A New Anthracycline Antibiotic, IT-62-B, Converts the Morphology of *ras*-Transformed Cells Back to Normal:

Taxonomy, Fermentation, Isolation, Structure Elucidation and Biological Characterization

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A new antibiotic, IT-62-B was isolated from the culture broth of *Streptomyces* sp. IT-62 by extraction with acetone and then with ethyl acetate, followed by conventional column chromatography using silica gel, Sephadex LH-20 and silica ODS. Its structure ($C_{39}H_{47}NO_{15}$, MW 769) was determined by ^{1}H, ^{13}C NMR, MS, IR and UV spectrometric techniques to be a new member of the baumycin-group anthracyclines. It showed moderate activity against Gram-positive bacteria and had antitumor activity against various tumor cell lines. Further, antibiotic IT-62-B converted the morphology of *ras*-transformed NIH3T3 cells and T-cells back to normal at concentrations inhibiting cell growth by 30% or more.

Research on oncogenes is proceeding rapidly. For example, many human tumors appear to be associated with the expression of activated *ras*-genes that encode the p21 protein. Further, many reports have supported the relationship between the level of expression of the *ras*-oncogene and tumorigenic potential. Hence, inhibitors of *ras*-gene functions have been widely sought for a possible new type of antitumor chemotherapy targeting the signaling transduction pathways where *ras* p21 is involved[1], *i.e.*, *ras*-specific chemotherapy. For this reason, as a means of finding potential antitumor antibiotics, we have started to screen the fermentation broth of various microorganisms for a new class of compounds that inhibits the function of such oncogenes and thereby suppresses the expression of transformed phenotypes.

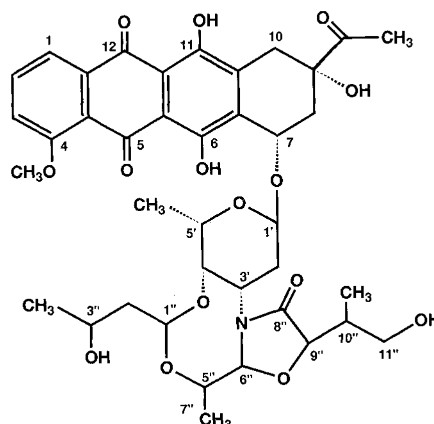
As a result of our screening for new antitumor antibiotics, we found a new anthracycline, IT-62-B (Fig. 1), mainly in the culture mycelia of *Streptomyces* sp. IT-62, a new soil isolate, which can convert the morphology of *ras*-transformed mouse NIH3T3 cells back to that of the normal cells. In this paper, we describe the taxonomic characteristics of the producing organism, fermentation, isolation, physico-chemical properties, structure elucidation and biological activities of IT-62-B.

Taxonomy of the Producing Strain

The strain IT-62 was isolated from a soil sample collected at Wulumuqi in Xinjiang Autonomous Province, China. Taxonomic properties were determined according to the method of the International Streptomyces Project (ISP)[2] by the use of the media recommended by WAKSMAN[3]. Color names and hue numbers were assigned according to the "Color Harmony Manual"[4].

Microscopic observation showed the aerial mycelium of strain IT-62 to be straight to flexuous and the mature spore chain to have 10 to 50 or more spores per chain

Fig. 1. Structure of antibiotic IT-62-B.

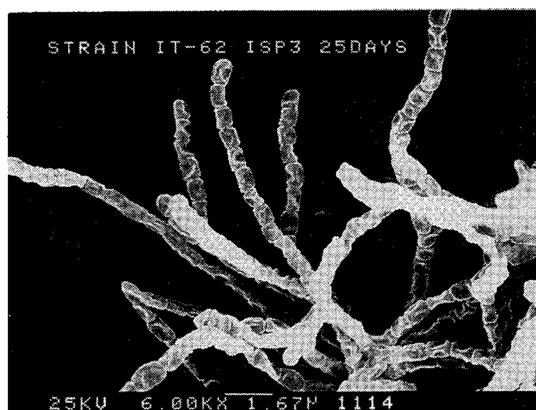


(Fig. 2). The spores were cylindrical in shape with a smooth surface and $0.5\sim0.8\times0.7\sim1.1\,\mu m$ in size. Sclerotia, sporangia and flagellated spores were not observed. The aerial mycelium was powdery and characteristically whitish to pale yellow or yellowish gray in color on inorganic salts - starch agar and oatmeal agar. The vegetative mycelium exhibited a reddish-orange series of color on most of the media. Melanoid pigment was produced, and a soluble pigment was slightly orangish.

