Six bryostatins were isolated from Japanese bryozoan by evaluating their binding to the C1B domain of protein kinase C\(\delta\) (PKC\(\delta\)). Structure-activity studies of bryostatins 4, 10, and 14 suggested that the ester group at C20 was not necessary for binding to and activating PKC\(\delta\). 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 C\(\delta\) (PKC\(\delta\)), 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 PKC\(\delta\)-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,\(^8,9\) 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 PKC\(\delta\) ligands in marine organisms by evaluating their binding to a C1B peptide of PKC\(\delta\) (\(\delta\)-C1B) based on competition with the potent PKC activator, \(^3\)H\]phorbol 12,13-dibutryrate (PDBu, Perkin-Elmer Japan, Yokohama, Japan).

Among over 200 CHC\(_1\)/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 \(\delta\)-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 CHCl\(_3\), MeOH, EtOAc, and 100% MeOH, to give two active fractions, EtOAc and MeOH eluates. These were purified again on the same silica gel with i-PrOH.
CHCl₃-hexane (2:20:78, 4:20:76, and 6:20:74) to yield two active fractions from the 4% and 6% i-PrOH-CHCl₃-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-12S05 and ODS-SIL12S05, respectively eluting with 90% MeOH. HPLC with silica-gel and ODS-gel Yamamura Chemical Laboratory, Kyoto, Japan) by column chromatography on YMC AA12S50 gel (ODS, two active fractions from the 4% and 6%)

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

<table>
<thead>
<tr>
<th>PKC5 and its C1 peptides</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole PKC5</td>
<td></td>
</tr>
<tr>
<td>δ-C1A</td>
<td>1.2</td>
</tr>
<tr>
<td>δ-C1B</td>
<td>42</td>
</tr>
</tbody>
</table>

Fig. 2. Activation of PKC5 and Induction of EBV-EA by Bryostatins. A, Kinase activity of PKC5 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 × 10⁻³ M) is statistically significant ($p < 0.01$, t-test).

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 PKC5 C1 peptides. In contrast, the $K_i$ values of bryo-14 for whole PKC5 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 PKC5, 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.

To evaluate the ability of these bryostatins to activate PKC5, 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)
EBV is activated by treating with a tumor-promoting activity 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 × 10⁻³ μM. In contrast, bryo-4, -10, and -14 showed almost no induction (1.0 × 10⁻⁷ M), even at 1.0 × 10⁻⁹ M (data not shown), and significantly attenuated the induction by TPA (3.16 × 10⁻³ M). Quite similar results have been reported for bryo-1. These results suggest that bryo-4, -10, and -14 had little tumor-promoting activity and that the inhibitory activity of EBV-EA was 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. These data do not reflect well the difference in the binding affinity to PKC⁰/C₀ values for PKC activity in antiproliferative assays against several cancer cell lines. These data do not reflect well the difference in the binding affinity to PKC⁰/C₀ values for PKC activation. These activation profiles are in good agreement with the results of the binding assay (Table 1). In summary, we screened and identified six bryostatins from the Japanese bryozoan by using a [⁸⁶⁵]PDBu binding assay with the PKC⁰/C₁ 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 PKC⁰, nor for the anti-tumor-promoting activity of bryostatin. As Wender has suggested, this means that one can utilize this position to introduce a photoaffinity label or a linker to identify new targets other than PKC isoforms.

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