CHEMICAL AND BIOLOGICAL PROPERTIES OF RUBIGINONE, A COMPLEX OF NEW ANTIBIOTICS WITH VINCRISTINE-CYTOTOXICITY POTENTIATING ACTIVITY

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A novel complex of isotetracenone (angucyclinone) group antibiotics was discovered from the cultured broth of *Streptomyces griseorubiginosus* No. Q144-2. The antibiotic consisted of six related factors, designated rubiginones A_1, A_2, B_1, B_2, C_1 and C_2. They significantly potentiated cytotoxicity of vincristine (VCR) against VCR-resistant P388 leukemia and Moser cells.

In our recent attempt to discover microbial metabolites which potentiate the cytotoxicity of vincristine (VCR) using Moser cells, *Streptomyces griseorubiginosus* strain No. Q144-2 isolated from a soil sample of Andhra Pradesh, India was found to produce a complex of antibiotics with the desired activity. The active principle named as rubiginone after the name of the producing strain was extracted from the fermentation broth by organic solvent extraction and separated into six components, rubiginones A_1, A_2, B_1, B_2, C_1 and C_2, by subsequent column chromatography. The structural studies showed that they are new members of the isotetracenone (the angucyclinone) group antibiotics without sugar substitution. All rubiginone components significantly potentiate the cytotoxicity of VCR against Moser cells. Rubiginone B_1, the most active component, was also effective in potentiating the cytotoxicity of VCR against VCR-resistant P388 leukemia in *in vitro* and *in vivo*. However, it showed no potentiation effect on doxorubicin.

Taxonomy of the Producing Organism

Source

Strain Q144-2 was isolated from a soil sample collected in Andhra Pradesh State, India.

Morphology

Both substrate and aerial mycelia are formed. The substrate mycelium is long, well-branched, partially zigzag, and not fragmented into short elements. Monopodially branched aerial mycelia wholly bear long straight chains of arthrospores, which include 20 to 100 spores per chain. The spores are cylindrical to oval ($0.5 \sim 0.8$ by $0.8 \sim 1.5$ μm), with a smooth surface. Atypical sclerotia, which are balloon-shaped in early growth turning to a viscid granule (5 to 20 μm i.d.), are observed in CZAPEK's sucrose-nitrate agar and oatmeal agar. Motile spore and sporangium are not formed.

Cultural Characteristics

Aerial mycelium is formed on most media. The color of spore mass is gray on ISP media Nos. 2, 3, 4, 5 and 7, grayish yellow on glucose-asparagine agar, and grayish pink on PAPAVIZAS' V-8 juice agar.

Table 1. Cultural characteristics of strain Q144-2.

Medium	Growth aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Poor None	Moderate yellow (87)	Pale yellow (89)
Tryptone-yeast extract broth (ISP No. 1)	Moderate, not turbid	Colorless	Strong brown (55)
Yeast extract-malt extract agar (ISP No. 2)	Good Good; light gray (264)	Strong yellowish brown (74)	Strong brown (55)
Oatmeal agar (ISP No. 3)	Moderate Good; medium gray (265)	Colorless to dark brown (59)	Light yellow (86)
Inorganic salts-starch agar (ISP No. 4)	Moderate Good; brownish gray (191)	Strong yellowish brown (74)	Light olive yellow (70)
Glycerol-asparagine agar (ISP No. 5)	Moderate Good; light gray (264)	Reddish brown (43)	Strong yellow (84)
Peptone-yeast extract-iron agar (ISP No. 6)	Scant None	Moderate yellowish brown (77)	Dark brown (59) to brown black (65)
Tyrosine agar (ISP No. 7)	Moderate Good; medium gray (265)	Dark brown (59)	Deep yellowish brown (75)
Glucose-asparagine agar	Moderate Poor; dark grayish yellow (91)	Dark yellowish brown (78)	Strong yellow (84)

Observation after incubation at 28°C for 3 weeks.
Color and number in parenthesis follow ISCC-NBC designation.

The reverse color is yellowish brown to dark brown. Melanin is formed in ISP media Nos. 1, 6 and 7. A dark brownish-red mycelial pigment is produced in PRIDHAM-GOTTLIEB's medium, supplmented with sugar such as trehalose. This pigment is a pH indicator (orange yellow in acid, and dark violet red in alkali). The cultural characteristics are summarized in Table 1.

