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Oximidine III, a New Antitumor Antibiotic against Transformed Cells from *Pseudomonas* sp.

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activity

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Our screening for antitumor antibiotics against transformed cells resulted in the isolation of a new active metabolite, oximidine III, from *Pseudomonas* sp. QN05727. This substance selectively inhibited the growth of rat 3Y1 fibroblasts transformed with various oncogenes. In *ras*- or *src*-transformed cells, oximidine III arrested the cell cycle at G1 phase and increased the expression of $p21^{WAF1}$.

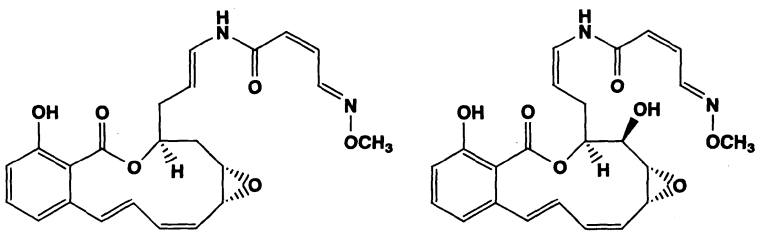
Recent studies have demonstrated that most of oncogenes are functionally linked to cell-cycle regulators including cyclins, cyclin-dependent kinase (CDK) inhibitors and retinoblastoma protein[1~3]. Thus, cell-cycle inhibitors in oncogene-transformed cells may act as selective anticancer agents. In the course of our screening for antitumor antibiotics against transformed cells, a new cell-cycle inhibitor structurally-related to oximidine I[4] was isolated from the culture of *Pseudomonas* sp. QN05727 and designated as oximidine III (Fig. 1). We report here the fermentation, isolation, physico-chemical properties and biological activity of oximidine III as well as the taxonomy of the producing organism. The structure elucidation of oximidine III is described in the accompanying paper[5].

Materials and Methods

Cells and Cell Culture

Rat 3Y1 fibroblasts and 3Y1-derived cell lines[6~8] were obtained from the Japanese Cancer Research Resources Bank (JCRB). The cells were cultured in DULBECCO's modified EAGLE's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose.

Fig. 1. Structures of oximidines III and I.



Oximidine III Oximidine I

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MTT Assay

Cells at 50% confluence were plated at one tenth lower cell density and incubated for 3 days with various concentrations of a sample. The growth was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C.

Flow Cytometry

One day after plating at 1×10^3 cells/cm^2, the cells were incubated with or without 4 nM of oximidine III for 3 days. The cells were trypsinized, fixed in 70% ethanol, and stained with 50 μg/ml of propidium iodide. Flow cytometric analysis was performed using a Coulter EPICS XL instrument.

Western Blotting

Expression of $p21^{WAF1}$ was assessed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Drug-treated cells were lysed with 100 μl of a lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 30 mM Na-pyrophosphate, 50 mM NaF, 1 mM Na-orthovanadate, 0.1 mM Pefabloc and 5 μg/ml leupeptin (pH 7.4). After 45 minutes of gentle agitation at 4°C, insoluble materials were cleared by centrifugation. An aliquot of each sample was loaded onto an SDS-PAGE system. The protein level was detected by Western blotting using a specific antibody against $p21^{WAF1}$ (C-19, Santa Cruz Biotechnology).

Vacuolar-type H^+-ATPase (V-ATPase) Activity

Intracellular acidic organelles were stained with acridine orange for V-ATPase activity. Cells were incubated at 37°C for 1 hour on coverslips with or without a test sample. The cells were further incubated for 1 hour with 5 μg/ml acridine orange. After three washes with a HANKS' solution, the coverslips were examined with a fluorescence microscope.

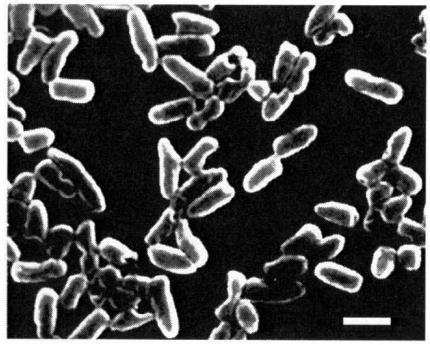
Results

Taxonomy

Bacterial strain QN05727 was isolated from a soil sample collected in Iriomote Island, Okinawa Prefecture, Japan. The strain was an aerobic Gram-negative, non-sporulating rod (0.7~0.9×1.1~2.9 μm) and was motile with polar flagella (Fig. 2).

The physiological characteristics are listed in Table 1. The strain grew between 4 and 32°C and produced a soluble fluorescent pigment. The oxidation-fermentation test revealed it to be of oxidative type. It gave positive results for nitrate reduction, citrate utilization, ammonium utilization, oxidase, catalase, Tween 80 hydrolysis, arginine dihydrolase and gelatin liquefaction. Acid formation was observed from L-arabinose, D-xylose, D-glucose, D-mannose, D-galactose and glycerol.

Fig. 2. Scanning electron micrograph of *Pseudomonas* sp. QN05727.



