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Synthesis and structure–activity studies of simplified analogues of aplysiatoxin with antiproliferative activity like bryostatin-1*

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Abstract: Protein kinase C (PKC) isozymes are promising targets for anticancer therapy. Bryostatin-1 (bryo-1), a unique PKC activator with little tumor-promoting activity, is currently in clinical trials for the treatment of cancer. However, its limited availability from natural sources and its synthetic complexity have hampered studies of its mode of action and structural optimization as a therapeutic agent. The development of synthetically more accessible compounds with bryo-1-like activities is thus needed. Recently, we developed a simple and less lipophilic analogue of tumor-promoting aplysiatoxin (ATX) (aplog-1) as a promising lead for bryo-1-like anticancer drugs. Structure–activity studies suggested that local hydrophobicity around the spiroketal moiety of aplog-1 is a crucial determinant of its antiproliferative activity. The hydrophobic analogue (12,12-dimethyl-aplog-1) displayed more potent antiproliferative activity. Moreover, it showed little tumor-promoting activity and even suppressed the tumor promotion by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in vivo and in vitro. Aplog-1 and bryo-1 bound selectively to novel PKC isozymes (δ, η, and θ) while tumor promoters bound to both conventional and novel PKC isozymes. These results suggest that the unique biological activities of aplog-1 and bryo-1 are ascribable in part to the ability to bind to PKCδ, but weak binding to conventional PKC isozymes might also be important.

Keywords: antiproliferative activity; antitumor activity; aplysiatoxin; bioactive molecules; biological activity; bryostatin; organic chemistry; organic synthesis; phorbol ester; protein kinase C; structure–activity; tumor promoter.

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INTRODUCTION

Tumor promoters themselves are non-carcinogenic, but they markedly increase tumor yields when applied repeatedly after the initial administration of a small amount of carcinogen. Nishizuka and colleagues suggested that the effects of tumor promoters are mediated by protein kinase C (PKC), a family of serine/threonine kinases that play a pivotal role in cell surface signal transduction [1]. Potent tumor promoters occurring in nature such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), teleocidin B-4, and aplysiatoxin (ATX), activate PKC regardless of their large structural differences (Fig. 1) [2,3]. On the other hand, bryostatin-1 (bryo-1), isolated from the marine bryozoan *Bugula neritina* [4], is a unique PKC activator with little tumor-promoting activity and antagonizes the effects of TPA [5,6]. It is currently undergoing clinical trials for the treatment of cancer, including solid tumors, leukemia, and other lymphomas [7–10]. However, its limited availability from natural sources and its synthetic complexity have hampered studies on its mode of action and structural optimization as a therapeutic agent.

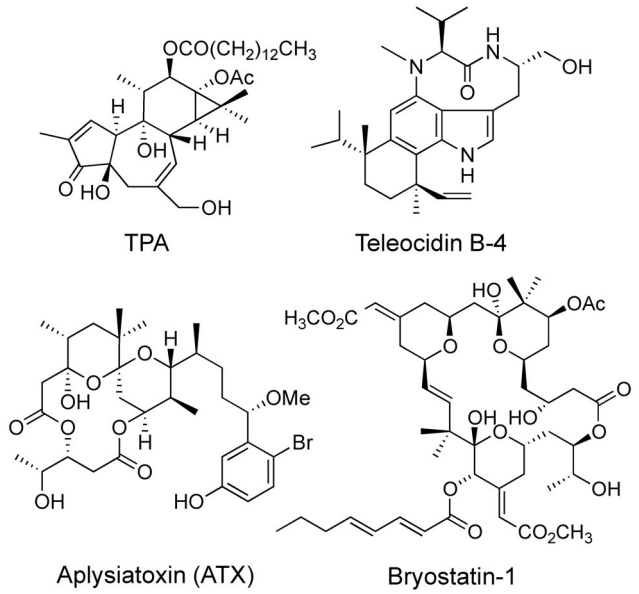

Fig. 1 Structure of naturally occurring tumor promoters and bryo-1.