The cultural characteristics, physiological properties, and utilization of carbon sources of strain IT-62 examined according to the method of PRIDHAM and GOTTLIEB[5] are shown in Tables 1, 2 and 3, respectively.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. IT-62.

Bar represents $1\,\mu m$.



Cultures were observed during incubation at 27°C for 21 days. The chemical analysis of whole-cell hydrolysate conducted by the method of NIMURA[6], showed the presence of LL-diaminopimelic acid.

Based on the taxonomic properties as described above, strain IT-62 was considered to belong to the genus *Streptomyces*. This strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as *Streptomyces* sp. IT-62 with accession number FERM BP-4666.

Fermentation

Strain IT-62 was inoculated into 100 ml of the seed medium in a 500-ml Erlenmeyer flask and incubated at 27°C for 2 days on a rotary shaker at 220 rpm. The seed medium (pH 7.2) consisted of glucose 0.5%, soluble starch 2.4%, beef extract 0.3%, yeast extract 0.5%, peptone 0.5%, corn steep liquor 0.4%, $CoCl_2\cdot 6H_2O$ 0.002% and $CaCO_3$ 0.4%. Two milliliters of the seed culture obtained was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the fermentation medium (pH 7.2) comprising glucose 0.5%, dextrin 2.5%, sesame meal 2.0%, corn steep liquor 0.5%, K_2HPO_4 0.05%, $MgSO_4\cdot 7H_2O$, 0.05%, KCl 0.03% and $CaCO_3$ 0.3%. The fermentation was carried out at 27°C for 5 days on a rotary shaker at 220 rpm. The production of IT-62-B was traced by measurement of its cytotoxic and antibacterial activities against KB carcinoma cells and

Table 1. Cultural characteristics of strain IT-62.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose - nitrate agar (Waksman med. No. 1)	Colorless	Colorless	None	None
Glucose - asparagine agar (Waksman med. No. 2)	Brite coral red ~ chinese red [6pa ~ 6pc]	Brite coral red ~ coral [6pa ~ 6lc]	None	Orangish
Yeast extract - malt extract agar (ISP med. No. 2)	Red wood [6ne]	Red wood [6ne]	White, scant	Orangish
Oat meal agar (ISP med. No. 3)	Copper [5lc]	Luggage tan [4ne]	Ivoly tint ~ lt citron gray [2cb ~ 1ec], powdery, thin	Orangish
Inorganic salts - starch agar (ISP med. No. 4)	Rust tan [5le]	Rust tan [5le]	Flesh pink [4ca], powdery, thin	Orangish
Glycerol - asparagine agar (ISP med. No. 5)	Mustard [2le]	Mustard [2le]	Whitish ~ lt ivoly [2ca], powdery, thin	None
Tyrosine agar (ISP med. No. 7)	Camel [3ie]	Golden brown [3pg], topaz [3ne]	Sand ~ biscuit [3cb ~ 2ec], powdery, thin	Brownish
Nutrient agar (Waksman med. No. 14)	Camel [3ie], copper [5lc]	Camel [3ie], postal orange [4ic]	Whitish, scant	Orangish
BENNET's agar	Chinese red ~ tomato red [6pc ~ 6½pc]	Lt lacquer red ~ chinese red [6nc ~ 6pc]	None	Orangish

Table 2. Physiological characteristics of strain IT-62.