Physiological Characteristics

Gelatin and starch are hydrolyzed. Tyrosinase is formed. Growth occurs between 14°C and 42°C, but not at 12°C or 45°C. All of the eleven diagnostic sugars cited in BERGEY's Manual (8th Ed. 1974) are utilized for growth.

Chemotaxonomy

Whole-cell hydrolysate contains LL-diaminopimelic acid, rhamnose, ribose and glucose, and hence the cell wall belongs to type I. The phospholipids include phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol, therefore the pattern belongs to P-II. The menaquinones have MK-9 (H_6) as the major component.

The major characteristics of strain Q144-2 reveal that the strain should be classified into *Streptomyces*. According to the descriptions of PRIDHAM and TRESNER[1], strain Q144-2 is placed in a species group; gray (GY) aerial spore mass, straight-flexuous (RF) spore chain, presence of melanin (C+), and smooth (SM) spore wall ornamentation. Among twenty-five species of the group, *Streptomyces achromogenes*[1,2] and *Streptomyces noboritoensis*[1,3] resemble strain Q144-2 in cultural characteristics, but differ in sugar utilization. *Streptomyces cacaoi* subsp. *asoensis*[1], *Streptomyces fulvoviolaceus*[1], *S. griseorubiginosus*[1,3] and *Streptomyces phaeopurpureus*[1] are similar to the strain in sugar utilization. However, *S. cacaoi* subsp. *asoensis* differs in the partial formation of spiral spore chain. The remaining

three species seem to constitute a taxon which forms distinct pigments with red or violet shade. Based on further comparative studies, strain Q144-2 was identified as *Streptomyces griseorubiginosus*.

Antibiotic Production

A slant culture of *S. griseorubiginosus* strain No. Q144-2 was propagated at 28°C for 10 days on a modified BENNETT's agar slant consisting of soluble starch 0.5%, glucose 0.5%, fish meat extract (Mikuni) 0.1%, yeast extract (Oriental Yeast) 0.1%, NZ-case (Sheffield) 0.5%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6%. A well-grown agar slant was used to inoculate a 500-ml Erlenmeyer flask which contained 100 ml of the seed medium composed of soluble starch (Nichiden Kagaku) 3%, lactose 1%, fish meal (Hokuyo Suisan) 1%, $CaSO_4 \cdot 2H_2O$ 0.6% and $CaCO_3$ 0.5% (pH 7.0 before autoclaving).

The seed flask was incubated at 28°C for 4 days on a rotary shaker (200 rpm). The seed culture (5 ml) was transferred into a 500-ml Erlenmeyer flask containing 100 ml of production medium with the same composition as the seed medium. The fermentation was carried out at 28°C for 7 days on a rotary shaker; the antibiotic production reached 150 μg/ml after 6 to 7 days cultivation. The fermentation was also carried out in stir-jar fermenters. A 500-ml portion of the above seed culture was inoculated into a 20-liter stir-jar fermenter (Marubishi, MSJ-20) containing 12 liters of production medium having the same composition as the flask fermentation medium. The fermentation was carried out at 28°C under agitation at 250 rpm and aeration rate of 12 liters per minute. Antibiotic production reached a maximum of 200 μg/ml after 90 hours cultivation.

Extraction and Purification

The fermentation broth (15 liters) was extracted with butanol (10 liters) by stirring for 1 hour. The extract was evaporated *in vacuo* to an aqueous concentrate (1 liter) which was diluted with 2 liters of water and extracted twice with 1.5 liters portions of ethyl acetate. Evaporation of the pooled extracts afforded a yellow amorphous solid (8.88 g). The solid was dissolved in methylene chloride (50 ml) and charged on a column of silica gel (Wakogel C-200, 600 ml) which had been equilibrated with methylene chloride. Elution was carried out with the same solvent; the eluate was collected in 18-ml fractions and monitored by HPLC (SSC-ODS-262, column, MeOH - H_2O, 70 : 30). Fractions No. 118 ~ 129 were pooled and evaporated to give yellow solid rubiginone B_2 (925 mg). Evaporation of fractions No. 130 ~ 147 yielded a complex of rubiginones B_1, C_1 and C_2 (1.68 g). The subsequent fractions containing homogeneous rubiginone A_2 (No. 267 ~ 311) were concentrated to 20 ml which upon standing in a cold room deposited yellow needles (rubiginone A_2, 181 mg). The fractions No. 324 ~ 420 were similarly worked up to isolate pure crystalline rubiginone A_1 (928 mg).

