PIPERAFIZINES A AND B, POTENTIATORS OF CYTOTOXICITY OF VINCRISTINE

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We have recently discovered a complex of new isotetracenone group antibiotics, rubiginones, in our screening for vincristine (VCR)-induced cytotoxicity potentiators[1]. In the continuation of the screening program, an actinomycete strain Q576-2 identified as *Streptoverticillium aspergilloides*, was found to produce a new active substance. Isolation and chemical studies of the responsible substance designated piperafizine showed that it was a complex of two components A and B structurally classified in the diketopiperazine family. This note presents a brief discussion of the producing organism, production, isolation, chemical properties and biological activities of piperafizines A and B.

Strain Q576-2 was isolated from a soil sample collected in the Fiji Islands. The aerial mycelia bore tufted short straight spore-chains on a biverticillate sporophore. The spores were oblong (0.6 by 1.0 to 2.5 μm) with a smooth surface. The color of aerial mycelium was white at first and later turned to light gray or light yellow. The strain grew between 16°C and 41°C and did not produce melanoid, prodigiosin or other diagnostic pigments. The following tests were positive: Tolerance to 7% NaCl; production of gelatinase, amylase and nitrate reductase; utilization of D-fructose, *myo*-inositol and D-mannitol. Negative responses were: Production of tyrosinase; utilization of L-arabinose, D-xylose, L-rhamnose, sucrose and raffinose. The whole-cell hydrolysate contained LL-diaminopimelic acid; the phospholipids had phosphatidylethanolamine indicating that the strain belonged to type I cell wall and type P-II phospholipid. Based on the above results, strain Q576-2 was identified as *S. aspergilloides*[2~4].

Piperafizine was produced by shake flask or tank fermentation using a medium composed of soluble starch 2%, soybean meal 1%, NaCl 2% and $CaCO_3$ 0.5%. In a tank fermenter (200 liters), fermentation was carried out at 28°C with agitation of 250 rpm and aeration of 120 liters/minute; 8 μg/ml of activity was obtained after 90 hours. The culture broth (220 liters) was extracted with BuOH (100 liters) and the extract was concentrated to an aqueous solution which was extracted with dichloromethane. After concentration, the active principle was purified by successive column chromatography on Diaion HP-20 (acetone), silica gel (dichloromethane and dichloromethane - hexane). With monitoring by TLC (SiO_2, dichloromethane), homogeneous solids of piperafizines A (1.20 g) and B (150 mg) were isolated and crystallized from MeOH (piperafizine A) and DMF (piperafizine B).

The physico-chemical properties of both components are summarized in Table 1. The data of piperafizine B were nearly identical with those of 3*Z*,6*Z*-dibenzylidene-2,5-dioxopiperazine[5,6] and identity was confirmed by a direct comparison with an authentic synthetic sample[7]. Piperafizine A showed molecular weight of 304, 14 mass units (a

Table 1. Physico-chemical properties of piperafizines A and B.

	Piperafizine A		Piperafizine B
Nature	Pale yellow needles		Light yellow plates
MP (°C)	181~182		299~300
Molecular formula	$C_{19}H_{16}N_2O_2$		$C_{18}H_{14}N_2O_2$
Microanalysis	Calcd:	Found:	
C	74.98	74.99	
H	5.30	5.28	
N	9.20	9.36	
EI-MS (*m/z*) (M^+)	304		290
UV λ_{max}^{MeOH} nm (ε)	260 (6,800), 326 (20,000)		234 (8,700), 338 (34,000)
TLC SiO_2, CH_2Cl_2	Rf 0.31		Rf 0.29
Color reaction			
+:	Iodine, H_2SO_4, $FeCl_3$, Dragendorff, Rydon-Smith		
−:	Ninhydrin, Sakaguchi		

Table 2. Potentiation of vincristine (VCR)-induced cytotoxicity.

Compound	Conc (μg/ml)	Vehicle	Potentiation index[a]		
			Moser	P388/S	P388/VCR
Piperafizine A	2.0	0.06% DMSO	14.8	3.1	4.1
Piperafizine B	10.0	0.5% acetone+ 0.5% DMSO	1.8	1.2	7.0
Di-*N*-methyl-piperafizine B	2.0	0.5% acetone+ 0.5% DMSO	4.4	1.5	5.4
Verapamil	2.0	H_2O	20.2	5.1	39.6

[a] Potentiation index: IC_{50} of VCR alone/IC_{50} of VCR in the presence of potentiator (the data are the mean of three experiments).

P388/S: VCR-sensitive P388. P388/VCR: VCR-resistant P388.

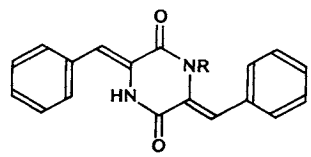
IC_{50} of VCR: 0.196 μg/ml (Moser), 0.0065 μg/ml (P388/S), 0.033 μg/ml (P388/VCR).

methyl) higher than that of piperafizine B and closely related physico-chemical and spectral properties. The methyl group was deduced to be on a nitrogen atom rather than on an oxygen atom based on its chemical shifts in the NMR (δ_H 2.83 and δ_C 36.8). In fact, piperafizine A was distinctly different from neihumicin, a cytotoxic antibiotic with 3*Z*,6*Z*-dibenzylidene-2-methoxy-3,6-dihydropyrazin-5-one structure by direct comparison[8].

Upon catalytic hydrogenation, piperafizine A yielded a complex of two tetrahydro derivatives (stereoisomers) which was hydrolyzed with 6 N HCl to afford phenylalanine and *N*-methylphenylalanine. When treated with dimethyl sulfate, both piperafizines A and B produced an identical di-*N*-methyl derivative. Thus 3*Z*,6*Z*-dibenzylidene-1-methyl-2,5-dioxopiperazine was assigned to piperafizine A (Fig. 1). Although a synthesis of this molecule was recently reported[9], this is the first isolation from a natural source.

Table 2 shows the effect of piperafizines A and B and verapamil on cytotoxicity of VCR. Since piperafizines A and B exhibited weak cytotoxicity with IC_{50}s of 6.6 and 23.8 μg/ml against Moser cells, 4.0 and 22.6 μg/ml against P388 cells, and 3.2 and 23.7 μg/ml against VCR-resistant P388 cells, respectively, practically non-cytotoxic concentrations of 2 and 10 μg/ml were used for potentiation tests of piperafizines A and B, respectively. Piperafizine A markedly enhanced the cytotoxicity of VCR against Moser cells and moderately against the VCR-sensitive and VCR-resistant P388, while piperafizine B showed a slight potentiation effect only against the VCR-resistant P388 cells. The di-*N*-methyl derivative synthesized from the natural components also exhibited slight potentiation effect. Although their activity was considerably lower than that of verapamil tested as a reference, piperafizines significantly increased the accumulation of vincristine in both Moser and P388/VCR cells in our preliminary experiments. In leukemia-bearing mice, piperafizine A significantly increased life span when used in combination with VCR.

Fig. 1. Structures of piperafizines A and B.



Piperafizine A R=$-CH_3$
Piperafizine B R=$-H$

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