Total synthesis of bryo-7 was first reported by Masamune and colleagues in 1990 [11], followed by that of bryo-2 and bryo-3 [12,13]. Recently, excellent practical methods for synthesizing bryo-1-related compounds have been developed by the groups of Wender, Keck, Hale, and Trost. Wender and colleagues developed simplified analogues of bryo-1 showing more potent antiproliferative effects than bryo-1 [14,15]. Keck and colleagues identified the structural factors responsible for the unique biological activities of bryo-1 [16,17]. Trost and colleagues established a practical route for producing bryo-16 as a common intermediate of various bryostatins [18,19]. More recently, Wender, Keck, Hale, and Kirsche have reported the total synthesis of bryo-9, bryo-1, and bryo-7, respectively [20–23]. In contrast, we attempted to identify more synthetically accessible compounds with bryo-1-like activities as another way to address the supply problem [24].

DESIGN AND SYNTHESIS OF APLOG-1

Although the origin of the biological difference between bryo-1 and tumor promoters remains unclear, the activation of PKCδ is proposed to be responsible for the unique biological activities of bryo-1 [6]. PKCδ, a PKC isozyme, is involved in apoptosis and plays a tumor suppressor role [25,26]. Tumor promoters as well as diacylglycerols bind to the tandem cysteine-rich domains in the regulatory region of PKC isozymes [27]. Recent investigations revealed that bryo-1 binds to both C1A and C1B domains of PKCδ and translocates it from the cytosol to the nuclear membrane (Fig. 2) [28–31]. In contrast, tumor promoters bind almost exclusively to the C1B domain and induce its translocation to the plasma membrane. The translocation was reported to correlate with the hydrophobicity of the ligand; tumor promoters are hydrophobic, while bryo-1 is rather hydrophilic [32]. Thus, we tried to develop new anticancer compounds based on these two factors, C1B selectivity and hydrophobicity.

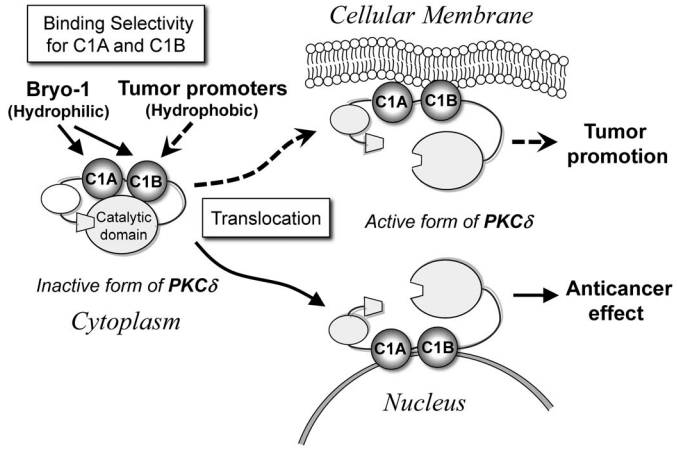

Fig. 2 Translocation of PKCδ by TPA and bryo-1.

However, determination of the selectivity for the C1B domain is almost impossible using the entire enzyme. Since the phorbol ester-binding sites are zinc fingers composed of only 50 amino acids [27], the synthetic approach is more advantageous than the DNA recombination method for the rapid and accurate evaluation of C1 domain selectivity. In collaboration with Prof. Wender, we established a binding assay using synthetic C1 peptides [33–35]. After folding with zinc, specific binding could be measured using tritium-labeled phorbol 12,13-dibutyrate (PDBu). PDBu showed a dissociation constant of 0.76 nM for whole PKCδ. In our assay system using C1 peptides, K_d values for the C1A and C1B peptides were 52 and 0.53 nM, respectively. By comparing with the value for PKCδ, the main binding site of PDBu can be identified as the C1B domain [35].

