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In situ photoaffinity labeling of the target protein for lembehyne A, a neuronal differentiation inducer

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Abstract **A C_{36} linear acetylene alcohol, lembehyne A (LB-A), induces neuronal differentiation against neuroblastoma cells morphologically and also functionally. The differentiation and cytostatic effect induced by LB-A was specific to neuroblastoma, Neuro 2A cells. To identify the target protein for LB-A, a radioactive photoaffinity probe, [^{125}I]18-(2′-azido-5′-iodo-benzoyloxy)-LB-18 ([^{125}I]azido-LB-18), was synthesized. As a result of in situ labeling experiments against Neuro 2A cells, a protein of M_r 30 kDa was photolabeled specifically. This labeling was inhibited in the presence of LB-A or the active analogs of LB-A, whereas the inactive analogs showed no inhibitory effect on this labeling. These results suggest that this protein of M_r 30 kDa is the target protein for LB-A and may play an important role for the neuronal differentiation in neuroblastoma, Neuro 2A cells.**

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Key words: Lembehyne A; Neuronal differentiation; G1 arrest; Photoaffinity labeling; Neuro 2A cell

1. Introduction

Differentiation inducers against tumor cells are expected to be new candidates for cancer chemotherapy, since tumor cells retain ability to differentiate in many cases [1]. These substances inducing differentiation may exhibit selective activity against tumor cells and minimal side effects against normal cells. Actually, clinical trials are in progress for retinoic acid or its synthetic analogs as chemoprevention and therapeutic agents in selected human cancers [2,3]. As the differentiation program might be disrupted in neuroblastoma cells, new differentiation inducers for chemotherapy of various neuronal diseases are expected to be worthwhile. So far, several differentiation inducers with low molecular weight for neuroblastoma have been reported [4–10]; however, the mechanism of cell differentiation induced by these inducers is still unclear.

A C_{36} long chain acetylene alcohol, lembehyne A (LB-A), and its analogs, which induce neuritogenesis in a murine neuroblastoma cell line, Neuro 2A, and a rat pheochromocytoma cell line, PC12, were found from a marine sponge of *Haliclona* sp. [11,12] (Fig. 1). After LB-A treatment, Neuro 2A cells brought out a morphological change with long bipolar processes emanating from opposite sides of the cell body and a functional change with increase of acetylcholinesterase [13]. LB-A was structurally a new type of differentiation inducer for these cells. LB-A also blocked the cell cycle of Neuro 2A at the G1 phase in a concentration-dependent manner [13]. The structure–activity relationship study of LB-A clarified that the terminal 1-yn-3-ol moiety including 3*R* configuration and the long alkyl chain in LB-A were required for neuronal differentiation-inducing activity [12]. In fact, the neuritogenic activities of several analogs (3-*O*-methyl or 1-methyl derivatives of LB-A, LB-10, having a short carbon-chain, and 3*S*-LB-A, having a 3*S* configuration) were much weaker than that of LB-A. On the other hand, LB-18 (C18 analog) and LB-20 (C20 analog) showed stronger activity than LB-A. The importance of the *R*-configuration at the C-3 chiral center for neuritogenic activity implied the presence of a target protein for LB-A. A crucial moiety of LB-A may be involved in primary recognition of the target protein. On the basis of this evidence, we undertook to identify the target protein for LB-A.

Judging from the chemical structure of LB-A, formation of a covalent bond between LB-A and the target protein seemed to be difficult. On the other hand, photoaffinity labeling, which forms a covalent bond between a ligand and a receptor protein forcibly under UV irradiation, is well known to be a powerful technology for identifying a target protein [14]. Thus, we synthesized a radioactive photoaffinity probe, [^{125}I]18-(2′-azido-5′-iodo-benzoyloxy)-LB-18 ([^{125}I]azido-LB-18), and performed a photolabeling experiment. This report communicates the in situ labeling of the target protein for LB-A.

