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Note

Identification and Biological Activities of Bryostatins from Japanese Bryozoan

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Six bryostatins were isolated from Japanese bryozoan by evaluating their binding to the C1B domain of protein kinase Cδ (PKCδ). Structure-activity studies of bryostatins 4, 10, and 14 suggested that the ester group at C20 was not necessary for binding to and activating PKCδ. These bryostatins showed significant anti-tumor-promoting activity in induction tests with the Epstein-Barr virus early antigen.

Key words: protein kinase C; bryostatin; *Bugula neritina*; tumor promotion; Epstein-Barr virus

Protein kinase C (PKC) isozymes play a crucial role in cellular signal transduction *via* second-messenger diacylglycerols.[1)] Protein kinase Cδ (PKCδ), one of the PKC isozymes, is involved in tumor suppression and apoptosis.[2,3)] Bryostatin 1 (bryo-1, Fig. 1), which was isolated from the marine bryozoan, *Bugula neritina*,[4)] showed strong anti-cancer and anti-tumor-promoting activity through PKCδ-dependent mechanisms.[5,6)] Bryo-1 has also attracted strong attention as a therapeutic lead against Alzheimer's disease and acquired immune deficiency syndrome.[7)] Although 20 bryostatin homologues have been identified,[6,8,9)] their limited availability from nature has hampered studies of their structure-activity relationships and molecular mechanisms of action. It is necessary to develop a convenient and reliable way to identify bryostatins and bryostatin-like compounds with high sensitivity and selectivity.

Bryo-1 binds to the cysteine-rich C1 domains of PKC isozymes (C1A and C1B) for their activation.[1)] Since whole PKC isozymes are highly unstable and precious, our group has developed a synthetic C1 peptide library that binds to PKC activators with an affinity comparable to that of whole PKC isozymes.[10,11)] We screened in this present study for PKCδ ligands in marine organisms by evaluating their binding to a C1B peptide of PKCδ (δ-C1B) based on competition with the potent PKC activator, [^{3}H]phorbol 12,13-dibutyrate (PDBu, Perkin-Elmer Japan, Yokohama, Japan).

Among over 200 $CHCl_3$/MeOH extracts of marine organisms (sponges, cyanobacteria, and bryozoan) collected by the OP-BIO Factory Co. (Okinawa), several extracts of *B. neritina* exhibited high affinity for δ-C1B. This bryozoan found in the Gulf of Imazu (Fukuoka, Japan) was harvested in the summer of 2010. Extraction of the animal (6 kg wet weight) was carried out twice with $CHCl_3$/MeOH (1:1). The extract after evaporation was partitioned three times between EtOAc and water. The resulting EtOAc extract (26.5 g) was chromatographed on Wakogel C-200 (silica gel, Wako Pure Chemical Industries, Osaka, Japan), sequentially eluting with 100% $CHCl_3$, 30% EtOAc in hexane, 100% EtOAc, and 100% MeOH, to give two active fractions from the 100% EtOAc and MeOH eluates. These were purified again on the same silica gel with *i*-PrOH-

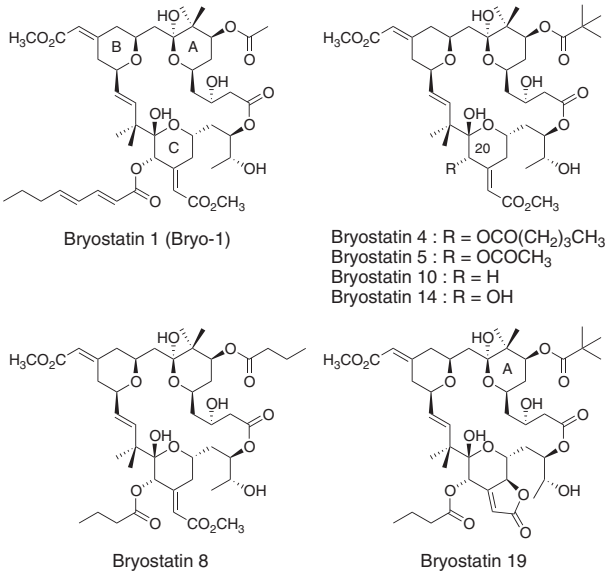

Fig. 1. Structure of Bryostatins.

The FAB-MS data (Jeol JMS-600, *m*-nitrobenzyl alcohol as a matrix, except glycerol for bryo-8 and bryo-10) for the compounds isolated in this study are as follows: bryo-4, m/z 917 $[M+Na]^+$; bryo-5, m/z 889 $[M+Na]^+$; bryo-8: m/z 863 $[MH-18]^+$, 845 $[MH-36]^+$, 827 $[MH-54]^+$); bryo-10, m/z 831 $[M+Na]^+$; bryo-14, m/z 831 $[M+Li]^+$ in the presence of LiI; bryo-19, m/z 879 $[MH]^+$.

