COMMUNICATIONS TO THE EDITOR

Reblastatin, a Novel Benzenoid Ansamycin-type Cell Cycle Inhibitor

Sir:

In the course of our screening for compounds which inhibit Rb protein phosphorylation at the cell level and cause cell cycle arrest at the G1 phase[1], reblastatin (Fig. 1) was found as a minor component of geldanamycin[2~4] from the culture of *Streptomyces hygroscopicus* subsp. *hygroscopicus* SANK 61995 deposited as FERM BP-5140.

One loopful of the producing strain was inoculated into 80 ml of the seed medium consisting of glycerol 2.5%, glucose 2.5%, pressed yeast 1%, soybean meal 1%, $CaCO_3$ 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and CB-442 (Nihon Yushi Co.) 0.005%, pH 7.0, in a 500 ml-Erlenmeyer flask. The seed culture was carried out for 4 days at 28°C on a rotary shaker (210 rpm). One milliliter of the culture was added to 80 ml of a medium consisting of the same composition as the seed medium in a 500 ml-Erlenmeyer flask and the fermentation was carried out for 4 days at 28°C on a rotary shaker (210 rpm).

An equal amount of acetone was added to the harvested culture (800 ml) after the pH was adjusted to 7.0 and the active substance was extracted for one hour by stirring. The filtrate was partitioned three times by EtOAc (800 ml) and the collected solvent layer was washed with satd. NaCl followed by drying over anhydr. Na_2SO_4 for one hour. The extract was concentrated *in vacuo* to dryness to yield a yellow oil (960 mg). Methanol (300 ml) was poured into the oil and the suspension was centrifuged at 3000 *g* to remove insoluble material that mainly contained geldanamycin. After extracting three times, the collected supernatant was evaporated to one milliliter followed by centrifuge at 3000 *g* to remove insoluble material. The concentrate was charged to a preparative HPLC column (Senshupak ODS H-5251, 20 mm×250 mm) equilibrated with 25% MeCN in water and developed with the same solvent system at the flow rate of 14 ml/minute. A peak from 25 minutes to 27 minutes under UV detection at 210 nm was obtained. The fraction was evaporated to remove MeCN and freeze-dried to yield 11 mg of reblastatin as a white powder.

Reblastatin, $[\alpha]_D^{20}$ +63.1° (*c* 1.0, MeOH), is soluble in MeOH and DMSO, and insoluble in *n*-hexane, $CHCl_3$ and water. The molecular formula was determined to be $C_{29}H_{44}N_2O_8$ based on HR-FAB-MS analysis of the molecular ion (found: *m/z* 548.3055 $(M)^+$, calcd: 548.3099). The UV spectrum in MeOH exhibited absorption maxima at 225 (ε 16000, sh), 250 (ε 6900) and 285 nm (ε 5800). The IR (KBr tablet) spectrum showed absorption bands at 3355, 2930, 1712, 1660, 1605, 1500, 1380, 1310, 1090, 1040 and 870 cm^{-1}. The ^1H and ^{13}C NMR (360 MHz, DMSO-d_6, TMS as the internal reference) signal assignments done by HMQC spectrum are shown in Table 1.

The structure of the ansa-bridge from the methyl residue at position 22 to 15-methylene was revealed directly by the ^1H-^1H spin couplings observed in DQFCOSY and HOHAHA spectra. The other connection was elucidated by the C-H long-range couplings observed in the HMBC spectrum (Fig. 2). The geometry of the two double bonds located at position 2 and 8 was both decided to be *E* according to the observed NOEs between the H-4 methylene (δ 2.12 and 2.19) and H-22 (δ 1.68) protons,

Fig. 1. Structures of reblastatin and geldanamycin.