2. Materials and methods

2.1. Materials

LB-A was isolated from a marine sponge of *Haliclona* sp., which was collected at Lembeh Island, Bitung, Indonesia, as described previously [11]. LB-A analogs, LB-10, LB-18, LB-20, and 3*S*-LB-18, were synthesized in our previous report [12,13].

2.2. Cell cultures

Murine neuroblastoma cell line, Neuro 2A, and fibroblast derived from Fischer rat embryos, 3Y1-B (clone 1-6), were grown in the Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and kanamycin (50 ng/ml). Human chronic myelogenous leukemia cell line, K562, was grown in RPMI1640 medium supplemented with 10% FBS and kanamycin (50 ng/ml). All cells were kept in an incubator at 37°C with 5% CO_2. The viability of cells was determined by trypan blue exclusion.

2.3. Cell cycle analysis

Cells were treated with LB-A for 24 h at 37°C with 5% CO_2. The harvested cells were washed with phosphate-buffered saline (PBS)

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twice, then dyed by DNA-Prep Reagents kit (Coulter) for 20 min. After removal of the supernatant by centrifugation ($500 \times g$ for 5 min), the resulting cell pellet was suspended in D-PBS(−) solution and filtered through 40-μm nylon mesh filter. The cell cycle analysis of the filtrate was carried out on FACSCalibur (Becton Dickinson, $\lambda_{ex} = 493$ nm, $\lambda_{em} = 630$ nm).

2.4. Western blotting of p21$^{cip1/waf1}$

Cells were treated with LB-A for the indicated time at 37°C with 5% CO_2. The harvested cells were lysed in lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1% protease inhibitor cocktail–DMSO solution (P-8340, Sigma)). Cellular lysates were mixed with SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) sample buffer (100 mM Tris–HCl, 4% SDS, 12% mercaptoethanol, 20% glycerol, 0.005% bromophenol blue), boiled for 5 min, and separated in 15–25% SDS–polyacrylamide gels. Then the proteins were electrotransferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The blot was incubated with monoclonal anti-mouse p21$^{cip1/waf1}$ antibody (Santa Cruz Biotechnology), and detection of p21 protein was carried out with the enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

2.5. Preparation of photoaffinity probe, 18-(2′-azido-5′-iodo-benzoyloxy)-LB-18 (azido-LB-18)

Azido-LB-18 was synthesized by the route as shown in Scheme 1. Benzoic acids 1 and 2 were synthesized from 5-iodoanthranilic acid as described in the previous report [15]. The treatment of 16-hydroxyhexadecanoic acid with *N*,*O*-dimethylhydroxylamine hydrochloride (MeONHMe·HCl), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) hydrochloride, and *N*,*N*-dimethylaminopyridine (DMAP) and subsequent protection of the hydroxyl group at C-18 afforded Weinreb amide 4 in 93% yield for two steps. The nucleophilic substitution for 4 with lithium (triisopropylsilyl (TIPS))acetylide provided an alkyne 5 in 98% yield. Then, the asymmetric reduction of 5 using (*R*)-2-methyl-CBS-oxazaborolidine [16,17] and borane methylsulfide complex followed by deprotection of the silyl group using tetrabutylammonium fluoride proceeded with high enantiomeric selectivity (100% ee) to afford an alcohol 7 in 86% yield for two steps. The alcohol 7 was finally coupled with the acid 1 and the resulting product was purified by silica gel column chromatography (*n*-hexane-EtOAc = 8:1) to furnish azido-LB-18 in 71% yield. The structure of azido-LB-18 was confirmed by the analysis of its nuclear magnetic resonance (NMR) and mass spectrometric (MS) spectrum. Azido-LB-18: Colorless powder, FAB MS: m/z 576 $(M+Na)^+$. HR-FAB MS: m/z 576.1699: calculated for $C_{25}H_{36}IN_3O_3Na$. Found: 576.1685. ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 8.12 (1H, d, $J = 1.8$ Hz, ArH), 7.79 (1H, dd, $J = 8.5$, 1.8 Hz, ArH), 6.98 (1H, d, $J = 8.5$ Hz, ArH), 4.37 (1H, td, $J = 7.0$, 1.8 Hz, H-3), 4.30 (2H, t, $J = 6.7$ Hz, H-18), 2.46 (1H, d, $J = 1.8$ Hz, H-1), 1.77–1.70 (4H, m, H-4, 17), 1.45–1.20 (H-5–16).

