The Structure–Activity Relationship between Synthetic Butyridenephthalide Derivatives Regarding the Competence and Progression of Inhibition in Primary Cultures Proliferation of Mouse Aorta Smooth Muscle Cells

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The inhibitory effects of synthetic butyridenephthalide (BP) derivatives on 10% fetal bovine serum-stimulated proliferation were assayed by measuring the proliferative cell number at an interval of 12 h in primary cultures of mouse aorta smooth muscle cells (SMC). Their potencies for the anti-proliferation effect were in the order BP-42 (4,5-dihydroxy BP) > BP-92 (4,5-dihydroxy butylphthalide) > BP-97 (6,7-dihydroxy-3-(3-bromo-1-octenyl)-phthalide) > BP-82 (6,7-dihydroxy BP) > BP-86 (5,6-dihydroxy BP) > BP-87 (4,5,6-trihydroxy BP) > BP-85 (4,7-dihydroxy BP) > BP-84 (5,7-dihydroxy BP) > BP-4C₃ (4-methoxy propylphthalide) > BP-7 (4-hydroxy BP) > BP-40 (4,5-dimethoxy butylphthalide) > BP-5C₃ (4-hydroxy propylphthalide). We divided these anti-proliferative effects into anti-competence and anti-progression effects by using a convenient assay. BP-42 had the greatest potency in used phthalides for competence inhibition of the SMC proliferation. BP-92 had small potency for competence inhibition. BP-97 had greater potency for competence inhibition than BP-82. These results demonstrated that the anti-proliferative effect of BP-42 was greatest in used phthalides in primary cultures of vascular SMC. The 4,5-dihydroxy group and 3-butyridene or 3-(3-bromo-1-octenyl) group in these synthetic BP derivatives contributed to the anti-competence effect on SMC. BP-42 may become a prototype of an anti-atherosclerotic drug.

Key words synthetic butyridenephthalide derivative; primary culture smooth muscle cell; competence inhibition

An abnormal proliferation of vascular smooth muscle cells (SMC) has been recognized to trigger the formation of atherosclerotic plaques.¹⁾ The proliferation of SMC has been reported to occur earliest in the media of an artery injured by an intraarterial balloon catheter technique.^{2,3)} Proliferative and non-proliferative SMC in the media migrate into the intima and proliferate there.^{1,3)} Cell proliferation is regulated by specific cellular phases, which involve both competence and progression.⁴⁾ Competence factors initiate the proliferative response of cells. They induce competent cells to respond to the progression factors required for the cell proliferation.⁴⁾ A specific inhibitory drug for the competence factors may be useful in treating atherosclerosis. The search for an anti-competence activity in primary cultured SMC is one of the approaches for finding a new type of an anti-atherosclerotic drug.

We have reported a convenient assay to determine relative indices of competence and progression by estimating the starting time and rate of DNA synthesis in primary cultured SMC.^{5,6)} The inhibitory effect of heparin on the progression index of DNA synthesis in the SMC is in parallel with that of proliferative cell numbers in SMC.^{5,7)} These results mean that the convenient assay is also applicable with data of proliferative SMC number. We have previously reported that cnidium rhizome-derived phthalides containing senkyunolide H, ligustilide, senkyunolide A and butyridenephthalide (BP) inhibit the proliferation of primary culture of mouse aorta SMC.^{7,8)} Analysis of proliferative SMC number by the convenient assay demonstrates that the inhibitory mode of BP is associated with the competence phase rather than the progression phase.⁷⁾ Since senkyunolide H, a 6,7-*cis*-dihydroxy ligustilide derivative, possesses a more potent

anti-competence effect than BP, the dihydroxy group is suggested to contribute to the anti-competence effect.⁷⁾ However, these ligustilide and senkyunolide derivatives in cnidium phthalides are unstable and susceptible to autooxidation in the presence of air.^{9,10)}

In the present study, the structure–activity relationship between synthetic BP derivatives for anti-proliferation were investigated by measuring the cell number of 10% fetal bovine serum (FBS)-proliferated SMC in primary cultures of mouse aorta. These effects were further analyzed for competence and progression inhibitions in the search for an anti-competence drug.