Bar represents 1 μm.

According to BERGEY's Manual of Systematic Bacteriology (1989), the strain was identified as a *Pseudomonas* sp. Strain QN05727 was deposited in the International Patent Organism Depositary, Japan, under the accession number of FERM P-18713.

Fermentation

The producing organism was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a seed medium and incubated on a rotary shaker at 28°C for 3 days. The seed medium was composed of glucose 1.0%, starch 2.0%, Polypepton 0.5%, yeast extract 0.5% and calcium carbonate 0.4% (pH 7.0). The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of glycerol 3.0%, glucose 0.1%, Polypepton 0.5%, meat extract 0.5% and sodium chloride 0.5% (pH 7.0). The fermentation was carried out at 28°C for 3 days on a rotary shaker at 220 rpm.

Isolation

The culture supernatant (2 liters) and the mycelial acetone extract were combined and extracted with ethyl acetate. The extract was chromatographed on a silica gel

Table 1. Physiological characteristics of strain QN05727.

Nitrate reduction	+	Acid production from	
Denitrification	-	L-arabinose	+
Methyl red test	-	D-xylose	+
Voges-Proskauer test	-	D-glucose	+
Indole production	-	D-mannose	+
H_2S production	-	D-fructose	-
Starch hydrolysis	-	sucrose	-
Citrate utilization	+	inositol	-
Nitrate utilization	-	D-mannitol	-
Ammonium utilization	+	D-galactose	+
Soluble fluorescent pigment	+	maltose	-
Urease	-	trehalose	-
Oxidase	+	lactose	-
Catalase	+	D-sorbitol	-
Growth temperature	4 ~ 32°C	glycerol	+
Optimum temperature	20 ~ 28°C	starch	-
Growth pH	5 ~ 9	Utilization of	
Optimum pH	6 ~ 8	L-arabinose	+
Anoxic growth	-	D-xylose	-
Oxidative-fermentative test	oxidative	D-glucose	+
Arginine dihydrolase	+	D-mannose	+
Growth in 3% NaCl	+	D-fructose	+
Tween 80 hydrolysis	+	sucrose	-
DNase	-	inositol	+
β-Galactosidase	-	rhamnose	-
Esculin hydrolysis	-	raffinose	-
		D-mannitol	+
		D-galactose	-
		maltose	±
		trehalose	+
		lactose	-
		D-sorbitol	-
		salicin	-
		melibiose	-
		glycerol	+
		starch	±
		xanthine	-
		chitin	-

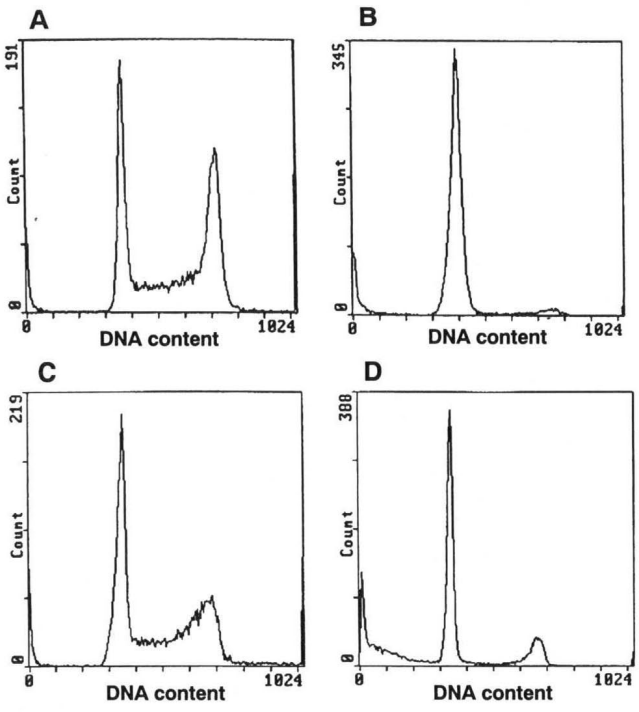
Table 2. Physico-chemical properties of oximidine III.

Appearance		colorless powder
MP		87 ~ 89°C
$[\alpha]_D^{22}$		-80° (*c* 0.027, MeOH)
Molecular formula		$C_{23}H_{24}N_2O_6$
HRFAB-MS (m/z)	Found:	425.1725 $(M+H)^+$
	Calcd.:	425.1713
UV λ_{max} nm (ε)	MeOH	276 (26,600)
	0.01M NaOH-MeOH	210 (23,400), 272 (28,000)
IR ν_{max} (KBr) cm^{-1}		3430, 1700, 1660

Table 3. IC_{50} values of oximidines III and I against normal and transformed 3Y1 rat fibroblasts (nM).