Neuritogenic activity of azido-LB-18 was examined according to the neurite outgrowth assay as described in the previous report [13].

2.6. Preparation of [^{125}I]azido-LB-18

The alcohol 7 was treated with the acid 2, EDCI, and DMAP, and the resulting product was purified by silica gel column chromatography (*n*-hexane-EtOAc = 8:1) to furnish a compound 8 in 35% yield. Compound 8: Colorless powder, FAB MS: m/z 592 $(M+H)^+$. HR-FAB MS: m/z 592.2561: calculated for $C_{28}H_{46}N_3O_3Sn$. Found: 592.2572. ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 7.89 (1H, d, $J = 1.2$ Hz, ArH), 7.61 (1H, dd, $J = 7.8$, 1.2 Hz, ArH), 7.20 (1H, d, $J = 7.8$ Hz, ArH), 4.37 (1H, td, $J = 6.9$, 1.8 Hz, H-3), 4.31 (2H, t, $J = 6.7$ Hz, H-18), 2.46 (1H, d, $J = 1.8$ Hz, H-1), 1.79–1.70 (4H, m, H-4, 17), 0.31 (9H, s, $-Sn(CH_3)_3$).

To a solution of 10 μg of the compound 8 in MeOH (30 μl) were added 0.7 mM PBS solution of chloramine T (36 μl, pH 7.5) and the water solution of Na^{125}I (17 μl, 0.5 mCi, Perkin-Elmer). The reaction mixture was stirred for 50 min at 45°C. Subsequently, the reaction mixture was treated with 0.7 mM MeOH solution of NaI (24 μl) at 45°C for 100 min. After evaporation of the solvent by argon gas, the residue was extracted with benzene, and the soluble portion was transferred to another vial. The production of [^{125}I]azido-LB-18 (estimated 11 Ci/mmol) was detected by autoradiography, which showed the same R_f value with azido-LB-18 on thin layer chromatography.

2.7. In situ labeling of cells with [^{125}I]azido-LB-18

The cell suspension of 2×10^6 cells in 5 ml of the culture medium was plated on a 10-cm dish and incubated for 3 h. Then, an ethanol solution of [^{125}I]azido-LB-18 was added to the cells at a final concentration of 53 nM. The ethanol solutions of the competitors were added 1 h or 7 h prior to the addition of [^{125}I]azido-LB-18. After incubation with [^{125}I]azido-LB-18 for 3 h at 37°C, the cells were washed with PBS three times and irradiated with a hand-held UV lamp (366 nm, 100 W, UVP) at a distance of 1 cm for 3 min × 2 (1 min pause) in PBS on ice. The photolabeled cells were harvested into a centrifuge tube and lysed in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1 mM Na_3VO_4, 0.1% Tween 80, 1% protease inhibitor cocktail–DMSO solution (P-8340, Sigma)). After stirring with a vortex mixer for 30 min, the lysate was centrifuged for 3 min at $500 \times g$, then the supernatants of the lysate were analyzed by 4–20% or 15–25% SDS–PAGE. The developed gel was dried and exposed to imaging plate BAS-IP MS2040 (Fuji Film) for 24 h. The radio-labeled protein bands were detected on a Bioimage Analyzer BAS-1500 (Fuji Film).

