VOL. 53 NO. 1, JAN. 2000 THE JOURNAL OF ANTIBIOTICS pp. 81 — 83

COMMUNICATIONS TO THE EDITOR

Belactosin A, a Novel Antitumor Antibiotic Acting on Cyclin/CDK Mediated Cell Cycle Regulation, Produced by *Streptomyces* sp.

Sir:

Cyclin/CDK complexes belong to a serine/threonine protein kinase family and play key roles as the positive regulators in cell cycle progression[1]. Overexpression of cyclins or CDKs, and loss or decreased level of endogenous CDK inhibitor proteins such as p16 and p27 in various tumors have been reported[2]. Flavopiridol, a specific small molecule-CDK inhibitor, showed potent antitumor activity in a series of experimental tumor models and is currently in clinical trial[3]. Thus, the CDKs are considered as new molecular targets for cancer chemotherapy. We established a novel cell-based assay using the budding yeast in which *Xenopus* cyclin A1 was induced and then CDK (Cdc28) kinase activity was elevated[4,5]. The hyper-activation of CDK in yeast resulted in showing growth arrest phenotype[4]. The compounds which can rescue the cyclin A1-induced growth arrest might be the new antitumor drug candidates acting on the cyclin/CDK-mediated cell cycle regulation. In the course of our microbial screening program, a novel *Streptomyces* metabolite belactosin A was identified as an active compound by which regrowth of the growth-arrested yeast was induced. Isolation, physico-chemical properties and biological activity of belactosin A are described.

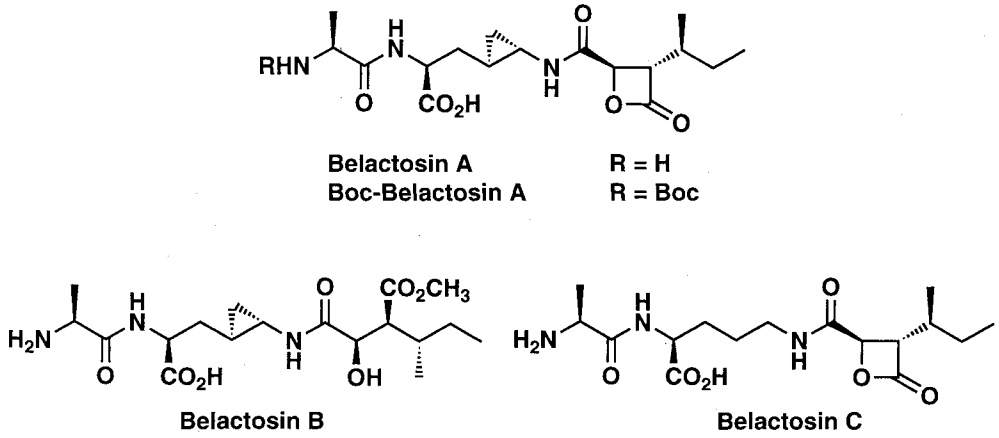
The producing organism KY11780 was isolated from a soil sample collected in Kanagawa prefecture, Japan and assigned to the *Streptomyces* sp. Fermentation was carried out at 28°C for 48 hours with appropriate aeration and agitation in 30-liter jar fermenters containing 15 liters of culture medium, consisting of 5% sucrose, 1.5% dry yeast 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$, pH 7.0. Belactosin A was accumulated in the culture medium. Which after filtration was applied to a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited). The column was washed with deionized water - MeOH (6 : 4) and eluted with deionized water - MeOH (5 : 5). The active eluate was concentrated and applied to a column of Diaion HP-20SS. The column was washed with deionized water - MeOH (7 : 3) and eluted with deionized water - MeOH (6 : 4). The active fraction was concentrated, and applied to a reverse phase column packed with ODS-AM 120-230/70 (YMC Inc.). The column was washed with deionized water - MeOH (7 : 3) and eluted with deionized water - MeOH (6 : 4). The active fraction was concentrated, and subjected to a silicagel column chromatography (C-200, Wako Pure Chemical Industries) developed and fractionated stepwise with BuOH - water - AcOH (50 : 1 : 0.1~6 : 1 : 0.1). Active fractions were combined, extracted with water and then lyophilized. 700 mg of crude belactosin A was obtained by this purification procedure. *t*-Butoxycarbonylation was effective in separating belactosin A from the hydrophilic impurities contained in the active fractions. The combined crude belactosin A was treated with di-*t*-butyl dicarbonate and $NaHCO_3$ in 50% aq THF followed by purification of resulting product using a silicagel column and eluting with $CHCl_3$ - MeOH - AcOH (50 : 1 : 0.1~5 : 1 : 0.1) to afford 382 mg of Boc-belactosin A. Deprotection with TFA gave intact belactosin A with 97% yield. The related minor products belactosin B and C were also obtained from the culture broth of KY11780. However, the production of these minor compounds was not reproducible under the same fermentation conditions. Physico-chemical properties are shown in Table 1. Each bioactive product was in quite water-soluble (>10 mg/ml). Spectral analysis revealed that belactosin A possessed an unique structure containing a novel amino acid, 3-(2-aminocyclopropyl)-alanine (AcpAla), and a ß-lactone, while belactosin C contained an ornithine instead of the AcpAla with ß-lactone and belactosin B contained a cleaved ß-lactone with AcpAla (Fig. 1). Belactosin B may be generated from belactosin A during the purification process, since belactosin A can be converted to belactosin B in the presence of MeOH under basic conditions. The details of the chemistry and structure elucidation will be reported elsewhere[6].

