from coagulation with 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 1,300 rpm for 10 minutes at room temperature. The PRP was diluted with platelet poor plasma obtained by further centrifugation of the blood at 3,000 rpm for 10 minutes. The platelet count in the PRP used for aggregation studies was about 4×10^5 platelets/mm^3. Platelet aggregation was measured by the nephelometric technique of BORN and CROSS[4] in a dual channel aggregometer (Sienco, DP-247 E). For most experiments PRP-saline/inhibitor mixtures (0.25 ml PRP plus 0.02 ml saline/inhibitor) were incubated in an aggregometer with stirring (1,000 rpm) at 37°C for 2 minutes, and then the aggregating agent (0.03 ml) was added. Platelet aggregation was measured turbidimetrically by recording changes in the light transmission of PRP during aggregation. Activities of inhibitors were expressed as IC_{50} values *i.e.* concentrations required to inhibit the platelet aggregation responses by 50%. Collagen (Tokyo Kasei Co., Ltd.) was used in amounts (2 to 20 μg/ml for PRP) sufficient to induce a response that was 80 to 90% of the maximum aggregation obtainable. Arachidonic acid (Sigma) was used at a final concentration of 100~150 μM. Similarly a final concentration of ADP (Boehringer Mannheim) and thrombin (Sigma), usually 1 to 5 μM and 0.3 U/ml, respectively, was chosen to induce approximately 75% of the maximum aggregation.

Pulmonary Thrombosis in Mice

The method of KOHLER *et al.*[5] was used to induce platelet aggregation and death in mice. Sodium arachidonic acid (0.2 ml volume) was administered by slow intravenous injection into the tail vein of male mice (15~20 g) at a dose of 50 mg/kg, 30 minutes after intraperitoneal administration of drugs. The mice were observed for signs of respiratory distress and sudden death, and drug effectiveness was expressed as the percentage of animals surviving two hours after provocation.

Results

Taxonomic Studies on Strain No. 5239

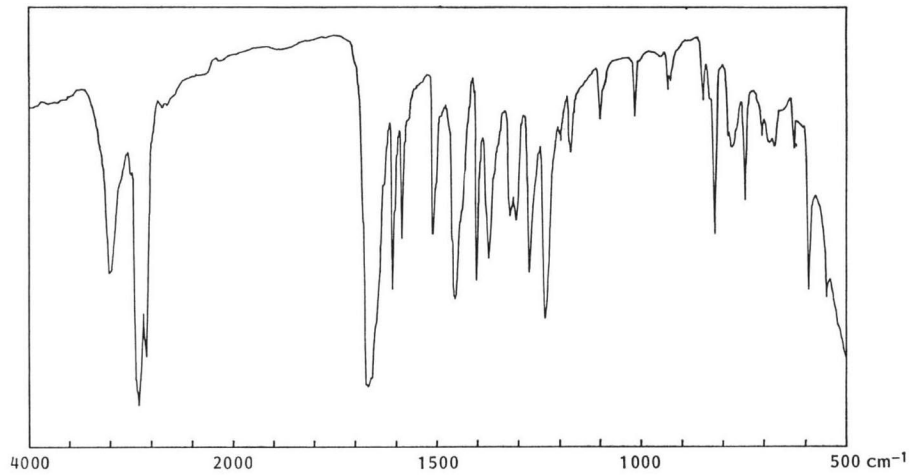
Strain No. 5239 which produces WF-5239 substance is a fungus isolated from a soil sample obtained at Fuji city, Shizuoka Prefecture. On the basis of its morphological characteristics the strain appears to belong to the hyphomycete genus *Aspergillus* Micheli. The morphological and cultural characters are as follows. Colonies on Czapek solution agar spread broadly and the surface is floccose, white at first and becomes green with the forming of conidial heads. This strain can grow at 45°C. A sexual reproductive organ was not observed. Conidial apparatus develops as erect conidiophores and heads from foot cells producing conidiophores. Tips of conidiophores enlarge and form vesicles with many phialides producing conidia in long dry chains. Conidial heads are compactly columnar, sometimes loosely cylindrical or clavate, 110~260 μm long and 20~60 μm in a diameter, green to dark green. Conidiophores are unbranched, smooth and pale green, 170~360 μm$\times$2~2.5 μm in size. Vesicles are flask-shaped, 5~13 μm in diameter. Phialides, formed on upper half parts of the vesicles without metulae, are $4\times2\sim2.5$ μm in size and pale green. Conidia are globose to subglobose, roughened, green in mass and 2~3 μm in diameter.

After comparing the characters of this strain with a description of RAPER and FENNELL[8], strain No. 5239 is considered to be *Aspergillus fumigatus* Fresenius.