3. Results and discussion

In our previous study, LB-A, a long chain acetylene alcohol, was revealed to induce neuronal differentiation against murine neuroblastoma, Neuro 2A cells, and rat pheochromocytoma, PC12 cells, morphologically and also functionally at low concentration [11,13]. LB-A was also found to arrest the cell cycle of Neuro 2A cells at the G1 phase specifically [13]. Neuronal differentiation closely relates to the cell cycle. The G1 arrest at the differentiation stage appears to be a normal cell response, since the endogenous nerve growth factor also arrests the cell cycle at the same phase [18].

We further examined the effect of LB-A on the cell cycles of the fibroblast and leukemia cells, which have quite different physiological features compared with neuroblastoma. For cell cycle analysis, flow cytometric analysis was carried out at 24 h after the addition of LB-A to Neuro 2A, rat fibroblast 3Y1, and human leukemia K562 cells grown by random culture. The treatment with 1–10 μM concentration of LB-A showed no effect on the cell cycle of 3Y1 cells or K562 cells, while the cell cycle of Neuro 2A cells was remarkably arrested at the G1 phase by the treatment with 1–3 μM concentration of LB-A (Fig. 2).

The cell cycle is regulated positively by cyclins associated with cyclin-dependent kinase (CDK) and negatively by cyclin-dependent kinase inhibitor (CDKI), which inhibits kinase activity of cyclin/CDK complex. CDKIs are classified into two groups of INK4 family (p15, p16, p18, and p19) and CIP/KIP family (p21, p27, and p57) based on their sequence similarities and putative target genes. The latter is concerned in arrest of

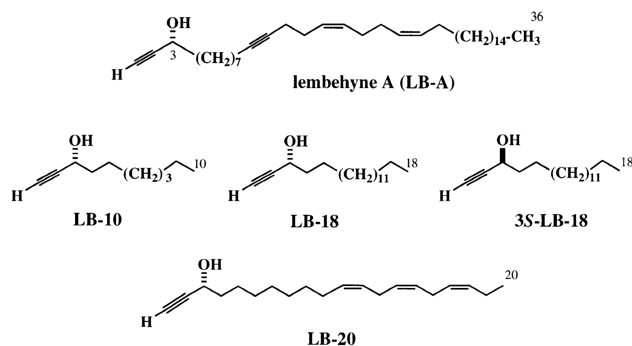
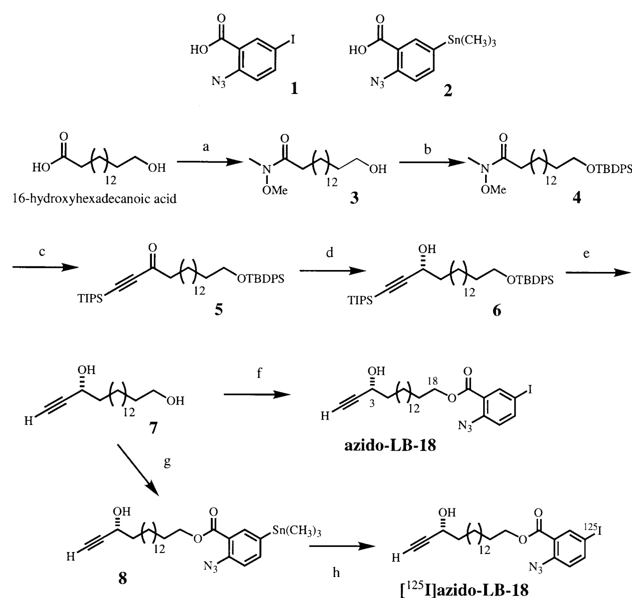


Fig. 1. Chemical structures of LB-A and its analogs.