Test	Results
Starch hydrolysis on ISP med. No. 4	Positive
Nitrate reduction (Difco, nitrate broth)	Positive
Milk (Difco, 10% skimmed milk)	
Coagulation	Positive
Peptonization	Positive
Gelatin liquefaction (20°C)	
On plain gelatin	Positive
On glucose-peptone gelatin	Positive
Gelatin liquefaction (27°C)	
On plain gelatin	Negative
On glucose-peptone gelatin	Negative
Melanin formation on ISP med. No. 7	Positive
on ISP med. No. 6	Positive
on ISP med. No. 1 +2% agar	Weakly positive
Temperature range for growth	20～37°C
Optimum temperature on ISP med. No. 4	20～27°C
Production of H_2S on ISP med. No. 6	Positive
on ISP med. No. 6 +yeast extract	Positive
Decomposition of cellulose	Negative
Growth in NaCl	1～7%

Table 3. Utilization of carbon sources by strain IT-62.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	−
Sucrose	−
D-Fructose	+
D-Mannitol	+
L-Rhamnose	−
Raffinose	−
D-Galactose	+
Salicin	−
Soluble starch	+
Dextrin	+
Glycerol	+
Maltose	+

+, Positive; −, negative.

Micrococcus luteus ATCC9341.

Isolation

The fermentation broth (40 liter) was centrifuged at 2800 rpm to separate the mycelial cake from the broth fluid. The mycelial cake was extracted twice with acetone and then the extract was concentrated to a small volume to remove acetone. This concentrate was adjusted to pH 8 and extracted twice with ethyl acetate. The ethyl acetate layer was dried with anhydrous Na_2SO_4 and evaporated to give an oily residue. The residue was dissolved in $CHCl_3$, and *n*-hexane was added to obtain the precipitate (2.7 g). This precipitate was dissolved in $CHCl_3$ and subjected to silica gel column (20 × 4.1 cm, i.d., Merck) chromatography. The column was washed with $CHCl_3$ and thereafter eluted with $CHCl_3$ - MeOH (50 : 1). The active fractions containing IT-62-B were combined and concentrated to give an oily residue (155 mg). A portion of the residue (75 mg) was chromatographed on a Sephadex LH-20 column (92 × 2.0 cm, i.d., Pharmacia AB) with $CHCl_3$ - MeOH (1 : 1) as an eluant. The eluted fractions containing IT-62-B were concentrated *in vacuo* to give an oily residue (26 mg). The crude residue was further purified by ODS column (Ultrapack ODS, 50 × 1.0 cm, i.d., Yamazen Co. Ltd.) chromatography with CH_3CN - H_2O (2 : 3) used as an eluant at a flow rate of 1.5 ml/minute. The fractions containing IT-62-B were collected, evaporated to remove the organic solvent, and lyophilized to give the purified IT-62-B (12 mg) as a red powder. The progress of purification as mentioned above was monitored by HPLC with an Inertsil ODS-2 column (GL Sciences) under the conditions indicated in the foot note of Table 4. During purification of IT-62-B, antibiotic TAN-1120[7] was simultaneously isolated as IT-62-A by conventional means from active fractions eluted stepwise with $CHCl_3$ - MeOH on silica gel column chromatography.

Table 4. Physico-chemical properties of IT-62-B.

Appearance	Red powder
Melting point	153～155°C
Molecular formula	$C_{39}H_{47}NO_{15}$
FAB-MS (*m/z*)	770 $(M+H)^+$
HR FAB-MS (*m/z*)	Found (Δ mmu) 792.2938 $(M+Na)^+$ (+9.5)
	Calcd. 792.2843
$[\alpha]_D^{23}$	+360° (*c* 0.015, MeOH)
UV λ_{max}^{MeOH} nm (ε)	233 (33,100), 251 (23,400), 289 (6,600), 480 (11,300), 495 (11,400), 530 (6,200 sh)
IR ν_{max} (KBr) cm^{-1}	3435, 2970, 2935, 1710, 1620, 1580, 1415, 1285, 1210, 1120, 1035, 995
Solubility	Soluble in MeOH, $CHCl_3$, dimethylsulfoxide
	Insoluble in H_2O, *n*-hexane
HPLC Rt (minute)[a]	6.9

[a] HPLC conditions: Column: Inertsil ODS-2 (150 × 4.6 mm, i.d.), mobile phase: CH_3CN - 0.05% TFA (50 : 50), flow rate: 1.0 ml/minute, detection: UV at 210 nm.

