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NOTE

Chaetoglobosin A, an Inhibitor of Bleb Formation on K562 Cells Induced by Phorbol 12,13-Dibutyrate

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Abstract In the course of screening for the substances suppressing bleb formation of K562 cell induced by phorbol 12,13-dibutyrate (PDBu), an inhibitor, chaetoglobosin A (CgA) was isolated from a cultured broth of unidentified fungus. CgA showed a strong inhibitory activity with the IC_{50} value of 60 pM against bleb formation on K562 cells induced by PDBu, but it did not inhibit the activity of protein kinase C (PKC) *in vitro*. The inhibitory activity of CgA might be due to the modulation of actin filaments on the cell membrane. CgA exhibited strong cytotoxicity against various human cancer cell lines.

Key words: Bleb formation, inhibitor, PDBu, K562 cells, chaetoglobosin A

Cell surface blebbing has been demonstrated in several different cell types exposed to anoxia/ischaemia, including cardiac myocytes, freshly dissociated hipocampal neurons, hepatocytes, ascites tumor cells, and endothelial cells [6, 7, 11, 12]. The blebbing is an early consequence of hypoxic and toxic injury to cells and leads ultimately to cell death upon rupture of the membrane [9, 11, 12, 25]. Bleb formation is associated with changes in the dynamics of actin filaments [3, 18]. Also, blebbing is a morphological characteristic corresponding to classical apoptosis or programmed cell death, two distinct cell death programs [13, 15], and is induced by various kinds of chemicals [14, 18, 26].

Osada *et al.* [16] have found that phorbol esters such as phorbol 12,13-dibutyrate (PDBu), a strong tumor promotor and protein kinase C (PKC) activator, induced many blebs on the cell surface of K562 human chronic myeloid leukemia cells. They have established a unique screening system called the bleb forming assay to detect PKC modulators based on the morphological change of K562 cells [16] and have reported novel PKC inhibitors [10, 17] and activators [14]. In our screening to search for the substances suppressing bleb formation of PDBu-induced K562 cells, a potent inhibitor was isolated from a cultured broth of fungus and was identified as chaetoglobosin A (CgA) which is known as a cytotoxic fungal metabolite [20]. The present paper describes the isolation, physicochemical properties, structure elucidation, and biological activities.

An unidentified fungus F60686 was isolated from a soil sample collected in Mooju, Korea. The strain was grown at 28°C and maintained on the medium (glucose 2%, malt extract 2%, polypeptone 0.1%, and agar 1.8%, not adjusted pH). For the production of active compound, the fungus was cultured in a 1 l Erlenmeyer flask containing 200 ml of fermentation medium (glucose 2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.1%, pH 7.0). The fermentation was carried out on a rotary shaker (150 rpm) at 28°C for 7 days. The fermentation broth of F60686 was extracted with the same volume of acetone. The acetone extract was concentrated *in vacuo* and suspended in distilled water. Then, the inhibitor was extracted with ethylacetate. The ethylacetate layer was concentrated *in vacuo* to dryness. The residue was applied onto a silica gel column chromatography and eluted stepwise by chloroform-methanol (100:1~10:1). The active fractions eluted with chloroform-methanol (30:1) were combined and concentrated *in vacuo* to dryness. The residue was applied onto a Sephadex LH-20 column chromatography and eluted with chloroform-methanol (1:1). The active residue was rechromatographed on an ODS RP-18 column using the solvent of methanol-water (70:30). The residue was dissolved in methanol and subjected to preparative HPLC (Shimazu LC-6AD, YMC J'sphere ODS-H80,

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150×20 mm; mobile phase: methanol:water=7:3, flow rate: 6 ml/min; UV detection at 210 nm). The active peak was collected and concentrated *in vacuo* to yield pure compound as a yellow powder. For the bleb formation assay, 10 μl of sample solution was added to 100 μl of K562 cell culture (1×10^5 cells/ml) in 96-multiwell plates, incubated at 37°C for 1 h in a CO_2 incubator, and then treated with PDBu at a final concentration of 1 μg/ml. Ten minutes after the addition of PDBu, the morphology of K562 cells was observed under a microscope. *In vitro* PKC assay was performed by the method of Huang *et al.* [8]. The cytotoxicity tests were carried out with the sulforhodamine B (SRB) microculture colorimetric assay described by Skehan *et al.* [23]. 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay for cell viability was performed by the method of Alley *et al.* [1].