Scheme 1. The synthesis of [^{125}I]azido-LB-18. Reagents: (a) MeONHMe·HCl, DMAP, EDCI·HCl, DMF, 95%; (b) TBDPSCl, imidazole, DMF, 99%; (c) Li (TIPS) acetylide, THF, 98%; (d) (*R*)-oxazaborolidine, $BH_3 \cdot (CH_3)_2S$, THF, 98% (100% ee); (e) TBAF, THF, 88%; (f) 1, DMAP, EDCI·HCl, THF; (g) 2, DMAP, EDCI·HCl, THF; (h) Na^{125}I, chloramine T, MeOH·H_2O.

cell cycle and induction of cell differentiation [19]. We further investigated whether the induction of CDKI, p21$^{cip1/waf1}$ protein, increases in response to the stimulus of LB-A or not. The induction of p21 protein was not observed in the untreated Neuro 2A cells, while the expression of p21 was remarkably raised after 24 h treatment with 3 μM concentration of LB-A (Fig. 3). The neurite sprouting in Neuro 2A cells treated with LB-A was synchronized with the expression of p21 protein. This result indicated that the expression of p21 was related to the differentiation of Neuro 2A cells induced by LB-A. On the other hand, the expression of p21 was not observed in K562 cells treated with LB-A. Besides, LB-A did not affect the differentiation and proliferation of K562 cells (data not shown), which have an ability to differentiate into erythrocyte, megakaryocyte or macrophage by the treatment with various low molecular differentiation inducers. Thus, the cytostatic effect of LB-A was unique to neuroblastoma.

Next, we turned our attention to identification of the target molecule of LB-A in neuroblastoma to examine the action mechanism of LB-A. The importance of the *R*-configuration at the C-3 chiral center in LB-A for the activity might indicate the presence of a target protein for LB-A. LB-A seems to be difficult to form a covalent bond with a target protein, since the reactive functional group attacked by a nucleophile in the target protein is absent in LB-A. A photoaffinity probe, which has a photo-reactive functional group such as an aromatic azide group, is able to form a covalent bond with the receptor protein forcibly under UV irradiation. So, the introduction of the 2′-azido-5′-iodo-benzoyloxyl group at C-18 in LB-18, which showed the strongest neuritogenic activity among lembehyne analogs, was designed. The non-radioactive photoaffinity probe, azido-LB-18 was synthesized from 16-hydroxyhexadecanoic acid efficiently (Scheme 1). This analog exhibited strong neuritogenic activity against Neuro 2A cells at 1 μM the same as LB-A, indicating that this probe has an affinity to the target protein for LB-A. Hence, the radioactive photoaffinity probe, [^{125}I]azido-LB-18, was used to photolabel the target protein for LB-A.

In situ photoaffinity labeling was carried out in Neuro 2A cells 3 h after the treatment with 53 nM concentration of [^{125}I]azido-LB-18. Labeled cells were lysed in lysis buffer, then the cell lysate was analyzed by SDS–PAGE and autoradiography. As a result of this labeling experiment, a protein of M_r 30 kDa was specifically photolabeled (Fig. 4). The labeling of this protein was completely inhibited by the pre-incubation of Neuro 2A cells with a 1–5 μM concentration of LB-A, LB-18, or LB-20 having neuritogenic activity (Fig. 4A,B). The pre-incubation time with LB-A required for the inhibition of labeling was much longer than that with LB-18 or LB-20. This result might be deduced to be due to the slow incorporation of LB-A into cells. The pre-incubation with the inactive analog, LB-10, at 1–5 μM concentration showed no inhibition for this labeling. The slightly active analog, 3*S*-LB-18, showed only weak inhibition for this labeling at 5 μM concentration. These results strongly suggest that this protein of M_r 30 kDa is the target protein, which mediates the neuronal differentiation in Neuro 2A cells.