Isolation of WF-5239

Fermentation broth (20 liters) was filtered with the aid of filter aid (Radiolite). The filtrate was extracted twice with 10 liters of ethyl acetate after acidification to pH 2.0 with 6 N HCl. The extracts

Fig. 1. IR spectrum of WF-5239 (Nujol).



were concentrated *in vacuo* and the oily matter obtained was applied to a 200-ml silica gel column. The column was washed with 500 ml of *n*-hexane and then eluted with ethyl acetate. The fractions containing WF-5239 were combined and concentrated to dryness. The residue was dissolved in a small amount of ethanol and again applied to a silica gel column. Active fractions eluted with ethyl acetate were concentrated and the crude solid obtained was crystallized from ethanol yielding 60 mg of WF-5239 as colorless needles.

Physico-chemical Properties of WF-5239

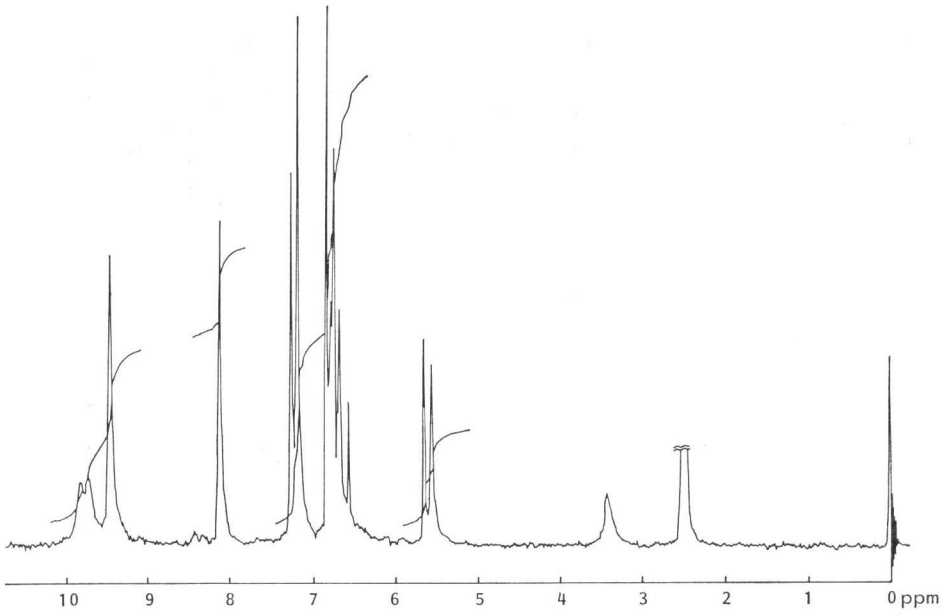
WF-5239 is a colorless crystal which melts at 142~145°C. It is soluble in methanol, ethanol and acetone, slightly soluble in chloroform, and ethyl acetate, substantially insoluble in water and *n*-hexane. Elemental analysis gave the following composition:

Anal Calcd for $C_9H_9NO_2 \cdot \frac{1}{8}H_2O$: C 65.34, H 5.63, N 8.47.
Found: C 65.27, H 5.42, N 8.38.

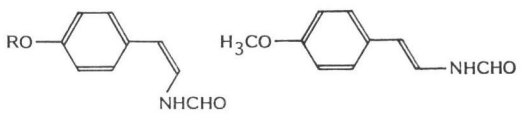
The molecular weight (163) required by this formula was confirmed by its mass spectrum. The observed specific rotation, $[\alpha]_D^{25}$ is 0° (*c* 1.0, ethanol). It gave a brown color with ferric chloride indicating a phenol group. The IR spectrum of WF-5239 (Fig. 1) disclosed a phenol (3300 cm^{-1}), a double bond (1655 cm^{-1}) in addition to the amide bond at 1670 cm^{-1}. The NMR spectrum (DMSO-d_6) of WF-5239 (Fig. 2) exhibited signals due to two exchangeable protons at δ 9.46 (phenolic hydroxyl) and 9.76 (d, J=10 Hz, amide NH). A singlet at δ 8.13 was attributed to *N*-formyl proton. An AB-quartet at δ 6.78 (2H, J=8 Hz) and 7.22 (2H, J=8 Hz) were assigned to the protons of 1,4-disubstituted phenyl ring. The olefinic protons appeared as a doublet at δ 5.60 (1H, d, J=10 Hz) and a double doublet at δ 6.65 (1H, dd, J=10, 10 Hz). Irradiation at δ 9.76 (NH) caused the double doublet (δ 6.65) to collapse to a doublet (J=10 Hz) and the signal at δ 9.76 exchanged in D_2O converting the signal at δ 6.65 into a doublet (J=10 Hz). These facts showed a double bond adjacent to a formamide group. The geometry about the double bond was assigned *cis* form on the basis of a coupling constant (J=10 Hz). The spectroscopic properties mentioned above allowed us to propose the structure (**1**) for WF-5239.