Employing the PDBu competition test using the PKC C1 peptides, the binding selectivity for the PKCδ C1 domains of various tumor promoters and their derivatives was examined. As reported previously [36], tumor promoters such as phorbol esters, ingenol esters, and indolactam derivatives bound mainly to the C1B domain with a ratio of 100–200. In contrast, bryo-1 bound to both of the C1 domains with a ratio of less than 10 [K_i(C1A)/K_i(C1B) = 8.8]. Unexpectedly, ATX isolated from the sea hare *Stylocheilus longicauda* [37,38] displayed low selectivity like bryo-1 with a ratio of 29. This led us to select ATX as a lead compound for new anticancer agents.

An interesting feature of ATX is the role of the bromine atom in the phenol ring. Loss of this atom did not affect the ability to bind to PKC, but reduced the tumor-promoting activity [39,40]. Moreover, 3-deoxy-debromo-ATX and debromo-ATX are equipotent as PKC activators, indicating that the hydroxyl group at position 3 is not indispensable to PKC activation [41,42]. Based on the results, we designed simple and less lipophilic analogues of ATX (aplog-1, 2 and DM-aplog-2) as potential candidates for synthetically accessible PKC activators with anticancer activities (Fig. 3) [24,43]. The chiral methyl and methoxy groups as well as the bromine atom were removed to decrease hydrophobicity, and the labile hemiacetal hydroxyl group at position 3 was also replaced with a hydrogen atom to increase chemical stability. While aplog-1 retains the geminal dimethyl substituents at the spiroketal moiety and the phenolic hydroxyl group at the side chain, aplog-2 lacks both. DM-aplog-2 lacks only the phenolic hydroxyl group. The lipophilicity of aplog-1 (ClogP = 2.3) was estimated to be similar to that of bryo-1 (ClogP = 1.9), 100 times less lipophilic than ATX (ClogP = 4.2) (Fig. 3).

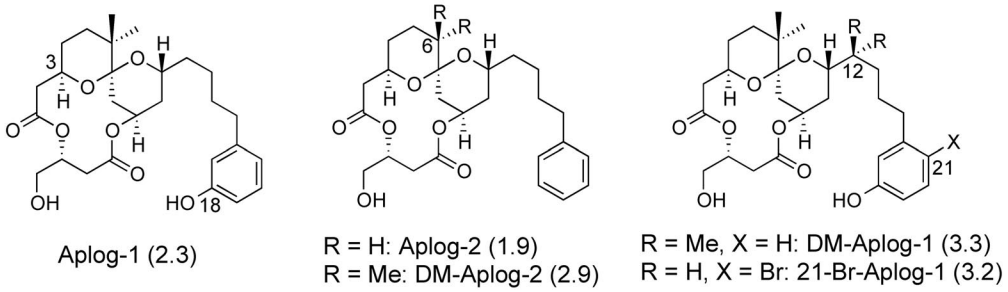

Fig. 3 Structure of aplog-1, aplog-2, DM-aplog-2, DM-aplog-1, and 21-Br-aplog-1, simplified analogues of tumor-promoting ATX, along with the calculated ClogP values. Those of bryo-1 and ATX are 1.9 and 4.3, respectively.

The three total synthesis of ATX and its analogues has been reported by Kishi, Katuski, and Yamamura's groups [44–46]. The synthesis of aplog-1 was carried out as shown in Fig. 4 [24]. Starting from 1-(benzyloxy)-3-(3-bromopropyl)benzene, the aldehyde **2** was obtained in three steps. Keck's asymmetric allylation of **2** [47], followed by Smith's iodocarbonate cyclization reaction [48] yielded the cyclic carbonate **3**, which was transformed into the epoxide unit **4** in two steps.