Further purification of rubiginone B_2 (860 mg) was effected by medium pressure chromatography on a column of reversed phase silica gel (Merck Kieselgel 60 silanized, 300 ml). Elution was performed with a mixture of methanol - water (3 : 2) and the peak fractions were concentrated *in vacuo* to deposit yellow needles of pure rubiginone B_2 (73 mg).

The mixture of rubiginones B_1, C_1 and C_2 (1.5 g) was subjected to medium pressure chromatography on reversed phase silica gel (300 ml). Fifty % methanol was used first and then the methanol content increased to 70% for elution of the components. The fractions containing rubiginone B_1 were pooled and evaporated to give 510 mg of yellow solid. Evaporation of the subsequent eluate yielded a mixture of rubiginones C_1 and C_2. The semi-pure rubiginone B_1 sample (490 mg) was again purified by similar medium pressure chromatography with 60% aqueous methanol elution to obtain 179 mg of pure yellow needles of

Table 2. Physico-chemical properties.

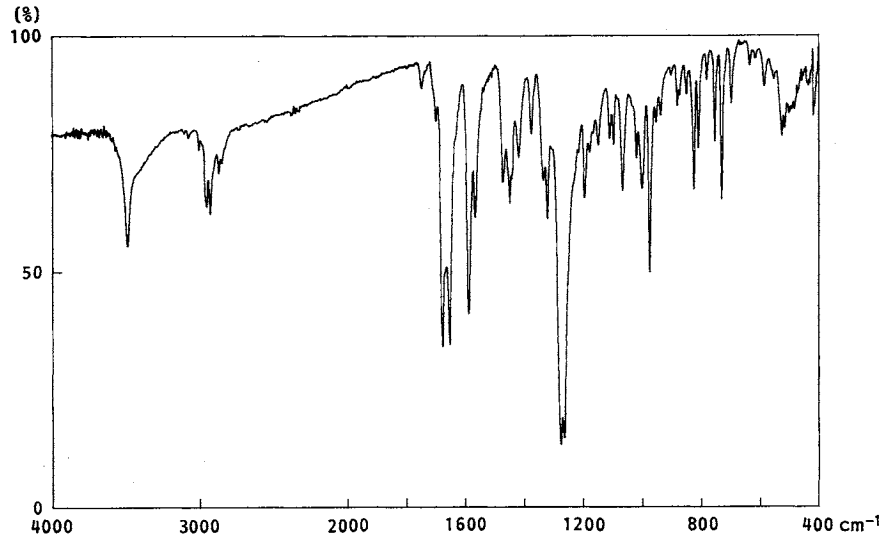
	Rubiginone A_1	Rubiginone A_2	Rubiginone B_1
Nature	Yellow needles	Yellow needles	Yellow needles
MP (°C)	198~199	>235 (dec)	164~166
$[\alpha]_D^{25}$ (*c* 0.5, $CHCl_3$)	+399°	+92°	+367°
Microanalysis	$C_{20}H_{18}O_5$	$C_{20}H_{16}O_5$	$C_{20}H_{18}O_4$
Calcd for:	C 70.99, H 5.36	C 71.42, H 4.80	C 74.52, H 5.63
Found:	C 70.74, H 5.27	C 71.21, H 4.80	C 74.39, H 5.66
EI-MS (*m/z*)	338 (M^+)	336 (M^+)	322 (M^+)
UV λ_{max}^{EtOH} nm (ε)	259 (36,000), 376 (6,100)	264 (34,000), 375 (5,400)	259 (34,000), 375 (6,100)
IR (KBr)ν cm^{-1} (OH)	3450, 3300	3450	3470
(=O)	1670	1690, 1660	1680, 1650
HPLC[a] (Rt, minutes)	4.16	3.2	20.2
TLC, SiO_2[b] (Rf)	0.04	0.05	0.27