Physico-chemical Properties and Structure Elucidation

Physico-chemical properties of IT-62-B are summarized in Table 4. Its UV-visible spectrum in MeOH solu-

Table 5. ^{1}H and ^{13}C NMR data of IT-62-B (400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$, 30°C).

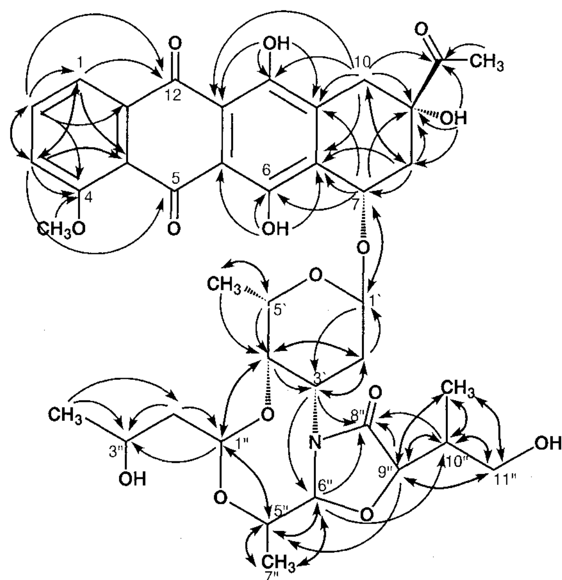
Position	^{1}H (ppm)		^{13}C (ppm)		HMBC (^{1}H-^{13}C)	NOE
1	8.01	d, 8 Hz	119.80	d	C-3, 4, 4a, 12	H-2
2	7.77	t, 8 Hz	135.65	d	C-1, 3, 4, 4a, 12, 12a	H-1, 3
3	7.38	d, 8 Hz	118.36	d	C-1, 2, 4, 4a, 5	H-2, OCH_3-4
4			161.03	s		
4a			120.95	s		
5			187.03	s		
5a			111.38	s		
6			156.41	s		
6a			134.14	s		
7	5.27	dd, 4, 1 Hz	69.68	d	C-6, 6a, 9, 10a, 1′	H-8, 1′
8	2.12, 2.36	dd, 15, 4 Hz dt, 15, 1 Hz	35.13	t	C-6a, 7, 9, 10	H-7, 5′
9			76.61	s		
10	2.91, 3.22	d, 19 Hz dd, 19, 1.5 Hz	33.36	t	C-6a, 8, 9, 10a, 11, 11a, 13	
10a			134.64	s		
11			155.92	s		
11a			111.21	s		
12			186.59	s		
12a			135.55	s		
13			212.19	s		
14	2.44	s	24.87	q	C-13	
OCH_3-4	4.07	s	56.64	q	C-4	H-3
OH-6	13.94	s			C-5a, 6, 6a	
OH-9	4.49	br s			C-8, 9, 13	
OH-11	13.23	s			C-10a, 11, 11a	
1′	5.60	d, 4 Hz	100.67	d	C-7, 3′	H-7, 2′
2′	1.61, 2.64	dd, 13.5, 4 Hz td, 13.5, 4 Hz	28.70	t	C-1′, 3′, 4′	H-1′, 3′, 6″
3′	4.61	dd, 13.5, 4 Hz	50.41	d	C-2′, 6″, 8″	H-2′, 4′, 5′
4′	3.61	s	79.18	d	C-2′, 3′, 1″	H-3′, 5′, 6′, 1″
5′	4.29	q, 6.5 Hz	69.51	d	C-4′, 6′	H-8, 3′, 4′, 6′
6′	1.26	d, 6.5 Hz	17.17	q	C-4′, 5′	H-4′, 5′
1″	4.74	dd, 7, 4 Hz	106.53	d	C-4′, 3″, 5″	H-4′, 2″, 5″
2″	1.78, 1.85	ddd, 14, 9.5, 4 Hz ddd, 14, 7, 3 Hz	45.64	t	C-1″, 2″, 3″	H-1″, 4″
3″	4.03	m	64.38	d		H-2″, 4″
4″	1.23	d, 6 Hz	23.97	q	C-2″, 3″	H-2″, 3″
5″	3.48	dq, 8, 6.5 Hz	80.37	d	C-1″, 6″, 7″	H-1″, 7″
6″	4.70	d, 8 Hz	91.03	d	C-5″, 7″, 8″, 10″	H-2′, 7″
7″	1.31	d, 6.5 Hz	17.77	q	C-5″, 6″	H-5″, 6″
8″			173.18	s		
9″	4.15	d, 4.5 Hz	80.20	d	C-5″, 8″, 10″, 11″, 12″	H-10″, 12″
10″	2.12	m	38.02	d	C-8″, 9″, 11″, 12″	H-9″, 11″, 12″
11″	3.58, 3.65	dd, 11, 7.5 Hz dd, 11, 4.5 Hz	64.21	t	C-9″, 10″, 12″	H-10″, 12″
12″	0.98	d, 7 Hz	12.44	q	C-9″, 10″, 11″	H-9″, 10″, 11″