Coupling of **4** with dithiane by the protocol of Ide and Nakata [49] yielded **5** which was converted to the spiroketal **6** in six steps. Condensation of **6** with carboxylic acid using Yamaguchi's method [50] yielded **7**. Oxidative cleavage of the double bond, followed by Yamaguchi's lactonization [50] and deprotection, gave aplog-1 in a 2.3 % yield (22 steps). Aplog-2 without the dimethyl group at the spiroketal moiety and hydroxyl group in the benzene ring was similarly synthesized [24]. DM-aplog-2 was obtained from the 18-*O*-triflate of aplog-1 with a transfer-hydrogenation using palladium(II) acetate, formic acid, and triethylamine [43].

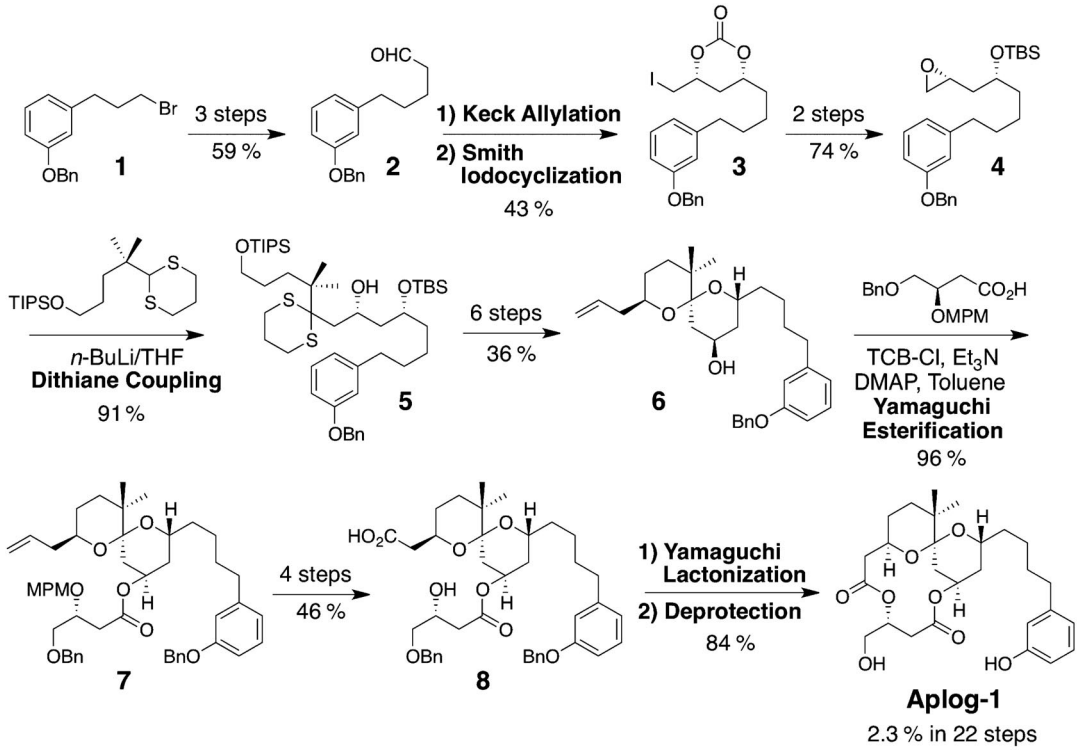

Fig. 4 Synthesis of aplog-1 [24].

BIOLOGICAL ACTIVITIES OF APLOG-1

The affinity of aplogs for the C1 domains of PKCδ was estimated. Aplog-1 showed strong binding to the C1B domain (K_i = 7.4 nM) along with significant binding to the C1A domain (K_i = 140 nM). The preference for the C1A domain of aplog-1 [K_i(C1A)/K_i(C1B) = 19] was similar to that of bryo-1 (8.8). The affinity of aplog-2 without the dimethyl and hydroxyl groups was about 20 times weaker than that of aplog-1 [K_i(C1A) = 6800 nM, K_i(C1B) = 170 nM]. In contrast, DM-aplog-2 [K_i(C1A) = 130 nM, K_i(C1B) = 9.8 nM] showed similar affinity to aplog-1, indicating that the dimethyl group at the spiroketal moiety plays a significant role in the binding to PKCδ [43].