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Abbreviations: Bryo, bryostatin; EBV-EA, Epstein-Barr virus early antigen; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

Table 1. K_i Values for the Inhibition of [^{3}H]PDBu Binding by Bryostatins

PKCδ and its C1 peptides	K_i (nM)		
	Bryo-4	Bryo-10	Bryo-14
Whole PKCδ	0.16	0.24	1.2
δ-C1A	5.2	6.6	42
δ-C1B	0.17	0.15	0.45

$CHCl_3$-hexane (2:20:78, 4:20:76, and 6:20:74) to yield two active fractions from the 4% and 6% *i*-PrOH-$CHCl_3$-hexane eluates. These were further purified by column chromatography on YMC AA12S50 gel (ODS, Yamamura Chemical Laboratory, Kyoto, Japan) by using 90% MeOH. HPLC with silica-gel and ODS-gel columns (YMC-packed SIL-SL12S05 and ODS-AM12S05), respectively eluting with *i*-PrOH-$CHCl_3$-hexane (5:15:80) and 80% acetonitrile in water and guided by the [^{3}H]PDBu inhibition assay with δ-C1B, finally yielded bryo-4[12] (1.2 mg), bryo-5[13] (0.3 mg), bryo-8[14] (0.5 mg), bryo-10[15] (2.3 mg), bryo-14[16] (0.9 mg), and bryo-19[8] (0.3 mg). These compounds completely inhibited the specific binding of [^{3}H]PDBu at 1 μg/mL. The ^{1}H- and ^{13}C-NMR data (AVANCE III 500, Bruker, Germany; ref. tetramethylsilane, 296 K), optical rotation (P-2200, Jasco, Tokyo, Japan), and FAB-MS (Jeol JMS-600, Tokyo, Japan) for these bryostatins were identical to those previously reported. Although Kamano *et al.* have identified bryo-4, 5, 6, 8, 9, and 10 from the Japanese bryozoan in the Gulf of Aomori, Ohzuchi and Sagami,[15,17,18] bryo-14 and 19 were alternatively identified in this study. Bryo-1, the major component of the American bryozoan,[4] could not be found at all in this sample. These results suggest that the geographical difference of *B. neritina* could affect the variability of the types and amounts of bryostatin homologues.

Bryo-4, 5, 10, and 14 all had a pivaloyl group on the A-ring, but had different substituents at C20 of the C-ring (Fig. 1). Little is known about the role of these substituents in the biological activities of bryostatins. Wender's group[19] have synthesized a simplified analogue of bryo-1 with several ester substituents at C20, and suggested a correlation between the number of carbons on the C20 ester and the affinity for a mixture of rat PKC isozymes. However, the role of the ester and hydroxyl groups at C20 in the various biological activities of bryostatins has remained elusive. We therefore examined the biological activities of bryo-4, -10, and -14. The biological evaluation of bryo-5 was almost impossible because of its small amount.

Since the unique biological properties of bryo-1 are considered to be correlated with the activation of PKCδ,[20] we evaluated the ability to bind to and activate PKCδ by using our PKCδ C1 peptides[10,11] and human recombinant PKCδ (Pan Vera LLC, Madison, USA). The 50% inhibition value (IC_{50}) for the specific binding of [^{3}H]PDBu to PKCδ was measured as previously reported.[10,11] The affinity of bryostatins for PKCδ is expressed by the K_i value calculated from the IC_{50} and K_d values of [^{3}H]PDBu reported by Sharkey and Blumberg.[21] Table 1 shows that the K_i values of bryo-4 and 10 for whole PKCδ were almost equal to each other and comparable to those of δ-C1B. Both compounds bound significantly to δ-C1A as reported for bryo-1 (K_i for δ-C1A and δ-C1B, 5.3 and 0.60 nM) unlike tumor promoters.[22] This suggests that the structural difference in the C20 side chain between bryo-1 and bryo-4 hardly changed the ability to bind to PKCδ C1 peptides. In contrast, the K_i values of bryo-14 for whole PKCδ and its C1 peptides were about 8 times larger than those of bryo-4 and 10, possibly due to the hydroxyl group at C20. These results indicate that the hydrophobicity at C20 slightly influenced the binding to PKCδ, but that the C20 ester group did not increase the binding ability. This conclusion is in contrast with that obtained when using the C20 esters of Wender's simplified analogue of bryo-1;[19] the hydrophobic C20 ester was necessary to potently bind to rat PKC isozymes in Wender's analogues.