	Rubiginone B_2	Rubiginone C_1	Rubiginone C_2
Nature	Yellow needles	Yellow needles	Yellow needles
MP (°C)	>236 (dec)	148~150	>215 (dec)
$[\alpha]_D^{25}$ (*c* 0.5, $CHCl_3$)	+78°	+125°	−61°
Microanalysis	$C_{20}H_{16}O_4 \cdot \frac{1}{3}H_2O$	$C_{24}H_{24}O_6$	$C_{24}H_{22}O_6$
Calcd for:	C 73.61, H 5.15	C 70.57, H 5.92	C 70.92, H 5.46
Found:	C 73.82, H 4.90	C 70.24, H 5.84	C 70.87, H 5.45
EI-MS (*m/z*)	320 (M^+)	320 ($M^+ - (CH_3)_2CHCOOH$)	406 (M^+)
UV λ_{max}^{EtOH} nm (ε)	264 (27,000), 375 (5,600)	258 (36,000), 380 (6,100)	262 (30,000), 378 (6,300)
IR (KBr)ν cm^{-1} (OH)		3400	
(=O)	1705, 1697, 1673	1720, 1670	1750, 1705, 1670
HPLC[a] (Rt, minutes)	8.0	41.6	18.4
TLC, SiO_2[b] (Rf)	0.47	0.24	0.53

[a] Column: Senshu Pak (SSC-ODS-262, 6 i.d. × 100 mm); mobile phase MeOH - H_2O (65 : 35); flow rate, 1 ml/minute; detector, UV 254 nm.

[b] CH_2Cl_2 - MeOH (100 : 1).

Fig. 1. IR spectrum of rubiginone B_1 (KBr disk).



rubiginone B_1. In a similar manner, the mixture of rubiginones C_1 and C_2 was separated and purified by reversed phase silica gel chromatography to obtain pure rubiginone C_1 (105 mg) and pure rubiginone C_2 (57 mg). All the purification processes were carried out under light-shielded conditions.

Physico-chemical Properties

All components of rubiginone (A_1, A_2, B_1, B_2, C_1 and C_2) were isolated as yellow needles. They are readily soluble in methanol, butanol, acetone, ethyl acetate and dimethyl sulfoxide, slightly soluble in hexane but insoluble in water. These components colored after spraying with iodine solution or sulfuric acid solution on TLC plate. They were negative to ninhydrin, Sakaguchi and Dragendorff reactions. The molecular formulae of rubiginone components were determined by microanalysis and mass spectra to be $C_{20}H_{18}O_5$ (A_1), $C_{20}H_{16}O_5$ (A_2), $C_{20}H_{18}O_4$ (B_1), $C_{20}H_{16}O_4$(B_2), $C_{24}H_{24}O_6$ (C_1) and $C_{24}H_{22}O_6$ (C_2). They showed UV absorption at 260 and 375 nm in methanol which did not shift in acidic or alkaline solution. The physico-chemical properties are summarized in Table 2. The IR spectrum of rubiginone B_1 is illustrated in Fig. 1. The ^1H NMR spectra (Table 3) of all rubiginone components show one $C-CH_3$

Table 3. ^1H NMR spectra of rubiginone (400 MHz, DMSO-d_6+D_2O).

	δ ppm (splitting, J=Hz)		
	Rubiginone A_1	Rubiginone A_2	Rubiginone B_1
1-H	5.61 (dd, 6.8, 7.6)		5.57 (dd, 6.5, 8.4)
2-H_{ax}	1.44 (ddd, 7.6, 11.1, 13.4)	2.53 (dd, 11, 16)	1.39 (ddd, 8.4, 11.4, 12.8)
2-H_{eq}	2.15 (ddd, 3.8, 6.8, 13.4)	2.91 (dd, 5.5, 16)	2.17 (ddd, 3.1, 6.5, 12.8)
3-H	1.64 (m)	2.2 (m)	1.84 (m)
3-CH_3	1.13 (d, 6.4)	1.14 (d, 6.6)	1.07 (d, 6.7)
4-H	4.23 (d, 9.6)	4.4 (d, 9.1)	2.55 (dd, 11, 17)
			2.82 (dd, 2, 17)
5-H	7.98 (d, 8.3)	8.02 (d, 8.4)	7.54
6-H	8.05 (d, 8.3)	8.22 (d, 8.4)	7.95 (d, 7.7)
8-OCH_3	3.95 (s)	3.95 (s)	3.93 (s)
9-H	7.53 (dd, 1, 8.5)	7.56	7.54
10-H	7.83 (dd, 7.7, 8.5)	7.84 (dd, 7.7, 8.5)	7.82 (dd, 7.7, 8.4)
11-H	7.76 (dd, 1, 7.7)	7.58	7.66 (dd, 1.1, 7.7)