As mentioned above, the activation of PKCδ is intimately coupled with its translocation from the cytosol to the membranous fraction. The binding of the tumor promoter TPA to inactive PKCδ in the cytoplasm induces its translocation to the plasma membrane, and subsequent partial redistribution to the nuclear membrane and internal membranes. Blumberg and colleagues [31,32] reported that bryo-1 with anticancer activities induced the translocation of PKCδ to the nuclear membrane rather than plasma membrane in CHO cells. For the evaluation of PKCδ's translocation by aplog-1, a translocation assay using GFP-tagged PKCδ was carried out involving CHO-K1 cells [24]. Aplog-1 as well as bryo-1 translocated PKCδ-GFP to the perinuclear region and nuclear membrane unlike TPA. These results strongly suggest aplog-1 to be a bryo-1-like compound rather than TPA.

The most likely adverse effect of aplog-1 is tumor-promoting activity since aplog-1 has the skeleton of ATX. We estimated the possible tumor-promoting activity of aplogs using Epstein–Barr virus early antigen (EBV-EA) [51,52]. EBVs, strictly controlled by host human lymphoblastoid Raji cells, are activated by tumor promoters to produce early antigen, which is detected by employing an indirect immunofluorescence technique. As shown in Fig. 5, the potent tumor promoters TPA and ATX significantly induced EBV-EA production at 100 nM, while bryo-1 and aplogs weakly induced it at this con-

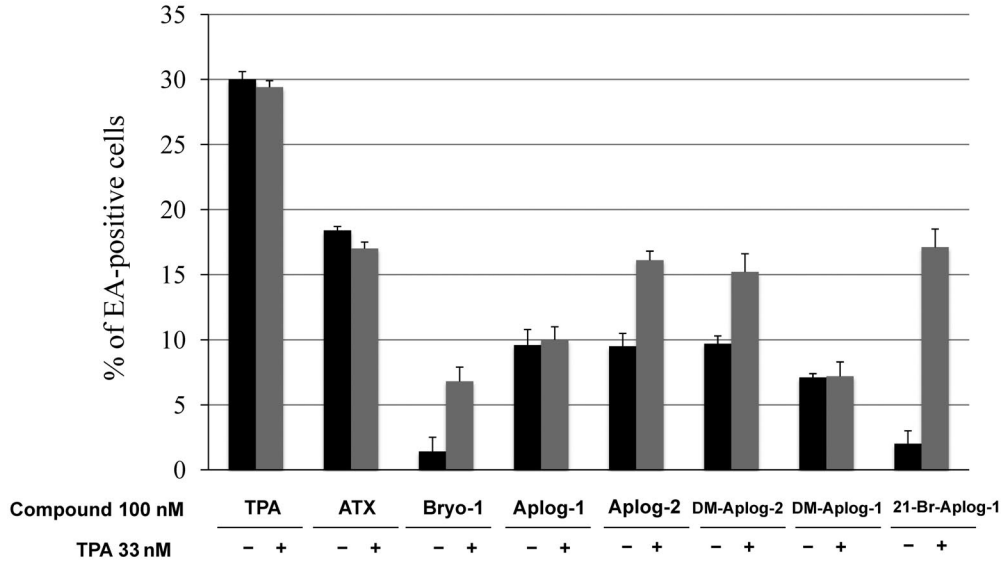

Fig. 5 EBV-EA induction test of TPA, ATX, aplog-1, aplog-2, DM-aplog-2, DM-aplog-1, and 21-Br-aplog-1 at 100 nM. In the anti-EBV-EA induction test, one of these compounds was added at 100 nM, before the addition of TPA (33 nM). Percentages of EA-positive cells are shown. Sodium *n*-butyrate (4 mM) was added to all samples to enhance the sensitivity of Raji cells. Only 0.1 % EA-induction was observed on addition of sodium *n*-butyrate. The final concentration of dimethyl sulfoxide was 0.4 %. Cell viability exceeded 60 % in each experiment except for ATX (50 %). Error bars represent standard errors of the mean (n = 3).

centration and even at 1 μM (data not shown). Moreover, the EA-induction by 33 nM TPA was significantly suppressed by aplog-1 and bryo-1. These results suggest aplog-1 to be an antitumor promoter like bryo-1, rather than a tumor promoter like TPA.