Belactosin A, B and C did not show antimicrobial activity against either Gram-negative and Gram-positive bacteria at the concentration up to 0.1 mg/ml. As for antitumor activity, belactosin A and C showed *in vitro* antiproliferateive activity against HeLa S3 cells with IC_{50} values of 51 μM and 200 μM, respectively, after 72 hours exposure. On the other hand, belactosin B showed no apparent activity (IC_{50}>300 μM). These results suggest that the ß-lactone is responsible for antiproliferative activity. However the potency of belactosins is lower than known

Table 1. Physico-chemical properties of belactosin A and C.

	Belactosin A	Belactosin C
Appearance	White powder	White powder
MP (°C)	184-185 °C	212-215 °C
$[\alpha]^{27}_D$	+4.8° [c 0.37, H_2O]	-8.1° [c 1.2, H_2O]
Molecular weight	369	357
Molecular formula	$C_{17}H_{27}N_3O_6$	$C_{16}H_{27}N_3O_6$
FAB-MS (m/z)	370 $(M+H)^+$	356 $(M-H)^-$
HRFAB-MS (m/z)	Found 370.1981 $(M+H)^+$	Found 356.1838 $(M-H)^-$
	Calcd. 370.1978	Calcd. 356.1821
UV λ_{max}^{DMSO} nm	End absorption	End absorption
IR ν_{max} (KBr) cm^{-1}	3261, 3078, 2964, 1834, 1668, 1558, 1456, 1389, 1271, 1111, 914	3388, 3086, 2966, 1834, 1662, 1568, 1412, 1269, 1115, 1016, 903

Fig. 1. Structures of belactosins.