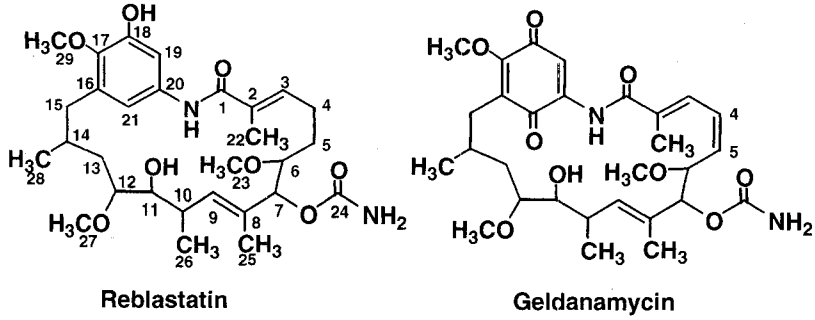
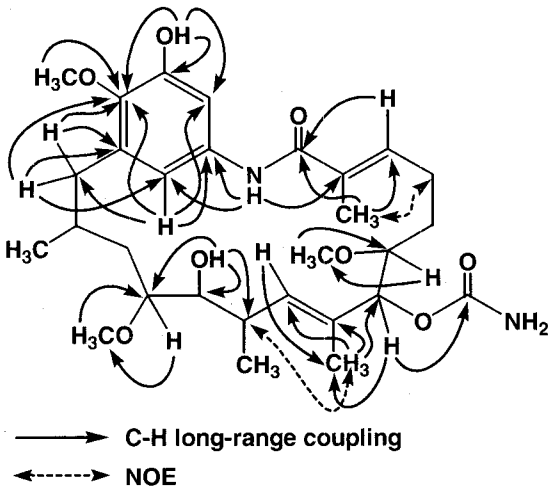


Table 1. ^{1}H and ^{13}C signal assignments of reblastatin.

Position	δ_C	δ_H	Position	δ_C	δ_H
1-NH_2		9.26 (1H, s)	15	35.8	2.35 (1H, m)
1	170.1				2.58 (1H, dd, 6.3, 13.4)
2	132.2		16	133.4	
3	134.6	5.87 (1H, br. t)	17	142.5	
4	23.6	2.12 (1H, m)	18	150.0	
		2.20 (1H, m)	18-OH		9.40 (1H, br. s)
5	29.8	1.29 (1H, m)	19	107.2	6.88 (1H, br. s)
		1.35 (1H, m)	20	134.5	
6	79.6	3.28 (1H, m)	21	114.6	6.30 (1H, br. s)
7	80.6	4.87 (1H, d, 7.5)	22	13.0	1.68 (3H, s)
8	129.7		23	58.2	3.33 (3H, s)
9	133.4	5.31 (1H, d, 9.8)	24	156.1	
10	33.6	2.38 (1H, m)	24-NH_2		6.51 (2H, br. s)
11	73.8	3.33 (1H, m)	25	11.6	1.44 (3H, s)
11-OH		4.37 (1H, br. d, 4.9)	26	15.9	0.91 (3H, d, 6.5)
12	81.1	3.03 (1H, m)	27	56.4	3.23 (3H, s)
13	34.4	1.17 (1H, m)	28	19.8	0.81 (3H, d, 6.5)
		1.55 (1H, m)	29	59.7	3.63 (3H, s)
14	31.1	1.73 (1H, m)			

Number of protons, multiplicity and coupling constants are shown in parentheses.

Fig. 2. C-H long-range couplings and NOEs.