To evaluate the antiproliferative activities of aplogs, a panel of 39 human cancer cell lines established by Yamori and colleagues [53] was employed. The growth inhibitory activity was expressed as the concentration required to inhibit cell growth by 50 % compared with an untreated control [GI_{50} (M)]. Table 1 summarizes the data for cell lines whose log GI_{50} values are greater than the full panel mean-graph midpoint (MG-MID) of aplog-1 (–4.98). Aplog-1 exhibited significant antiproliferative activities comparable to bryo-1. Aplog-2 without the dimethyl group at the spiroketal moiety and the hydroxyl group at the side chain showed one-order weaker activities (MG-MID = –4.27). On the other hand, the activities of DM-aplog-2 (MG-MID = –5.09) and aplog-1 were similar. These results indicate that the dimethyl group at the spiroketal moiety is critical to the biological activities of aplogs. Moreover, the affinity for PKCδ and antiproliferative activity correlated well, suggesting PKCδ to be required for bryo-1-like activities.

Taken together, it is concluded that aplog-1 is a new candidate for bryo-1-like anticancer agents.

Table 1 Growth-inhibitory effect [logGI_{50} (M)] on human cancer cell lines by aplogs.

	Aplog-1	Aplog-2	DM-aplog-2	DM-aplog-1	21-Br-aplog-1	Bryo-1[b]
MG-MID[a] of 39 cancer cell lines	–4.98	–4.27	–5.09	–5.16	–5.20	NT[c]
HBC-4 (breast)	–6.33	–5.32	–6.20	–6.67	–7.01	NT
MDA-MB-231 (breast)	–5.61	–4.56	–5.67	–5.92	–6.33	–5.20
SF-295 (CNS)	–5.06	–4.57	–5.14	–5.32	–5.26	–5.20
HCC2998 (colon)	–5.43	–4.57	–5.53	–6.06	–6.10	–5.30
NCI-H460 (lung)	–5.60	–4.70	–5.83	–6.05	–5.78	–5.60
A549 (lung)	–5.32	–4.48	–5.49	–5.51	–5.30	–5.20
LOX-IMVI (melanoma)	–5.74	–4.66	–5.17	–6.04	–6.10	NT
St-4 (stomach)	–5.55	–5.04	–6.05	–6.20	–6.02	NT
MKN45 (stomach)	–5.33	–4.74	–6.09	–5.33	–5.74	NT

[a]MG-MID: the full panel mean-graph midpoint.
[b]Data are cited from ref. [14].
[c]Not tested.

STRUCTURE–ACTIVITY STUDIES OF APLOG-1 AND ITS TUMOR-PROMOTING ACTIVITY IN VIVO

Based on these results, we are trying to develop superior analogues of aplog-1. As mentioned above, hydrophobicity around the spiroketal moiety of aplog-1 is critical to the ability to bind to PKCδ and antiproliferative activity. This suggests the biological effect of aplog-1 to be enhanced by increasing local hydrophobicity around the spiroketal moiety, and led us to design 12,12-dimethyl-aplog-1 (DM-aplog-1) with a geminal methyl group proximal to the spiroketal moiety. DM-aplog-1 was synthesized from the bromide **1** in 22 steps with an overall yield of 2.4 % [54]. Biological assays revealed that DM-aplog-1 was more effective than aplog-1 in binding to PKCδ (K_i = 5.9 nM), suppressing TPA-induced EA-production (Fig. 5), and inhibiting cancer cell growth (Table 1, MG-MID = –5.16).