MATERIALS AND METHODS

Cell Culture of Aortic Smooth Muscle Primary cultured SMC of mouse aorta were prepared by the method previously reported.^{5,7,8)} Thoracic aortas of ddY strain male mice (6–7 weeks old, Japan Shizuoka Laboratory Center, Hamamatsu) were placed in Hanks' solution (pH 7.3, 136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 4.2 mM NaHCO₃, 5.6 mM glucose), and the blood, fat and connective tissues were removed. They were then incubated in Hanks' solution containing 1 mg/ml collagenase (type I, Sigma, St. Louis, MO, U.S.A.) and 3.3 unit/ml elastase (Sigma) for 30 min at 37 °C. The adventitia was cleanly stripped from each aorta, and the remaining medial tissue was incubated in Hanks' solution containing collagenase and elastase for 60 min at 37 °C with gentle shaking in order to obtain single cells and small cell clumps. The suspension of SMC was centrifuged at 150 × *g* for 5 min at 4 °C, and resuspended at the density of 3.0 × 10⁴ cells/0.5 ml/16 mm-well in Dulbecco's modified Eagle's medium

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Chart 1

(DMEM, Nissui, Tokyo) supplemented with 10% heat-inactivated FBS (Whittaker Bioproducts, Walkersville, MD, U.S.A.), penicillin G potassium (160 unit/ml, Banyu Seiyaku, Tokyo) and streptomycin sulfate (0.1 mg/ml, Meiji Seika, Tokyo). The cells were cultured in a 24-well plate (16-mm well, Corning, NY, U.S.A.) under a humidified atmosphere of 5% CO₂ in air at 37 °C. The cultured medium was changed every other day. The cells were identified as SMC because the cells exhibited hill and valley patterns at confluence under an optical microscope.¹¹⁾

Assay of SMC Proliferation 10% FBS-proliferated SMC were detached in 0.25% trypsin (Difco, Detroit, MI, U.S.A.) and 0.2% EDTA in Ca²⁺, Mg²⁺-free Hanks' solution and collected by centrifugation at 150 × *g* for 5 min. The number of SMC was counted with a hemocytometer every 12 h from the first to 11th day in culture.

Agents Used synthetic BP derivatives and BP-7 (4-hydroxy BP) derived from cnidium rhizome were provided by Tsumura Research Institute for Pharmacology (Tokyo). The chemical structures of BP-7, BP-5C₃ (4-hydroxy propylphthalide), BP-4C₃ (4-methoxy propylphthalide), BP-42 (4,5-dihydroxy BP), BP-92 (4,5-dihydroxy butylphthalide), BP-40 (4,5-dimethoxy butylphthalide), BP-86 (5,6-dihydroxy BP), BP-82 (6,7-dihydroxy BP), BP-97 (6,7-dihydroxy-3-(3-bromo-1-octenyl) phthalide), BP-84 (5,7-dihydroxy BP), BP-85 (4,7-dihydroxy BP) and BP-87 (4,5,6-trihydroxy BP) are presented in Chart 1. All phthalides were dissolved in a final concentration of 0.05% ethanol, diluted with 10% FBS-DMEM and administered to the culture medium from the start of SMC culture. The culture media with these drugs were changed every other day. These phthalides at the concentrations used did not damage and/or have a toxic effect on the SMC during the 11-day treatment (data not shown), because no uptake of trypan blue was observed during these treatments.¹²⁾

Analysis of Indices of Competence and Progression Inhibition The inhibitory effects of these BP derivatives on SMC proliferation were analyzed by separating them into competence and progression inhibition using the convenient assay previously reported.⁷⁾ Proliferation lines of the SMC number from 3 × 10⁴ to 6 × 10⁴ cells/well as a function of culture day were fitted to the individual data of SMC number by the least-squares method. Their anti-proliferative effects were expressed as a ratio (TIT₂) of total time required for cell number doubling from the

day when the SMC were plated (day 0) in the presence of drug to that in the absence of it (Table 1). The index of competence inhibition was estimated as the relative difference ((C_i - C₀)/C₀) of the starting time of SMC proliferation between with (C_i) and without drug (C₀). The index of progression inhibition was the relative difference ((P_i - P₀)/P₀) between the doubling time and the starting time, which was calculated by the slope of the proliferation line, between SMC with (P_i) and without drug (P₀).⁷⁾