CgA was purified as a yellow powder. The inhibitor was soluble in ethylacetate, chloroform, methanol, and dimethylsulfoxide (DMSO) but not in *n*-hexane and water. UV spectrum obtained in methanol contained peaks at λ_{max} 228, 270, 285, and 291 nm. The absorptions around 270~291 nm indicated the presence of indole chromophore [21]. The IR spectrum of the compound exhibited a broad absorption between 3445 and 3250 cm^{-1} indicating the presence of hydroxyl groups. Additional bands appeared at 1695~1610 cm^{-1} (lactam and conjugated carbonyl group) and at 980~890 cm^{-1} (double bonds). The molecular weight of the compound was determined to be 528 (*m/z* 529, M+ H^+) by FAB-MS. ^1H and ^{13}C NMR assignments are listed in Table 1, along with those of CgA for comparison. It was revealed that the compound had all 32 carbons in the molecule; four methyl carbons ($-CH_3$), two methylene

Table 1. ^1H and ^{13}C NMR data for chaetoglobosin A (in DMSO-d_6).

Carbon No.	MT686-A[a]		Chaetoglobosin A[b]	
	^{13}C (δ, ppm)*	^1H (δ, ppm)	^{13}C (δ, ppm)	^1H (δ, ppm)
20 (C=O)	200.37 (201.90)	-	200.26	-
23 (C=O)	199.42 (198.89)	-	199.20	-
1 (C=O)	172.64 (175.54)	-	172.50	-
17	137.57 (140.91)	5.33 (d)	137.55	5.39
1'a	136.08 (138.08)	-	136.03	-
21	135.90 (136.08)	6.65 (d)	135.34	7.33
22	133.74 (134.24)	7.22 (d)	133.74	6.55
14	133.48 (133.79)	5.07 (ddd)	133.20	5.13
18	131.62 (133.31)	-	131.54	-
3'a	127.36 (129.73)	-	127.24	-
13	127.13 (128.86)	6.04 (dd)	127.24	6.07
5'	124.06 (125.78)	7.00	123.94	6.9~7.4
2'	120.86 (122.39)	7.07 (d)	120.72	7.02
6'	118.48 (120.06)	6.92	118.34	6.9~7.4
4'	118.02 (119.62)	7.36 (d)	117.95	6.9~7.4
7'	111.42 (112.60)	7.27 (d)	111.29	6.9~7.4
3'	109.15 (109.55)	-	109.05	-
19	81.58 (82.94)	4.88 (d)	81.46	4.87
9	62.97 (65.10)	-	62.88	-
7	61.28 (63.67)	2.65 (d)	61.25	2.69
6	57.11 (59.28)	-	57.02	-
3	52.20 (54.20)	3.65 (t)	52.13	3.67
8	45.98 (50.23)	2.50	46.04	2.16
4	45.88 (47.90)	2.22 (dd)	45.90	[c]
15	40.17 (42.86)	1.85, 2.24 (m)	40.16	1.8~2.4
5	35.29 (37.67)	1.75	35.32	1.72
10	32.69 (33.61)	2.48, 2.74 (m)	32.64	2.58, 2.77
16	31.80 (33.21)	2.52	31.67	[d]
16-CH_3	20.97 (21.29)	0.93 (d)	20.82	0.96
12	19.14 (19.76)	1.08 (s)	19.02	1.14
11	12.19 (13.19)	0.64 (d)	12.11	0.77
18-CH_3	10.60 (10.74)	1.33 (s)	10.48	1.34
1'(NH)	-	10.86 (d)	-	10.8
2(NH)	-	8.29 (s)	-	7.92
19-OH	-	5.22 (d)	-	[c]