As another approach to developing more potent analogues of aplog-1, the side chain was modified. The introduction of a bromine atom on the phenol moiety enhanced the antiproliferative activity as shown in 21-Br-aplog-1 (MG-MID = –5.20). In addition, the ability of 21-Br-aplog-1 to generate EBV-EA was weaker than that of aplog-1. However, its anti-EBV-EA-inducing activity was not stronger than that of aplog-1 (Fig. 5).

As mentioned above, most critical to developing derivatives of aplog-1 is confirmation that the structural modifications will not increase tumor-promoting activity. Having confirmed the weak tumor-promoting activity of aplogs in vitro by the EBV-EA induction test (Fig. 5), an in vivo tumor-promotion assay was carried out for DM-aplog-1 (Fig. 6). The skin on the back of imprinting control region (ICR) mice was treated with a single dose of 390 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA) and from one week later, with 8.5 nmol of DM-aplog-1 twice a week. DM-aplog-1 did not induce any tumor at week 20. In a control experiment using TPA (1.7 nmol twice a week), the first tumor appeared in week 7, and the proportion of tumor-bearing mice reached 100 % at week 12. The number of papillomas/mouse was 7.9 in week 20. Moreover, DM-aplog-1 was shown to be an antitumor promoter like bryo-1 [5]. The application of 8.5 nmol of DM-aplog-1, 1 h before the application of 1.7 nmol of TPA, reduced significantly the number of tumor-bearing mice and the tumor yield (70 % and 4 papillomas/mouse).

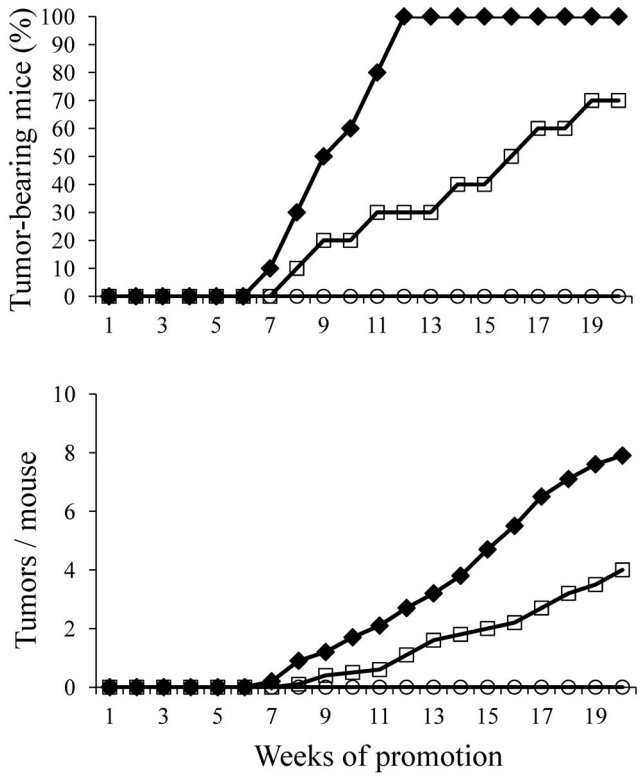

Fig. 6 Tumor-promoting activity of TPA, and DM-aplog-1. The back of each female 6-week-old ICR mouse was shaved with surgical clippers. From a week after initiation by a single application of 390 nmol of DMBA in 0.1 mL acetone, 8.5 nmol of DM-aplog-1 in 0.1 mL acetone was applied twice a week from week 1 to 20 (○). The control group was treated with DMBA and 1.7 nmol TPA (◆). To estimate antitumor promoting activity of DM-aplog-1, 8.5 nmol of DM-aplog-1 in 0.1 mL acetone was applied, 1 h before the application of 1.7 nmol of TPA (□). Each group consisted of 10 mice. Difference in papillomas/mouse between the positive control (TPA) and TPA + DM-aplog-1 at week 20 was statistically significant ($P < 0.01$).