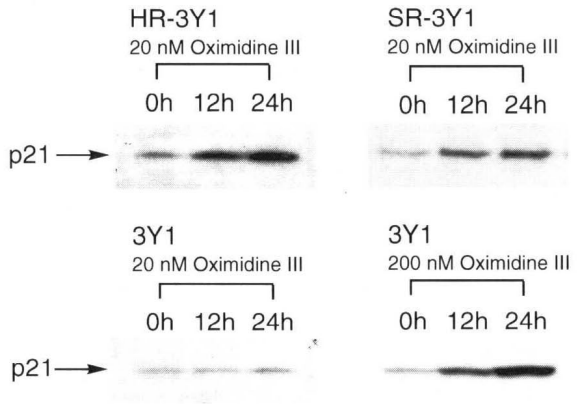
Cell line	Oncogene	Oximidine III	Oximidine I
3Y1		140	610
HR-3Y1	v-H-*ras*	14	49
SR-3Y1	v-*src*	4.5	38
SV-3Y1	SV40 large T	19	85
E1A-3Y1	E1A	24	110
Ad12-3Y1	E1A, E1B	31	210

Fig. 3. Flow cytometric cell-cycle analysis of oximidine III-treated cells.



HR-3Y1 cells (A, B) and SR-3Y1 cells (C, D) were incubated with (B, D) or without (A, C) 4 nM of oximidine III for 3 days and then propidium iodide-stained cells were analyzed with a flow cytometer.

Fig. 4. Effect of oximidine III on $p21^{WAF1}$ expression in normal and transformed 3Y1 cells.



column with chloroform - methanol (50 : 1). The active eluate was purified by HPLC using a PEGASIL-ODS column (20 mm×250 mm) with 65% methanol. The active fraction was evaporated to dryness to yield a colorless powder of oximidine III (1.5 mg).

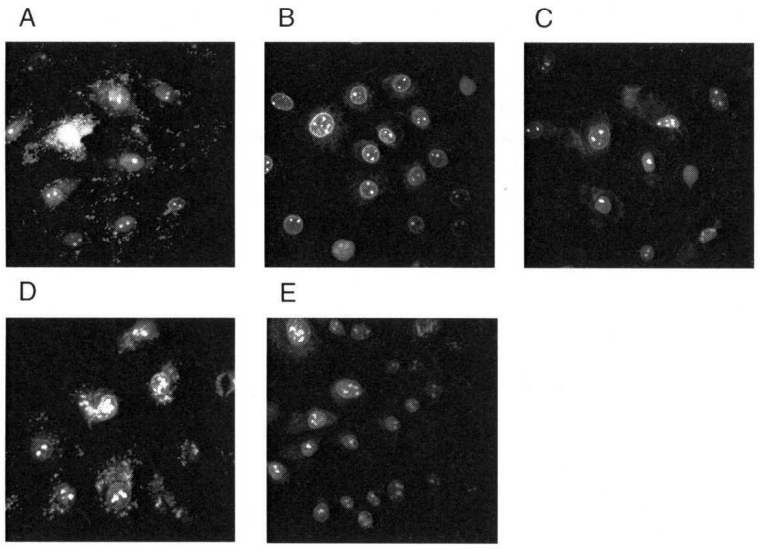
Physico-chemical Properties

The physico-chemical properties of oximidine III are summarized in Table 2. The molecular formula was established to be $C_{23}H_{24}N_2O_6$ from high-resolution FAB-MS. The IR spectrum revealed the presence of hydroxyl (3430 cm^{-1}), ester carbonyl (1700 cm^{-1}) and amide carbonyl (1660 cm^{-1}) groups.

Biological Activity

The antitumor effects of oximidines III and I were investigated using normal and transformed 3Y1 rat fibroblasts (Table 3)[6~8]. Oximidines III and I inhibited the

Fig. 5. Acridine orange-stained acidic organelles in 3Y1 and HR-3Y1 cells.



3Y1 cells (A, B, C) and HR-3Y1 cells (D, E) were incubated with 200 nM of oximidine III (B, E), with 1 μM of bafilomycin A1 (C) or without drugs (A, D) for 1 hour and then acridine orange-stained cells were observed with a fluorescence microscope.

growth of 3Y1 cells transformed with various oncogenes at lower concentrations than that of the parent 3Y1 cells. Flow cytometric analysis revealed that oximidine III arrested the cell cycle of *ras*-transformed cells (HR-3Y1) and *src*-transformed cells (SR-3Y1) at G1 phase as shown in Fig. 3.

We have reported that the cyclin-dependent kinase inhibitor $p21^{WAF1}$ was up-regulated by vacuolar-type H^+-ATPase (V-ATPase) inhibitors including oximidine I[9,10]. As shown in Fig. 4, increased expression of $p21^{WAF1}$ was observed in oximidine III-treated cells. Transformed cells reveled higher sensitivity to $p21^{WAF1}$ induction by oximidine III. V-ATPase maintains low pH of intracellular acidic organelles and the activity is detected as orange fluorescent pigments by staining with acridine orange[11]. Intracellular acidic organelles in 3Y1 cells or HR-3Y1 cells were clearly decreased by treatment with oximidine III as observed in the cells treated with bafilomycin A1[11], a structurally unrelated V-ATPase inhibitor (Fig. 5). Studies on further biological activity of oximidine III are in progress.

Acknowledgments

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