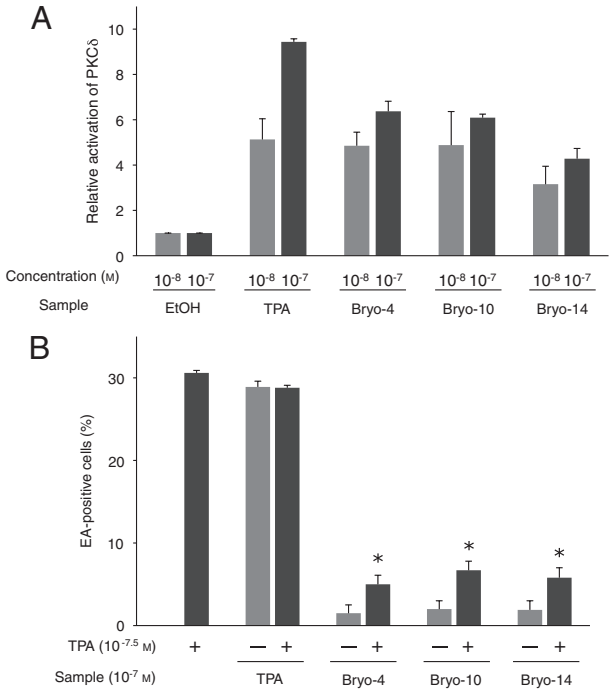

Fig. 2. Activation of PKCδ and Induction of EBV-EA by Bryostatins.
A, Kinase activity of PKCδ stimulated by bryostatins at the indicated concentrations. The values are expressed as a percentage relative to the control (EtOH). The final concentration of EtOH was 2.0%. The results are presented as the average of duplicated points when all compounds were simultaneously tested. Error bars represent the standard error of the mean ($n = 2$). Another experiment gave similar results. B, Induction of EBV-EA by bryostatins. The percentages of EA-positive cells are shown. Each point is presented as the mean of triplicate experiments. Sodium *n*-butyrate (4 mM) was added to all samples to enhance the sensitivity of the Raji cells (B-lymphocytes). Only 0.1% EA-induction was observed at 4 mM sodium *n*-butyrate. The final concentration of dimethyl sulfoxide was 0.4%. Error bars represent the standard error of the mean ($n = 3$). *The difference between each sample and the positive control (TPA 3.16×10^{-8} M) is statistically significant ($p < 0.01$, *t*-test).

To evaluate the ability of these bryostatins to activate PKCδ, the phosphorylation levels were measured by using a fluorescence-labeled peptide substrate (PepTag® non-radioactive protein kinase assay; Promega, Tokyo, Japan) according to the manufacturer's instructions (Fig. 2A). After the reaction had been stopped by heating at 95 °C for 10 min, the samples were loaded on to 0.8% agarose gel (Nippon Gene, Toyama, Japan)

which was then quantified by densitometry software (NIH ImageJ, Bethesda, MD, USA). Bryo-4 and -10 both activated PKCδ even at 1.0×10^{-8} M, comparable to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a positive control, while bryo-14 showed slightly weaker activation. These activation profiles are in good agreement with the results of the binding assay (Table 1).

Anti-tumor-promoting activity is characteristic of bryo-1[23] while most PKC activators in nature are tumor promoters. The anti-tumor-promoting activity was evaluated *in vitro*, based on the inhibitory activity of the Epstein-Barr virus early antigen (EBV-EA) induced by TPA.[24,25] EBV is activated by treating with a tumor promoter like TPA to generate EA which can then be detected by an indirect immunofluorescence technique. Figure 2B shows that TPA generated about 30% of EA-positive cells at 3.16×10^{-8} M. In contrast, bryo-4, -10, and -14 showed almost no induction (1.0×10^{-7} M), even at 1.0×10^{-6} M (data not shown), and significantly attenuated the induction by TPA (3.16×10^{-8} M). Quite similar results have been reported for bryo-1.[23] These results suggest that bryo-4, -10, and -14 had little tumor-promoting activity and that the inhibitory activity of EBV-EA was not related to the structure at C20. This was consistent with the finding that 20-*epi*-bryo-7, with an acetyloxy group at C20, exhibited significant biological activity in antiproliferative assays against several cancer cell lines.[26] These data do not reflect well the difference in the binding affinity to PKCδ, possibly because these data were measured at a higher concentration (1.0×10^{-7} M) when compared with the K_i values for PKCδ (1.0×10^{-9} M).

In summary, we screened and identified six bryostatins from the Japanese bryozoan by using a [^{3}H]PDBu binding assay with the PKCδ-C1B peptide developed by us. Structure-activity studies on bryo-4, -10, and -14 clearly indicated that the C20 ester was not necessary for binding to and activating PKCδ, nor for the anti-tumor-promoting activity of bryostatins. As Wender has suggested,[19,27] this means that one can utilize this position to introduce a photoaffinity label or a linker to identify new targets other than PKC isozymes. Since bryostatins are not tumor promoters, conventional assays for PKC ligands such as EBV-EA induction are not applicable. The assay presented in this paper would be useful for finding new PKC ligands with antagonistic activities like bryostatins.

Acknowledgments

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