We are also examining the origin of the unique biological activities of aplog-1, and found that aplog-1 and bryo-1 differed in affinity for PKC isozymes from tumor promoters (Fig. 7). The C1A peptides were used as conventional PKC surrogates, and the C1B peptides were employed as novel PKC surrogates since these domains are the main binding sites of tumor promoters [24,35,55]. Tumor promoters like PDBu and ATX bound significantly to both conventional and novel PKC isozymes. In contrast, antiproliferative compounds like aplog-1 and bryo-1 exhibited selectivity for novel PKC isozymes other than PKCε, that is PKCδ, η, and θ. These results suggest that the activities of aplog-1 are ascribable in part to the ability to bind to PKCδ, but weak binding to conventional PKC isozymes might also be important for the unique biological activities of aplog-1 and bryo-1.

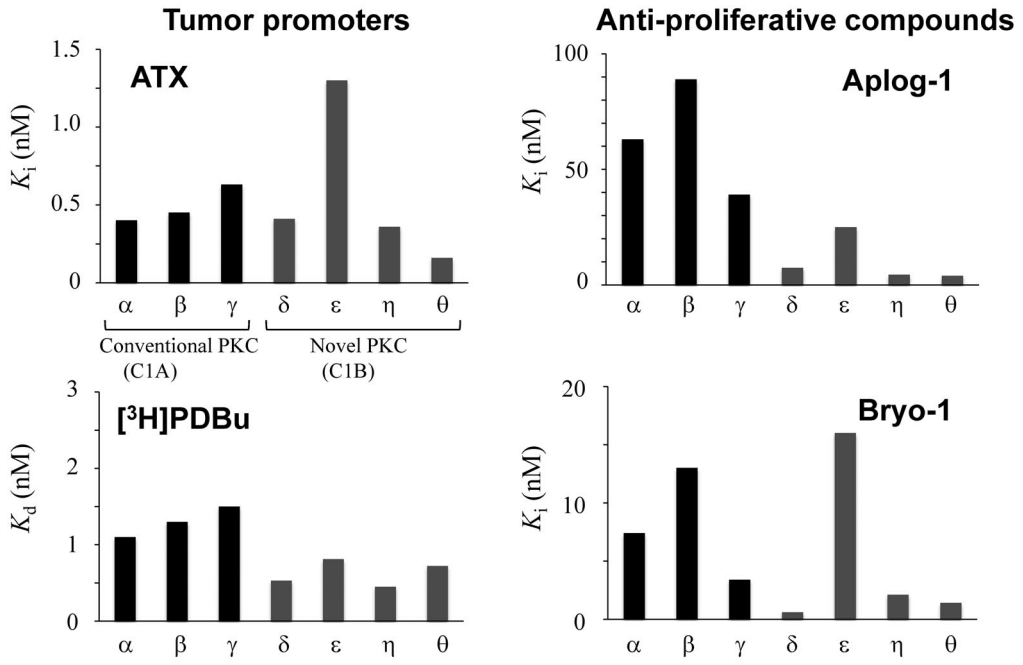

Fig. 7 PKC isozyme selectivity of aplog-1 and bryo-1 along with ATX and PDBu. The C1A peptides of conventional PKC isozymes and the C1B peptides of novel PKC isozymes were used [35].

CONCLUSIONS

We developed synthetically accessible simple analogues of ATX as possible anticancer compounds on the basis of the activation mechanism of PKCδ. The importance of aplog-1 as a therapeutic lead for cancer was introduced in *Science-Business Exchange* [56]. Although PKCδ might play a critical role for in the unique biological activities of aplog-1 and bryo-1, the antiproliferative activity of aplog-1 cannot be fully explained only by PKC isozymes. Further studies of its mode of action are in progress using the FLAG-tagged aplog-1.

Recently, Blumberg and colleagues have reported that the plasma membrane translocation of PKCδ and lipophilicity of the ligands did not correlate with the divergent effects of tumor promoters and that active phorbol esters are not all equivalent [57]. They also suggest that bryo-1-like compounds may be obtained from other structural templates. The aplogs presented in this paper may be one such example.

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