Statistical Analysis Data were expressed as means ± S.E. and analyzed by one-way analysis of variance (ANOVA, Scheffe or Tukey test) at *p* = 0.05 or 0.01.

RESULTS

Inhibitory Effects of Synthetic Dihydroxy Derivatives of BP, BP-42 and BP-86 on the Proliferation of Primary Cultured SMC The inhibitory effects of *o*-dihydroxy BP derivatives (BP-42, BP-86) on 10% FBS-stimulated proliferation were compared in primary cultured SMC. BP-42 (0.1, 0.3, 0.5 μg/ml) inhibited the FBS-stimulated proliferation of SMC in a concentration-dependent manner and showed 19.1, 71.1 and 91.9% decreases on day 11 at the corresponding concentrations, respectively. BP-42 significantly inhibited the proliferation from day 5 at 0.1 μg/ml and from day 3.5 at 0.3 and 0.5 μg/ml. BP-42 (0.1, 0.3 μg/ml) prolonged the starting times of SMC proliferation and decreased the slopes of the proliferation curve (Fig. 1). BP-42 (0.5 μg/ml) prolonged the starting time of SMC proliferation completely during 11-day exposure.

BP-86 (0.1, 0.3, 0.5, 1.0 μg/ml) also inhibited SMC proliferation in a concentration-dependent manner and showed 13.8, 45.7, 62.3 and 92.5% decreases on day 11 at the corresponding concentrations. BP-86 significantly inhibited the proliferation from day 5.5 at 0.3 and 0.5 μg/ml and from day 3.5 at 1.0 μg/ml. BP-86 affected the slopes of the proliferation curve rather than the starting time of SMC proliferation (Fig. 2). These results demonstrated that BP-42 had a more potent anti-proliferative effect than BP-86.

Inhibitory Effects of BP Derivatives on the Competence and Progression Phases of SMC Proliferation The anti-proliferative effects of BP derivatives were expressed as ratios of TIT₂ and were divided into competence and

Fig. 1. Time-Dependent Effects of BP-42 (0, 0.1, 0.3, 0.5 µg/ml) on the Proliferation of Primary Cultured Mouse Aortic SMC Stimulated by 10% FBS

The values are expressed as means $\pm$ S.E. of 4–14 experiments. The values with BP-42 were significantly different from those without it on the corresponding day from day 5 to day 10 at 0.1 µg/ml ($p < 0.05$: days 8.5–10; $p < 0.01$: days 5–8) and from day 3.5 to day 11 at 0.3 µg/ml ($p < 0.05$: day 3.5; $p < 0.01$: days 4–11) and 0.5 µg/ml ($p < 0.05$: days 3.5–4; $p < 0.01$: days 4.5–11).

Fig. 2. Time-Dependent Effects of BP-86 (0.1, 0.3, 0.5, 1.0 µg/ml) on the Proliferation of Primary Cultured Mouse Aortic SMC Stimulated by 10% FBS.

The values are expressed as means $\pm$ S.E. of 3–12 experiments. The values with BP-86 were significantly different from those without it on the corresponding day at 0.3 µg/ml ($p < 0.05$: days 5.5–6; $p < 0.01$: days 7–11), 0.5 µg/ml ($p < 0.05$: day 5.5; $p < 0.01$: days 6–11) and 1.0 µg/ml ($p < 0.05$: day 3.5; $p < 0.01$: days 4–11).

progression inhibition (Table 1). We have previously reported that BP (4 µg/ml)-induced increases in the values of TIT_2 , competence and progression indices are 1.11, 0.13 and 0.03, respectively.⁷⁾ BP-7 (4 µg/ml), which introduces a hydroxy group to the C4 position of BP, increased the three values more greatly than the same concentration of BP. BP-5C₃ (4 µg/ml), in which is substituted a propyl group for a butylidene group in BP-7, presented smaller values for the TIT_2 and competence index than BP-7. BP-4C₃ (4 µg/ml), in which is substituted a methoxy group for a hydroxy group in BP-5C₃, more greatly increased the three values than BP-5C₃. These results demonstrated that 4-hydroxy and 3-butyldiene groups in BP derivatives are involved in the inhibitory effect on SMC proliferation.