tion, and intense absorptions (1710, 1620, 1580 cm^{-1}) in IR spectrum were characteristic for anthracyclines such as daunomycin. It showed a quasi-molecular ion peak at m/z 770 $(M+H)^+$ by FAB-MS. The molecular formula was determined to be $C_{39}H_{47}NO_{15}$ from high-resolution FAB-MS and ^{13}C NMR spectra.

The ^{1}H and ^{13}C NMR data of IT-62-B are summarized in Table 5. These data showed the partial structure corresponding to its aglycone and amino sugar moieties to be identical with those of daunomycin or baumycins. Furthermore, the spectra showed close similarity to those of the known anthracycline TAN-1120, which was simultaneously isolated as a related component during this study. The COSY and HOHAHA experiments revealed the presence of an additional C5 unit in the molecule, which was assigned to be a 2,4-dihydroxy-isovaleryl group. NOZAKI *et al.*[7] reported TAN-1120 to be a labile compound due to its characteristic substructure, *i.e.*, a carbinolamine group. However, IT-62-B is considerably more stable; in addition, its proton signals at H-3′ (4.61 ppm) and H-6″ (4.70 ppm) were significantly shifted lower than those of TAN-1120. These results indicate a structural change in the carbinolamine group in IT-62-B.

Finally, detailed HMBC analysis proved the presence of an oxazolidine ring consisting of a nitrogen atom of daunosamine, methine carbon at C-6″, and a 2,4-dihydroxyisovaleryl group, as illustrated in Fig. 3.

Fig. 3. Correlations of C-H long-range couplings observed in the HMBC experiment of IT-62-B.



Arrows indicate the correlation from ^{1}H to ^{13}C.

Observed C-H long-range coupling from H-3′ to C-8″ clearly indicated the 4-oxo-oxazolidine structure. Consequently, the novel structure of IT-62-B was determined to be that shown in Fig. 1.

Biological Characterization

The *in vitro* antimicrobial activity of IT-62-B was determined by a serial agar dilution method using Mueller-Hinton agar (Difco) after incubation for 18 hours at 37°C. As shown in Table 6, IT-62-B showed activity against most of the Gram-positive bacteria tested, but no activity against Gram-negative bacteria except *E. coli* NIHJ. When it was tested with recombination-deficient mutants of *Bacillus subtilis* by the *rec* assay for the detection of DNA-damaging substances, the repair mutant (M-17) was more sensitive to IT-62-B than the parent strain (M-45). The cytotoxicity activity (IC_{50}) was estimated by the dye-uptake method against P388, L1210, doxorubicin-resistant P388 leukemia cells (P388/ADR), and KB carcinoma cells in EAGLE's minimal essential medium (Flow Laboratories) supplemented with 10% fetal calf serum (Gibco Laboratories, MEM·FCS) at 37°C. When the cells ($1 \sim 2 \times 10^3$ cells/well) were exposed to the antibiotic for 3 days in 96-well multiplates, the results shown in Table 7 were obtained and compared with those of doxorubicin. The cytotoxicity of IT-62-B resembled that of doxorubicin, although the cytotoxicity of IT-62-B against doxorubicin-resistant P388 cells is more potent than that of doxorubicin. The antitumor effect *in vivo* was examined by a single intraperitoneal administration to CDF_1 mice intraperitoneally implanted with 1×10^6 P388 cells. IT-62-B showed a significant antitumor effect of 119% (Increased Life Span) at a dose of 0.25 mg/kg, whereas doxorubicin was required at 7 mg/kg for a similar effect (116% ILS).