[a]Chaetoglobosin A isolated in this work. [b]from Sekita S *et al.* [19]. [c,d]Overlapping with H_2O and the solvent signals, respectively. *Chemical shifts in CD_3OD.

carbons ($-CH_2$), seven methine carbons (-CH), carbons showing ten doublets (=CH) and four singlets (-C=), and three carbonyl carbons (-C=O). In the ^1H NMR spectrum, there were four methyl protons (δ_H 0.93~1.33), two methylene protons (δ_H 1.85~2.74), seventeen methine protons, two -NH protons (δ_H 10.86 and 8.29), and an -OH proton (δ_H 5.22). Among the methine protons, there were five protons (δ_H 6.92~7.36) corresponding to the indole ring moiety and also olefinic protons (δ_H 7.22 and 6.65, δ_H 6.04 and 5.07) originating from the lactam structure. From searching the chemical databases of natural products [4], the physicochemical properties and spectral data of UV, IR, FAB-MS, and NMR of the compounds were very similar to those of CgA. In order to define the structure, ^1H-^1H connectivities and ^1H-^{13}C long-range couplings were determined from a correlated spectroscopy (COSY) and a heteronuclear multiple-bond correlation (HMBC), respectively. Thereafter, it was confirmed that the structure of the compound is identical with that of CgA (Fig. 1). However, it might be different in the protons of H-21 (δ_H 6.65) and H-22 (δ_H 7.22). That is, the two protons are interconverted, suggesting the possibility of conformational change in this part [19]. CgA is a fungal metabolite belonging to the family of cytochalasin, and had been first isolated from *Chaetomium globosum* [20].

When K562 cells were treated with 1 μg/ml of PDBu, blebs appeared within 10 minutes on the cell surface (Fig. 2b) and were maintained for more than 2 h. Thereafter, the cells exhibiting blebs began to rupture and flatten on the bottom of wells. CgA completely suppressed the bleb formation at the concentration of 200 pM (Fig. 2e) and the IC_{50} value was 60 pM (Fig. 2d). This value was one hundred times higher than that of staurosporine (about 6 nM), which is known as a strong inhibitor of PKC [24]. When CgA was added after pretreatment of PDBu to K562 cells, the blebs also disappeared within 10 minutes. Although no cells showed a morphological change in response to CgA alone at the low concentrations showing the inhibitory activity on bleb formation, interestingly, at higher concentrations (above 2 μM) the cells were transformed into spindle-like shapes (Fig. 2c). No significant change in cell viability was observed up to a concentration of 200 nM but the viability was rapidly decreased above the concentration. This result suggested that the cell viability correlated with the morphological change of K562 cells to spindle-like shapes. It is known that bleb formation on the K562 cell surface is closely related

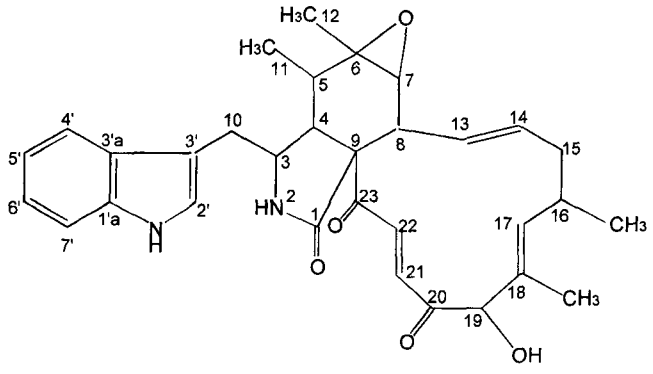

Fig. 1. Structure of chaetoglobosin A.