	δ ppm (splitting, J=Hz)		
	Rubiginone B_2	Rubiginone C_1	Rubiginone C_2
1-H		5.72 (dd, 7.3, 6.2)	
2-H_{ax}	2.51 (dd, 11, 15.7)	1.6 (ddd, 7.3, 10, 13.6)	2.69 (dd, 10, 16)
2-H_{eq}	2.85 (dd, 5, 15.7)	2.21 (ddd, 4.8, 6.2, 13.6)	2.98 (dd, 5.8, 16)
3-H	2.36 (m)	1.96 (m)	2.53 (m)
3-CH_3	1.11 (d, 6.4)	1.04 (d, 6.4)	1.04 (d, 7)
4-H	2.72 (dd, 11, 16.5)	5.83 (d, 9.1)	5.88 (d, 8.1)
	3.04 (dd, 2.8, 16.5)		
5-H	7.56 (d, 8.1)	7.50 (d, 8.1)	7.57 (d, 8.1)
6-H	8.12 (d, 8.1)	8.06 (d, 8.1)	8.23 (d, 8.1)
8-OCH_3	3.95 (s)	3.95 (s)	3.95 (s)
9-H	7.56 (d, 8.1)	7.55 (dd, 1, 8.4)	7.58 (dd, 1, 8)
10-H	7.84 (dd, 8, 8.1)	7.84 (dd, 7.7, 8.4)	7.85 (dd, 8, 8.1)
11-H	7.71 (d, 8)	7.68 (dd, 1, 7.7)	7.70 (dd, 1, 8.1)
4-OCOC*H*		2.71 (m)	2.73 (m)
4-OCOCH(CH_3)$_2$		1.18 (d, 7.3), 1.19 (d, 6.8)	1.16 (d, 7.0), 1.17 (d, 6.9)

Table 4. ^{13}C NMR spectra of rubiginone (100 MHz, DMSO-d_6).

	δ ppm					
	Rubiginone A_1	Rubiginone A_2	Rubiginone B_1	Rubiginone B_2	Rubiginone C_1	Rubiginone C_2
C-1	64.3	196.9	64.8	198.0	63.5	195.9
C-2	38.3	44.5	40.8	46.9	37.1	43.4
C-3	35.1	37.8	27.0	30.4	33.4	34.9
3-CH_3	18.9	18.2	21.7	21.0	18.9[b]	17.6[c]
C-4	72.7	71.6	38.8	46.9	74.2	73.0
C-4a	148.6	152.6	144.4	149.5	142.3	146.1
C-5	132.0	131.1	134.1	133.6	130.9	131.2
C-6	125.4	129.0	125.2	128.9	125.9	129.5
C-6a	134.8	133.7	134.5	134.4[a]	135.5	134.2[d]
C-7	181.2	180.1	181.2	180.2	181.0	179.9
C-7a	119.9	120.0	119.9	119.9	119.9	119.9
C-8	159.1	159.5	159.1	159.5	159.2	159.6
8-OCH_3	56.3	56.4	56.3	56.4	56.3	56.4
C-9	118.0	118.4	118.0	118.4	118.1	118.5
C-10	135.3	135.7	135.4	135.8	135.4	135.9
C-11	118.9	118.4	118.9	118.4	118.9	118.5
C-11a	137.1	134.7	137.2	134.5[a]	137.1	135.4[d]
C-12	186.2	183.7	186.4	183.8	185.8	183.4
C-12a	131.2	133.6	132.3	134.3[a]	131.6	134.1[d]
C-12b	141.4	136.8	141.6	136.9	142.1	136.8
4-OCOCH(CH_3)$_2$					18.7[b], 18.5[b]	18.8[c], 18.7[c]
4-OCO*C*H					32.5	33.2
4-O*C*O					176.2	175.7

[a~d] Assignment may be interchanged.

(around δ 1.1), one OCH_3 (δ 3.9), and five aromatic protons (δ 7.5~8.3). Their ^{13}C NMR (Table 4) exhibited in common two methyls (δ 17.6~21.7 and 56.3~56.4), twelve aromatic carbons (δ 115~160) and two carbonyl carbons (δ 181.2 and 186.4). These physico-chemical and spectral properties suggested that rubiginone components belong to the isotetracenone group[4] (or the angucycline/angucyclinone group[5]) having no sugar moiety. Rubiginones A_2 and B_2 were found to have physico-chemical and spectral properties very similar to those of fujianmycin B[6] and X-14881C[7], respectively. However, since authentic samples were not available, a direct comparison has not yet been carried out.