Evidence for cell morphological reversion was sought by microscopic observation of *ras*-NIH3T3 cells in DULBECCO's modified EAGLE's medium supplemented with 10% heat-inactivated calf serum (Gibco) at 37°C and of human bladder carcinoma T-24 cells in MEM·FCS in 5% CO_2-containing humidified atomosphere in air at 37°C, according to the method described by KANBE *et al.*[16]. As shown in Fig. 4, IT-62-B caused a flattening of NIH3T3 cells that had been transformed by human

Table 6. Antimicrobial activity of IT-62-B.

Test organism	MIC (μg/ml)
	IT-62-B
Staphylococcus aureus Smith	6.25
Staphylococcus aureus (MRSA) 70	12.5
Staphylococcus epidermidis ATCC 12228	25
Enterococcus faecalis IFO 12968	50
Micrococcus luteus ATCC 9341	3.13
Micrococcus luteus ATCC 10240	1.56
Bacillus subtilis ATCC 6633	3.13
Bacillus subtilis (rec⁺)	6.25
Bacillus subtilis (rec⁻)	0.39
Escherichia coli NIHJ	100
Proteus vulgaris IID OX-19	>100
Klebsiella pneumoniae ATCC 10031	>100
Serratia marcescens IFO 12648	>100
Pseudomonas aeruginosa NCTC 10490	>100

The MIC was determined by the serial dilution method using Mueller-Hinton agar at an inoculum of 10^6 cells/ml after an 18-hour incubation at 37°C.

Table 7. *In vitro* cytotoxicity of IT-62-B and doxorubicin (ADR).

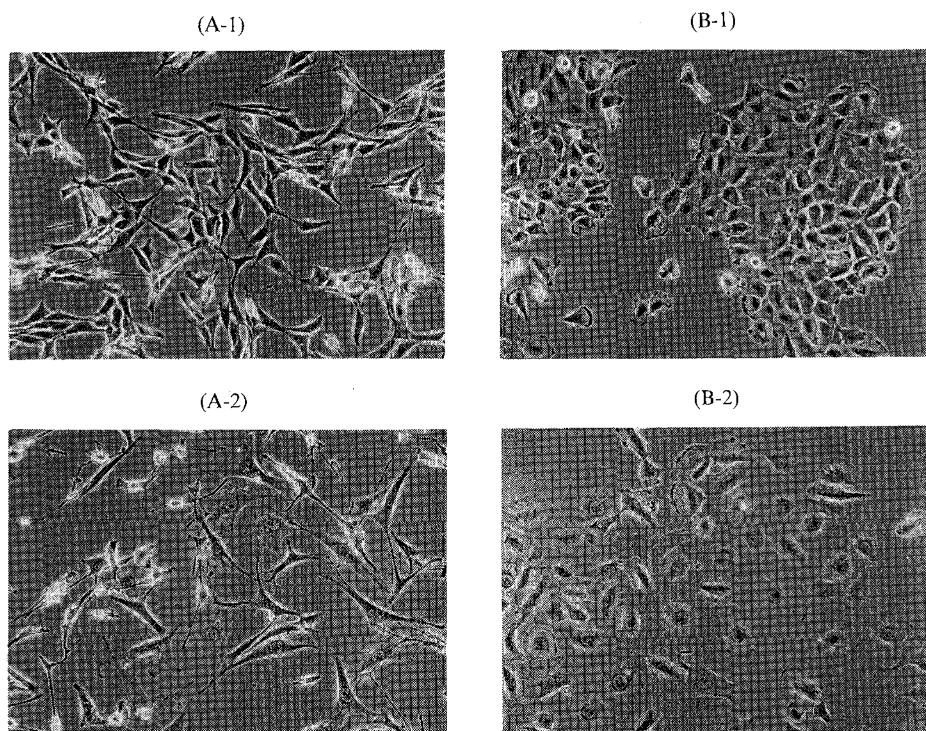
Compound	IC_{50} (μg/ml)			
	L1210	P388	P388/ADR[a]	KB
IT-62-B	0.04	0.006	0.04	0.006
Doxorubicin (ADR)	0.02	0.003	0.1	0.004

L1210 (murine leukemia cells), P388 (murine lymphocytic leukemia cells), KB (human nasopharyngeal carcinoma cells).