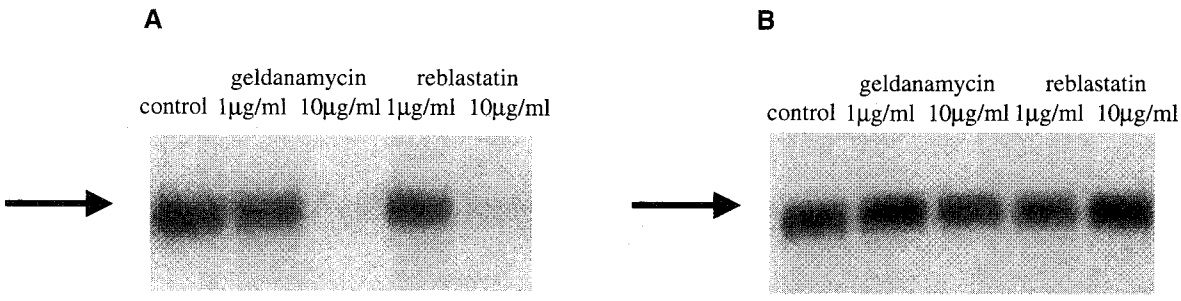


and the H-10 (δ 2.38) and H-25 (δ 1.44) protons in the NOESY spectrum (Fig. 2). Therefore, the planar structure of reblastatin was elucidated as shown in Fig. 1. Although the structure is related to the major component of geldanamycin and classified as a benzenoid-type ansamycin, reblastatin possesses a unique structure, *i.e.* the chromophore is neither a benzoquinone nor a hydroquinone but a phenol, and the double bond at position 4 is hydrogenated.

Geldanamycin and reblastatin strongly inhibited proliferation of cell lines determined by tritium thymidine incorporation. The IC_{50} values of geldanamycin and reblastatin with treatment for 24 hours were calculated as 0.0011 and 0.43 μg/ml against human histiocytic lymphoma U-937. On the other hand, viability of U-937 cells determined by the XTT method[5], which reflects reduction activity in mitochondria, did not decrease under the same conditions up to 1 μg/ml and 10 μg/ml, respectively. These results indicate that these compounds do not induce apoptosis but rather arrest the cell cycle, resulting in a decrease in cell numbers after long term culture.

After treatment of human lung carcinoma, A-549 cells with geldanamycin or reblastatin for 24 hours, cell extracts were immunoprecipitated with anti-cdk2 antibody and kinase activities were measured using histone H1 as a substrate[6]. Cdk2-associated H1 kinase activities were decreased with geldanamycin or reblastatin treatment of

Fig. 3. Effect of geldanamycin and reblastatin on cdk2-associated H1 kinase activities.



(A) A-549 cells were treated with indicated concentrations of geldanamycin or reblastatin for 24 hours. Cell extracts were obtained by extracting Tris-buffered saline containing 0.5% NP-40 and immunoprecipitated with anti-cdk2 antibody (Santa Cruz M-2). Immunoprecipitates were incubated with γ-32P-ATP and histone H1 as a substrate, separated on SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (B) Untreated A-549 extracts were immunoprecipitated with anti-cdk2 antibody and immunoprecipitates were incubated in the presence of indicated concentrations of geldanamycin or reblastatin. Arrows indicate the position of histone H1.

A549 cells (Fig. 3A). At this condition, no decrease of the viability was observed indicating that these compounds are not toxic to A-549. These compounds inhibit Rb protein phosphorylation in A-549 cells and cause cell cycle arrest at the G1 phase.

On the other hand, addition of these drugs to kinase reaction *in vitro* did not change H1 kinase activity (Fig. 3B), indicating that neither geldanamycin nor reblastatin directly affects cdk2-associated kinase activity but affects upstream regulation of cdk2.

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References

1) WEINBERG, R. A.: The retinoblastoma protein and cell cycle control. Cell 81: 323~330, 1995
2) DEBOER, C.; P. A. MEULMAN, R. J. WNUK & D. H. PETERSON: Geldanamycin, a new antibiotic. J. Antibiotics 23: 442~447, 1970
3) SASAKI, K.; K. L. RINEHART Jr., G. SLOMP, M. F. GROSTIC & E. C. OLSON: Geldanamycin. I. Structure assignment. J. Am. Chem. Soc. 92: 7591~7593, 1970
4) JOHNSON, R. D.; A. HABER & K. L. RINEHART Jr.: Geldanamycin biosynthesis and carbon magnetic resonance. J. Am. Chem. Soc. 96: 3316~3317, 1974
5) SCUDIERO, D. A.; R. H. SHOEMAKER, K. D. PAULL, A. MONKS, S. TIERNEY, T. H. NOFZIGER, M. J. CURRENS, D. SENIFF & M. R. BOYD: Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 48: 4827~4833, 1988
6) KOFF, A.; M. OHTSUKI, K. POLYAK, J. M. ROBERTS & J. MASSAGUE: Negative regulation of G1 in mammalian cells: Inhibition of cyclin E-dependent kinase by TGF-β. Science 260: 536~539, 1993