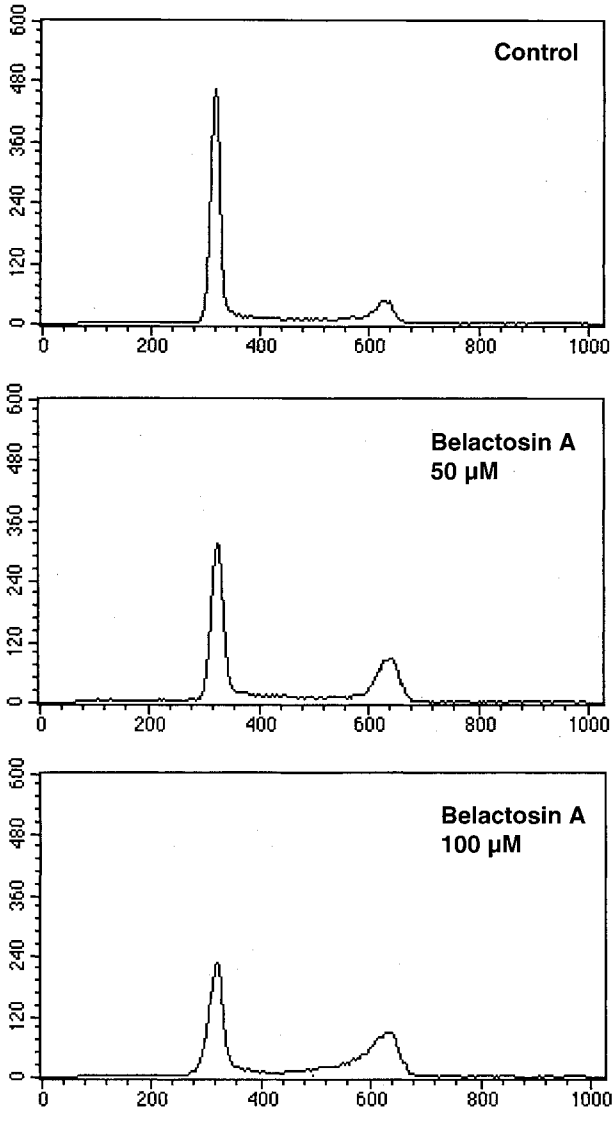


antitumor drugs. Uptake enhancement into HeLa S3 cells with the treatment of electroporation resulted in potentiation of antiproliferative activity of belactosin A (data not shown). Thus, low potency of belactosin A on antiproliferative activity was considered to be due to low permeability into human cells. Further examination revealed that protection of the carboxylic acid in belactosin A with several esters potentiated antiproliferative activity and antitumor activity *in vivo*[5]. Futhermore the effect of belactosin A on the cell cycle distribution was examined on HeLa S3 cells according to the propidium iodide staining method using a flow cytometer reference[7]. A decrease in G1 phase and increase in G2/M phase of cycling HeLa S3 cells in a dose dependent manner were observed after 24 hours exposure. Thus, we carried out microbial screening for their ability to restore the cyclin A1-induced growth arrest in budding yeast and identified a novel natural product belactosin A which inhibited cell cycle progression of human tumor cells at G2/M phase. The details on the mode of action and further evaluation of belactosin A will be reported elsewhere[5].

Acknowledgment

The authors are grateful to Mr. Naoyuki Hiraoka, Ms. Machi Kusunoki for their technical assistance.

Fig. 2. Effect of belactosin A on cell cycle distribution of HeLa S3 cells.



The cells were harvested after 24 hours treatment with belactosin A (50 and 100 μM) or without (Control). Cell fixation, RNA hydrolysis, and DNA staining with propidium iodide were performed as previously described[7]. DNA content of the cells was analyzed by FACSCalibur (BECTON DICKINSON).

AKIRA ASAI*
ATSUHIRO HASEGAWA
KEIKO OCHIAI
YOSHINORI YAMASHITA
TAMIO MIZUKAMI†

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, Japan

(Received September 7, 1999)

References

1) SHERR, C. J.: Cancer cell cycles. Science 274: 1672~1677, 1996
2) SHERR, C. J. & M. ROBERTS: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes & Dev. 13: 1501~1511, 1999
3) MEIJER, L.: Chemical inhibitors of cyclin-dependent kinases. Trends Cell Biol. 6: 393~397, 1996
4) FUNAKOSHI, M.; H. SIKDER, H. EBIHARA, K. IRIE, K. SUGIMOTO, K. MATSUMOTO, T. HUNT, T. NISHIMOTO & H. KOBAYASHI: Xenopus cyclin A1 can associate with Cdc28 in budding yeast, causing cell-cycle arrest with an abnormal distribution of nuclear DNA. Genes to Cells 2: 329~343, 1997
5) ASAI, A.; T. TSUJITA, Y. YAMASHITA, T. AKIYAMA, S. AKINAGA, M. FUNAKOSHI, H. KOBAYASHI & T. MIZUKAMI: in preparation
6) HASEGAWA, A.; A. ASAI, R. KATAHIRA, Y. SAITO, S. IKEDA & M. YOSHIDA: in preparation
7) OKAMOTO, A; A. ASAI, H. SAITO, M. OKABE & K. GOMI: Differential effect of duocarmycin A and its novel derivative DU-86 on DNA strand breaks in HeLa S3 cells. Jpn. J. Cancer Res. 85: 1304~1311, 1994

† Present address: Pharmaceutical Research & Development Center, Kyowa Hakko Kogyo Co. Ltd., 1-6-1 Ohte-machi, Chiyoda-ku, Tokyo 100-8185, Japan.