[a] ADR-resistant cell lines.

Fig. 4. Phase-contrast micrographs of K-*ras* NIH3T3 cells and T-24 cells.

(A-1): K-*ras* NIH3T3 cells as control, (A-2): 150 ng/ml of IT-62-B, (B-1): T-24 cells as control, (B-2): 100 ng/ml of IT-62-B.



The respective cells were incubated for 48 hours in the absence or presence of the indicated concentration of IT-62-B.

H-*ras* and K-*ras* and that of T-24 cells. The effect was evident in almost all of the cells, as early as 2 days following exposure to IT-62-B at the concentrations inhibiting cell growth by 30% or more.

Discussion

We have searched for novel antitumor antibiotics from microbial metabolites that can reverse the transformed phenotype of H-*ras* transformants to normal, and which could also show inhibitory effects on the growth of tumor cells. In order to discover antitumor substance with selective cytotoxicity against transformed cells, we established the direct primary screening system for culture broth by using *ras*-transformed NIH3T3 cells and/or T-cells. As a result of screening, we isolated a novel baumycin-group anthracycline antibiotic, IT-62-B, from the culture mycelia of *Streptomyces* sp. IT-62 along with the already known anthracycline TAN-1120[7], which was isolated as a comparative compound IT-62-A in this study, IT-62-B inhibited moderately the growth of several tumor cell lines much like doxorubicin, as well as that of Gram-positive bacteria, but was ineffective against most of Gram-negative bacteria (Tables 6, 7). While antibiotic IT-62-A exhibited high antibacterial activity against most of Gram-positive and -negative bacteria tested, with MIC values 10- to 100-fold lower than those of IT-62-B (data not shown). Among the baumycin-group anthracyclines, baumycins[8], carminomycins[9,10] barminomycins[11], and TAN-1120 are known to show more potent cytotoxic activity than the other anthracyclines such as doxorubicin, however, IT-62-B showed similar cytotoxic activity to that of doxorubicin. Although IT-62-B and TAN-1120 have aglycone and daunosamine as the same fundamental structure, the former possesses an oxazolidine ring in its side chain, on the other hand, the latter has a carbinolamine group in daunosamine moiety. A similar relationship in structure regarding carbinolamine moiety is seen in the comparison of barminomycin I with carminomycin III. So, barminomycin I has potent activity but carminomycin III does not. The presence of the carbinolamine moiety attached to daunosamine in baumycin-group anthracyclines affords more extensive antimicrobial, typically against Gram-negative bacteria, and potent cytotoxic activities, as described in the report of TAN-1120 by Nozaki *et al.*[7].

Several compounds, such as herbimycin[12] and epiderstatin[13], which can reverse the transformed phenotype, have previously been isolated from microbial sources. Among them, Hori *et al.*[14] have reported that 2-demethylsteffimycin D altered the morphology of NRK cells expressing the *ras*-oncogene. Further, in addition

to the efforts to find new antibiotics, they examined some clinically used antitumor antibiotics to determine whether they have any selective activity against *ras* oncogene-expressing cells (referred to as anti-*ras* activity). They recently found that some of the anthracycline antibiotics such as doxorubicin and pirarubicin, but not aclarubicin, selectively reversed the transformed phenotype of *ras*, but not that of *sar* oncogene-expressing cells *in vitro*[15], and inhibited the growth of solid tumors of K-*ras*-NIH3T3 cells transplanted into nude mice[16]. Thus, better chemotherapy may be attainable with anthracycline IT-62-B having strong anti-*ras* activity, if the target tumors have been diagnosed to have the activated *ras*-oncogene. Furthermore, this antibiotic could become a useful chemical tool to examine the possible correlation between anti-*ras* activity and effectiveness in inhibiting the growth of solid tumors. The actions of antibiotic IT-62-B on H-*ras* transformed cells will be reported in detail elsewhere.

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