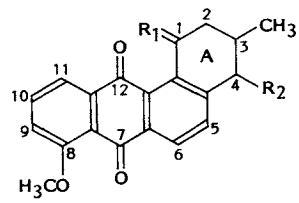
Structure Determination

The UV spectra of rubiginones A_1, A_2, B_1, B_2, C_1 and C_2 indicated that these compounds belong to the isotetracenone (or the angucycline/angucyclinone) group antibiotics. The five aromatic protons of the components appeared as ABX (δ 7.5 (dd), 7.7 (dd), 7.8 (dd)) and AB (δ 7.5 (dd) and 8.0 (dd)) patterns and one OCH_3 (δ 3.95) was observed in the ^1H NMR of all components suggesting an 8-methoxyisotetracenone nucleus. Comparison of their ^1H NMR spectra further suggested that the six components are close structural analogs differing from each other only at the A-ring moiety of the molecules. Upon irradiation with fluorescent light in methylene chloride solution, rubiginones A_1, B_1 and C_1 were rapidly converted to rubiginones A_2, B_2 and C_2, respectively. The ^1H NMR spectrum of rubiginone A_1 containined one methyl (δ 1.13 (d)), one non-equivalent methylene (δ 1.44 (ddd) and 2.15 (ddd)), three methines (δ 1.64 (m), 4.23 (d) and 5.61 (dd)) in addition to the five aromatic protons (Table 3). On

acetylation, rubiginone A_1 yielded a diacetate (EI-MS: m/z 422 M^+). The two low field methine protons (δ 4.23 and 5.61) of rubiginone A_1 underwent a downfield shift (to δ 5.76 and 6.61, respectively) in the acetate indicating that these methine carbons bear the hydroxyl groups. First order analysis of their resonance patterns (Table 3) combined with the ^1H-^1H COSY experiments revealed a $-CHOH-CH_2CHCH_3-CHOH-$ unit in the A-ring. A long range interaction was observed between the proton at δ 4.23 (4-H) and one of the AB type aromatic protons (δ 7.98, 5-H). Therefore, rubiginone A_1 was assigned the structure 1,2,3,4-tetrahydro-1,4-dihydroxy-3-methyl-8-methoxybenz[*a*]anthraquinone. As noted previously, rubiginone A_2 was obtained by the photo-oxidation of rubiginone A_1. In the ^1H NMR spectrum of rubiginone A_2, the methine proton assigned to 1-CH of rubiginone A_1 was missing and the 2-CH_2 resonated with simpler splittings at lower field (δ 2.53 (dd) and 2.91 (dd)). The ^{13}C NMR spectrum of rubiginone A_2 showed the presence of a new carbonyl carbon (δ 196.9) at the expense of a CHOH carbon (δ 64.3) of rubiginone A_1. Thus, rubiginone A_2 was determined to be the 1-keto analog of rubiginone A_1. The physico-chemical data of rubiginone A_2 are consistent with those reported for fujianmycin B[6] except for a significant difference in their $[\alpha]_D$ values.

The IR spectrum of rubiginone C_1 indicated an ester carbonyl absorption at 1720 cm^{-1} in addition to the quinone carbonyl (1670 cm^{-1}) commonly observed in rubiginones A_1, B_1 and C_1. When hydrolyzed in 0.6 N sodium hydroxide at room temperature, rubiginone C_1 yielded rubiginone A_1. In the ^1H NMR spectrum, 4-H appeared at δ 5.83, 1.60 ppm lower field than that of rubiginone A_1, indicating that the C-4 hydroxyl group of rubiginone C_1 was acylated. The acid attached was identified as isobutyric acid by ^1H NMR analysis as shown in Table 3 and the molecular formula or rubiginone C_1. Rubiginone C_2 was determined to be the 1-keto analog of rubiginone C_1 since the fluorescent oxidation of rubiginone C_1 afforded rubiginone C_2 in good yield. All the spectral data supported this assignment.