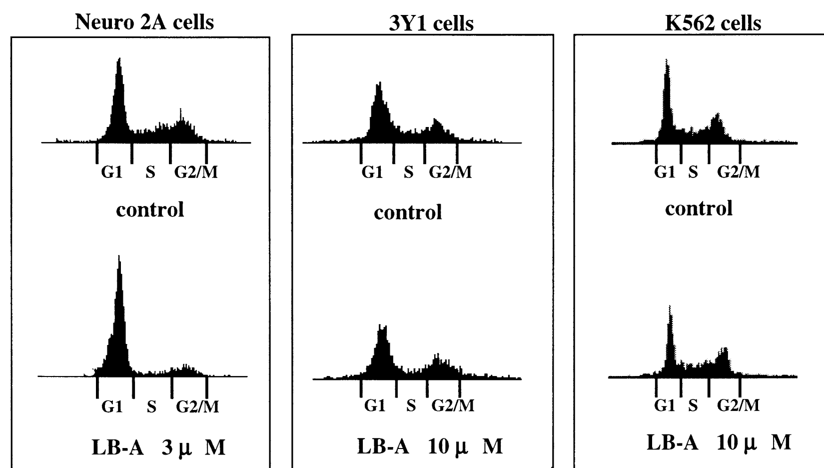


Fig. 2. The effect of LB-A on the cell cycle of various cells. Various cells were exposed to LB-A at the indicated concentration for 24 h, and cell cycle distribution was determined. Triplicate experiments concurred with the same result.

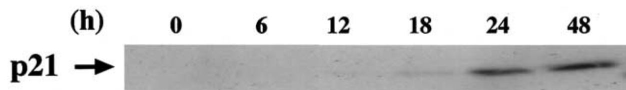


Fig. 3. The effect of LB-A on the expression of p21$^{cip1/waf1}$ in Neuro 2A cells. Whole cell extract was prepared at the indicated times after treatment with 3 μM of LB-A and subjected to SDS–PAGE. Immunoblotting was performed with anti-p21$^{cip1/waf1}$ antibody. Triplicate experiments concurred with the same result.

In general, cell lysate has been used as the labeling source for photoaffinity labeling experiments, since the efficacy of photolabeling using whole cells was very poor. Fortunately, we succeeded in in situ photoaffinity labeling in Neuro 2A cells. Then, this labeled protein was strongly supported to be a target protein for LB-A. Furthermore, the known differentiation inducers for Neuro 2A cells, mevinolin [4], ganglioside GD1a [5], retinoic acid [6], staurosporine [7], and lactacystin [9], did not inhibit this labeling by [^{125}I]azido-LB-18 up to 10 μM concentration. We also tried the photolabeling of

Fig. 4. In situ photoaffinity labeling of the target protein for LB-A. Neuro 2A cells were photolabeled with 53 nM of [^{125}I]azido-LB-18 after 1 h (panel A) or 7 h (panel B) incubation with various competitors. Whole cell extracts were analyzed by 15–25% SDS–PAGE and autoradiography. K562 cells or 3Y1 cells were treated the same as Neuro 2A cells in the absence of competitors, and whole cell extracts were analyzed by 15–25% SDS–PAGE and autoradiography (panel C). Triplicate experiments for panels A and B and duplicate experiments for panel C concurred with the same result.

the target protein using K562 cells and 3Y1 cells; however, no protein was labeled by [^{125}I]azido-LB-18 under the same conditions for Neuro 2A cells (Fig. 4C). These facts show that the action mechanism of LB-A in Neuro 2A cells is different from those of the known differentiation inducers and the target protein for LB-A seems to be not ubiquitous in various cells.

In conclusion, the photoaffinity labeling by [^{125}I]azido-LB-18 clarified that the protein of M_r 30 kDa is a molecular target for LB-A. This labeled protein may be responsible for the neuronal differentiation induced by LB-A, since this protein was not labeled in other cell lines, fibroblast or leukemia, which are non-responsive to LB-A. The photoaffinity probe, [^{125}I]azido-LB-18, is expected to be a useful tool for studying the action mechanism of LB-A, which is different from those of the known differentiation inducers. LB-A has a simple chemical structure and potent neuronal differentiation activity, so it might be a good lead compound for differentiation therapy. Further study is needed for the characterization of the protein of M_r 30 kDa and the elucidation of its role for the neuronal differentiation in Neuro 2A cells.

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