The three values of the dihydroxy BP derivatives (BP-42,

Table 1. Inhibitory Effects of Synthetic Butylidenephthalide-Derived Compounds on the Competence and Progression Phases in Proliferation of Primary Cultured SMC of Mouse Aorta

	Concentration (µg/ml)	TIT_2	$(C_t - C_0)/C_0$	$(P_t - P_0)/P_0$	n
BP-7	4.0	1.32	0.38	0.22	2
BP-5C ₃	4.0	1.07	0	0.23	2
BP-4C ₃	4.0	1.65	0.38	1.37	2
BP-42	0.1	1.22	0.25	0.29	4
	0.3	1.73	0.43	1.63	4
	0.5	∞	∞	∞	4
BP-92	0.1	1.19	0	0.69	4
	1.0	∞	∞	∞	4
BP-40	4.0	1.13	0.14	0.16	4
BP-86	0.1	1.02	0	0.10	4
	0.3	1.13	0	0.41	4
	0.5	1.55	0.17	1.55	4
BP-82	1.0	3.05	1.14	3.59	4
	0.5	1.23	0	0.64	4
	1.0	4.37	0	11.46	2
BP-97	0.5	1.94	0.57	2.12	4
	1.0	∞	∞	∞	4
BP-84	1.0	1.29	0.29	0.31	4
BP-85	1.0	1.77	0.57	1.54	4
BP-87	0.1	1.27	0.29	0.21	4
	1.0	2.78	0.71	5.58	4

TIT_2 : ratio of total time required for SMC doubling from the day SMC were plated with drug to that without it; $(C_t - C_0)/C_0$: relative difference between starting time of SMC proliferation with drug and that without it (competence index); $(P_t - P_0)/P_0$: relative difference between proliferation rate of SMC with drug and that without it (progression index); n : number of experiments.

BP-86, BP-82, BP-84, BP-85) were greater than those of the monohydroxy BP (BP-7). *O*-dihydroxy BP derivatives (BP-42, BP-86, BP-82) presented greater values of TIT_2 than *p*-dihydroxy BP (BP-85) and *m*-dihydroxy BP (BP-84). The potencies of these *o*-dihydroxy BP derivatives for TIT_2 were in the order BP-42 > BP-82 > BP-86. The potencies of dihydroxy BP derivatives for the competence index were in the order BP-42 > BP-86 > BP-85 > BP-84 > BP-82. The potencies of these derivatives for the progression index were in the order BP-42 > BP-82 > BP-86 > BP-85 > BP-84. The effects of trihydroxy BP (BP-87) on the TIT_2 , competence and progression indices were not greater than those of BP-42. These results demonstrated that BP-42 had the greatest potency among those hydroxy BP derivatives used for the competence and progression inhibition of SMC proliferation.

BP-92 (0.1 µg/ml), in which is substituted a butyl group for a butylidene group in BP-42, presented a similar value of TIT_2 to that of BP-42 (0.1 µg/ml). BP-92 had a smaller value of competence index and a greater value of progression index than BP-42 (Table 1). BP-40 (4 µg/ml), in which is substituted a dimethoxy group for a dihydroxy group in BP-92, had smaller values of the three parameters than BP-92. BP-97 (0.5, 1.0 µg/ml), in which is substituted a 3-(3-bromo-1-octenyl) group for a butylidene group in BP-82, increased the three values more greatly when used at the same concentrations as BP-82.