Tuberin (**3**) is reported by SUZUKI *et al.* as an antimycobacterial antibiotic[7]. Although tuberin structually resembles WF-5239, it has been determined that the former has *trans*-styrylamine[8]. Treat-

Fig. 2. ^{1}H NMR spectrum of WF-5239 (DMSO-d_6).



ment of WF-5239 with ethereal diazomethane gave the methoxy compound (**2**), mp 72~74°C, $C_{10}H_{11}NO_2$, whose IR spectrum is very similar to that of tuberin, but it revealed the absence of the characteristic band at 965 cm^{-1} region due to *trans*-double bond.



1 R = H
2 R = CH_3
3

Biological Properties of WF-5239

WF-5239 shows no antimicrobial activity at a concentration of 100 μg/ml by agar dilution method against yeasts, fungi and bacteria including mycobacteria.

Each of five *dd*Y mice (15~20 g in weight) was given a single intraperitoneal dose of 10 mg of WF-5239 and all survived. During 7 days of observation after injection, no toxic symptom was observed.

The effect of WF-5239 on the aggregation of rabbit platelet induced by arachidonic acid, collagen,

Table 1. Inhibition of rabbit platelet aggregation by WF-5239 and aspirin.

Aggregating agent	IC_{50} value (μg/ml)	
	WF-5239	Aspirin
Arachidonic acid	1.25	50.0
Collagen	5.0	25.0
ADP	>50.0	>50.0
Thrombin	>50.0	>50.0

Platelet aggregation was determined as described in the text. Results are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

Table 2. Effect of WF-5239 and aspirin on arachidonic acid induced pulmonary thrombosis in mice.

Drug	Percent survival at a dose of		
	30 mg/kg (n)	10 mg/kg (n)	3 mg/kg (n)
WF-5239	80 (10)	40 (10)	10 (10)
Aspirin	100 (10)	100 (10)	80 (10)

Control=10% survival, n=20

Pulmonary thrombosis was determined as described in the text. Drugs were administered intraperitoneally to mice at 30 mg/kg, 10 mg/kg or 3 mg/kg 30 minutes prior to induction of thrombosis.

ADP and thrombin is illustrated in Table 1. The effect of aspirin on these assay is included for comparison. WF-5239 had activities against arachidonic acid and collagen, but was less active against ADP and thrombin. Aspirin also had activities against arachidonic acid and collagen but was less active than WF-5239. The platelet anti-aggregant activity of the methyl ether of WF-5239 (**2**), is less than 25% that of WF-5239 (not illustrated). The ability of drugs to block arachidonic acid-induced pulmonary thrombosis was determined 30 minutes after intraperitoneal administration to mice. WF-5239 was able to prevent arachidonic acid-induced sudden death in at least 80% of the mice at 30 mg/kg (Table 2). Aspirin was a more effective drug in this model.

Discussion

WF-5239 is a potent inhibitor of platelet aggregation induced by arachidonic acid and collagen (Table 1). Its mechanism of action is not certain, but the spectrum of activity suggests that WF-5239 is aspirin-like in action. The mechanism of action of aspirin is believed to involve inhibition of cyclo-oxygenase, the enzyme that converts arachidonic acid to the prostaglandin endoperoxide precursors of thromboxane A_2[9]. To ascertain the mechanism of action of WF-5239 we need further investigation by using *in vitro* prostaglandin synthetase system.

As shown in Table 2, arachidonic acid aggregates platelets *in vivo* and like aggregation induced *in vitro*, such aggregation is inhibited by WF-5239. However, aspirin has more potent activity *in vivo* than WF-5239, despite its weak effect on *in vitro* aggregation.

Evidence is accumulating indicating that drugs with potent antiplatelet activity may also be anti-thrombogenic. Thromboxane A_2, an extremely potent inducer of platelet aggregation, can be produced by activated platelets themselves. It greatly accelerates the growth of an incipient platelet thrombosis[10]. Therefore thromboxane A_2 synthesis inhibitors such as imidazole derivatives[11] may be antithrombogenic. Aspirin, a well known inhibitor of platelet aggregation, which inhibits thromboxane A_2 formation by inactivating platelet cyclo-oxygenase is now used clinically as antithrombotic agent. A platelet inhibitory compound as described in this paper might well be expected to have antithrombotic properties in man.

NINOMIYA *et al.*, reported that pyrrothine, a broad spectrum antibiotic produced by *Streptomyces* had potent membrane stabilization and platelet anti-aggregant activities[12,13]. However, clear differences were found between physico-chemical and biological properties of the two. Recently xanthocillin X monomethyl ether, and thielavins A and B were isolated from cultures of fungi as potent inhibitors of prostaglandin biosynthesis[14,15]. Differences between WF-5239 and the latter are seen in their physico-chemical properties.

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