Rubiginone B_1 showed two methines and two sets of non-equivalent methylenes (δ 1.39 (ddd), 2.17 (ddd), 2.55 (dd) and 2.82 (dd)) in the ^1H NMR spectrum. The methine proton attached to the hydroxyl-bearing carbon appeared at δ 5.57 (m) which sharpened to a double doublet after D_2O addition. When oxidized by light, rubiginone B_1 yielded rubiginone B_2 which lacked the lowfield methine in the ^1H NMR. Consequently, rubiginones B_1 and B_2 are 4-deoxy analogs of rubiginones A_1 and A_2, respectively. The physico-chemical data of rubiginone B_2 are nearly identical with those reported for X-14881C[7]. The structures of rubiginones A_1, A_2, B_1, B_2, C_1 and C_2 are shown below. Absolute stereochemistry of rubiginones is currently being elucidated and the results will be reported in a separate paper.



	R_1	R_2
Rubiginone A_1	H, OH	OH
Rubiginone A_2	O	OH
Rubiginone B_1	H, OH	H
Rubiginone B_1	O	H
Rubiginone C_1	H, OH	$OCOCH(CH_3)_2$
Rubiginone C_2	O	$OCOCH(CH_3)_2$

Potentiation of Antitumor Activity

In Vitro Effect

Six components of rubiginone were tested for potentiating effect on VCR-induced cytotoxicity against logarithmically growing Moser cells which are naturally occurring multi-drug resistant human colon carcinoma cells. Moser cell suspension (180 μl, 6×10^4 cells/ml) in RPMI-1640 medium supplemented with fetal calf serum (10%), streptomycin (100 μg/ml) and benzylpenicillin (100 U/ml), test compounds (20 μl) and VCR (10 μl) were mixed in

Table 5. Potentiating effect of rubiginone on VCR-induced cytotoxicity against Moser cells.

Compound	Conc (μg/ml)	IC_{50} of VCR (μg/ml)	P.I.[a]	Compound	Conc (μg/ml)	IC_{50} of VCR (μg/ml)	P.I.[a]
Vehicle	—	0.59	—	Rubiginone C_1	30	0.18	3
Rubiginone A_1	30	0.23	3		10	0.13	5
	10	0.21	3	Rubiginone C_2	30	0.05	12
Rubiginone A_2	30	0.02	30		10	0.08	7
	10	0.05	12	Verapamil	4	0.0088	67
Rubiginone B_1	30	0.007	84		2	0.045	13
	10	0.010	59		1	0.098	6
Rubiginone B_2	30	0.10	6				
	10	0.12	5				

[a] Potentiation index: IC_{50} of VCR alone/IC_{50} of VCR and test compound (the data are the mean values of three experiments).

Table 6. Potentiating effects of the VCR- and ADR-induced cytotoxicities of rubiginone B_1 against VCR- and ADR-resistant and sensitive P388 leukemia cells.

Compound	Conc (μg/ml)	Antitumor drug	P.I.[a]		
			P388/VCR	P388/ADR	P388/S
Rubiginone B_1	10	VCR	34	—	29
	10	ADR	—	1.4	1.7
	5	VCR	16	—	7.1
	5	ADR	—	0.9	1.0
Verapamil	2	VCR	44	—	64
	2	ADR	—	5.9	2.2

[a] Potentiation index: IC_{50} of VCR (or ADR) alone/IC_{50} of VCR (or ADR) and test compound (the data are the mean values of three experiments).

wells of a 96-well microplate and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 3-day incubation, viable cells were stained with 0.006% neutral red and determined at 540 nm by a microplate autoreader. As shown in Table 5, all six components, rubiginones A_1, A_2, B_1, B_2, C_1 and C_2 were found to potentiate the VCR-induced cytotoxicity against Moser cells. Each component alone did not show cytotoxicity at 30 μg/ml (data not shown). Among them, rubiginone B_1 was the most potent; it enhanced the cytotoxicity of VCR 59- and 84-fold at 10 and 30 μg/ml, respectively. The order of potency was $B_1 > A_2 > C_2 > B_2 = C_1 > A_1$. The potentiation activity of rubiginone B_1 was a half or less than half that of verapamil which is known to reverse multi-drug resistance by increased accumulation of the drug in cells[8~10]. The potentiating effects on the VCR- and doxorubicin (ADR)-induced cytotoxicity against VCR-resistant and ADR-resistant leukemia P338 (multi-drug resistant cell lines[8,9]) kindly provided by Prof. T. TSURUO of Institute of Applied Microbiology, The University of Tokyo) and sensitive P388 leukemia cells were evaluated using rubiginone B_1. Under similar culture conditions as used for Moser cells, the cell suspension (900 μl, 1.3×10^4 cells/ml) was treated with the test compounds and the number of cells was directly counted by a cell counter (Sysmex microcellcounter CC-130A). Rubiginone B_1 markedly enhanced the VCR-induced cytotoxicity against both VCR-resistant and sensitive P388 cells (Table 6). In contrast, it did not show potentiating effect on the ADR-induced cytotoxicity against both ADR-resistant and -sensitive P388 cells. However, verapamil enhanced both the VCR- and ADR-induced cytotoxicities