DISCUSSION

A new type of an anti-atherosclerotic drug was investigated for its anti-competence activity of proliferation of

SMC in primary culture. We have previously reported that the (Z)-6,7-dihydroxy isomer of dihydroligustilide in cnidium rhizome-derived phthalides is essential for the anti-competence effect on SMC proliferation.^{7,8)} The anti-proliferative effect of cnidium derived phthalide is selective for vascular SMC and not for vascular endothelial cells.⁸⁾ In the present study, the chemical structure-activity relationship of synthetic hydroxylated derivatives of BP was investigated in terms of the anti-proliferation of aortic SMC. 4-hydroxy BP showed a greater effect on the anti-proliferation of SMC than BP.⁷⁾ The potencies of dihydroxy BP derivatives were greater than those of monohydroxy BP derivatives. The potencies of dihydroxy BP derivatives for anti-proliferation were in the order $o > p > m$ -dihydroxy derivative of BP. In these o -dihydroxy BP derivatives, the potency of 4,5-dihydroxy BP (BP-42) was greater than that of either 5,6-dihydroxy BP (BP-86) or 6,7-dihydroxy BP (BP-82). These results demonstrated that the 4,5-dihydroxy group in BP derivatives was essential for the anti-proliferation of primary cultured SMC. These results suggested that the conformation of 4,5-dihydroxy and 3-butyldiene groups might be crucial for its anti-proliferative effect.

In the present study, the effect of anti-proliferation was divided into anti-competence and anti-progression effects by using a convenient assay.⁷⁾ The potencies for competence inhibitions were in the order BP-42 > BP-97 > BP-86 > BP-87 > BP-85 > BP-84 > BP-7 = BP-4C₃ > BP-40. BP-92, BP-82 and BP-5C₃ had no effect on the competence index. The substitution of a butyldiene group with butyl group and propyl group in BP derivatives decreased the anti-competence activity, but the substitution of a butyldiene group with a 3-bromo-1-octenyl group increased the anti-competence activity. These results demonstrated that a butyldiene or a 3-bromo-1-octenyl group at the C3 position in phthalide derivatives was also important to the anti-competence effect. The potencies of these hydroxy BP derivatives for the progression inhibition was BP-92 > BP-42 > BP-97 > BP-82 > BP-87 > BP-86 > BP-85 > BP-84 > BP-7. The order of potencies differed from that for competence inhibition. The substitution of a butyldiene group with a butyl group in dihydroxy BP increased the progression inhibition. These results demonstrated that the 3-butyldiene group in BP derivatives was not necessary for the anti-progression effect. It is suggested that the site of action of synthetic phthalides for the competence phase was different from that for the progression phase. These results supported a previous suggestion that cnidium-derived phthalides have two independent sites of action for both competence and progression.⁷⁾

We have previously reported that cnidium rhizome-derived natural phthalides inhibit the proliferation of primary cultured SMC.^{7,8)} These potent natural phthalides are unstable, are susceptible to autoxidation in the presence of air, and are transformed into natural stable phthalides containing BP.^{9,10)} The inhibitory mode of BP

is associated with the competence phase rather than the progression phase.⁷⁾ In the present study, BP-42 showed the most potent anti-competence effect in the synthetic BP derivatives used. The anti-competence effect was greater than that of BP.⁷⁾

Many growth factors are essential for the abnormal proliferation of SMC. Platelet-derived growth factor (PDGF) has competence and progression activities,^{5,6,13)} and epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) accelerate the progression phase.^{14,15)} Tyrphostins, which have chemical structures similar to these dihydroxy BP derivatives, effectively block PDGF- and EGF-dependent proliferation.^{16,17)} These BP derivatives might interact with these growth factors as similarly to tyrphostins.

In conclusion, the anti-proliferative effect of BP-42 was greatest among the phthalides used in the primary cultures of vascular SMC. The 4,5-dihydroxy group, and 3-butyldiene or 3-(3-bromo-1-octenyl) group in these synthetic BP derivatives contributed to its anti-competence effect on SMC. BP-42 may become a prototype for use as an anti-atherosclerotic drug.

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