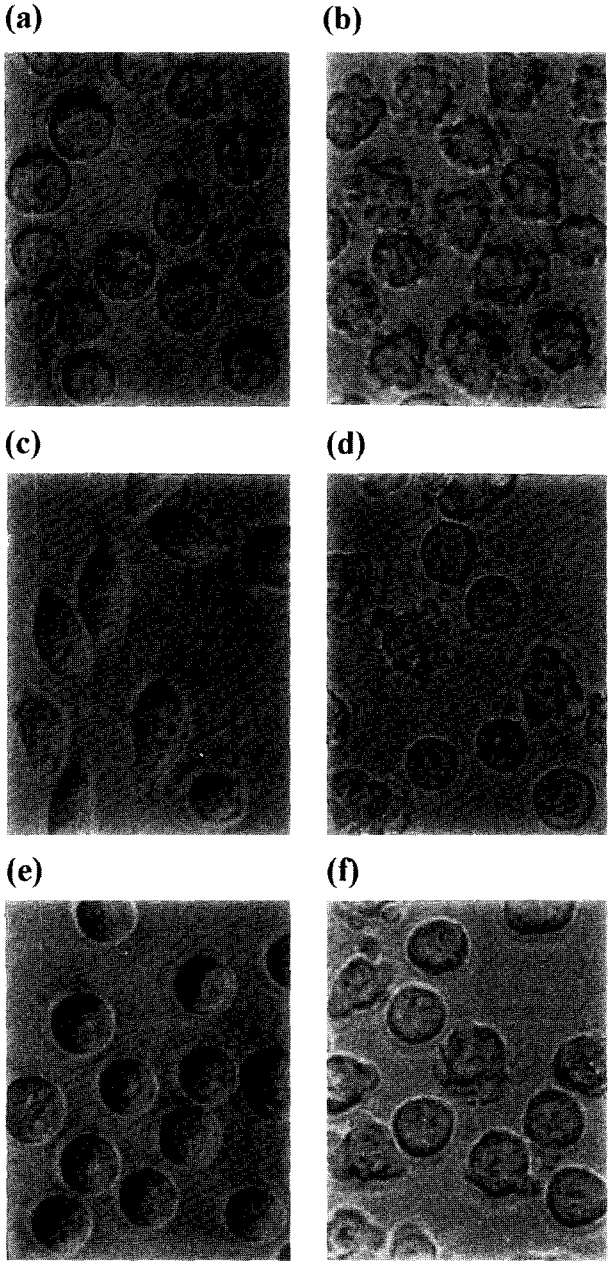

Fig. 2. Morphology of K562 cells (×400) treated with various compounds.

(a) Control (cultured on RPMI-1640 supplemented with 10% FBS), (b) treated with PDBu (1 μg/ml) for 10 min, (c) treated with CgA (2 μM) for 60 min, (d~f) pretreated with the following inhibitors for 60 min and then treated with PDBu (1 mg/ml) for 10 min, (d) CgA (60 pM), (e) CgA (200 pM), (f) staurosporine (6 nM).

with the activity of PKC [16]. Moreover, most of the inhibitors of bleb formation were reported as inhibitors of PKC. Unfortunately, even at the concentration of 200 μM, however, CgA did not inhibit *in vitro* PKC activity (data not shown). This result suggested that the inhibitory activity of CgA might be independent of the inhibition of PKC activation by PDBu. At first, CgA was reported as a cytotoxic metabolite of fungi to HeLa cells [20]. The metabolite also induced polynucleation in the cells and blocked the salt-induced polymerization of G-actin [19, 22]. Also, it was well known that cytochalasins potently inhibit the polymerization of actin filaments [2, 5]. Therefore, the inhibitory activity of CgA on bleb formation might be due to the inhibition of polymerization of actin filaments on the membrane [16]. In contrast, there were reports that cytochalasin B and D did not inhibit, but induced the bleb formation on K562 cells like PDBu [14, 18]. Thus further experiments are needed to determine the inhibition mechanism of bleb formation by CgA.

CgA exhibited strong cytotoxicity *in vitro* against various human cancer cell lines such as melanoma, colon, lung, and leukemia.

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