Table 7. Chemosensitizing activity of rubiginone B_1 in VCR-resistant P388-bearing mice.

Compound and dosage	MST (days)	T/C (%)	Compound and dosage	MTS (days)	T/C (%)
VCR (0.4)[a]	14.5	132	Rubiginone B_1 (20)	11.0	100
VCR (0.2)	14.5	132	Rubiginone B_1 (10)	12.0	109
VCR (0.1)	13.0	118	VCR (0.05)+verapamil (100)	Toxic	Toxic
VCR (0.05)	11.0	100	VCR (0.05)+verapamil (75)	15.0	136
VCR (0.03)	11.0	100	VCR (0.05)+verapamil (50)	14.0	127
VCR (0.05)+rubiginone B_1 (40)	14.0	127	Verapamil (100)	13.0	118
VCR (0.05)+rubiginone B_1 (20)	14.5	132	Verapamil (75)	10.0	91
VCR (0.05)+rubiginone B_1 (10)	14.5	132	Verapamil (50)	10.0	91
Rubiginone B_1 (40)	12.5	114	Vehicle	11.0	100

[a] mg/kg/day by qd × 10 (from 1 to 10 days after tumor inoculation).

in P388/VCR and P388/ADR cells, respectively. These results suggest that rubiginone B_1 is a specific potentiator of VCR.

Combination Chemotherapy in VCR-resistant P388 Leukemia (P388/VCR)-bearing Mice

P388/VCR cells were used in this experiment. Female CDF_1 mice (5-week old) were intraperitoneally inoculated with 0.4 ml of diluted ascitic fluid containing 1.5×10^6 P388/VCR cells. The mixture of test compound and VCR was intraperitoneally administered daily for 10 days from 1-day after the tumor inoculation. Table 7 summarizes the chemosensitizing activity of rubiginone B_1 by combination chemotherapy with VCR in P388/VCR-bearing mice. Although 0.05 mg/kg/day of VCR did not show significant prolongation of life span of P388/VCR-bearing mice, rubiginone B_1 administered daily at doses of 40, 20 and 10 mg/kg/day with 0.05 mg/kg/day of VCR slightly enhanced the chemotherapeutic effect of VCR with maximum T/C of 132%. When verapamil was tested in the same regimen, it also slightly enhanced antitumor activity of VCR with maximum T/C of 136% at 75 mg/kg/day. Although the chemosensitizing activity of rubiginone B_1 and verapamil were weak in these tests, the above results indicated that rubiginone B_1 appeared to be somewhat more potent than verapamil in terms of the effective dose.

Discussion

Rubiginone, a complex of new antibiotics produced by *Streptomyces griseorubiginosus* No. Q144-2, was screened as a potentiator of VCR-induced cytotoxicity. Rubiginone components are yellow crystalline compounds having UV absorption maxima at 260 and 380 nm. A combination of photo-oxidation and spectral studies established that they are new isotetracenone (angucyclinone) group antibiotics and differ from each another in the A-ring. It is unique that rubiginones A_1, B_1 and C_1 have a hydroxyl group at C-1 position of the A-ring and are converted to rubiginones A_2, B_2 and C_2, respectively, by photo-oxidation. Among the six components, rubiginone B_1 showed the highest potentiating effect on VCR-induced cytotoxicity. However, it did not exhibit potentiation of cytotoxicity of doxorubicin. In *in vitro* tests, the potentiating activity of rubiginone B_1 was half or less than half that of verapamil. On the other hand, it slightly enhanced the chemotherapeutic effect of VCR in the VCR-resistant P388 leukemia-bearing mice with activity somewhat greater than that of verapamil in terms of optimal dose. This antibiotic will be further evaluated for its potentiating effect on antitumor activity of other drugs and for its mode of action.

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