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Potent inhibitors of amyloid β fibrillization, 4,5-dianilinophthalimide and staurosporine aglycone, enhance degradation of preformed aggregates of mutant Notch3

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations in human *NOTCH3*. We have recently reported that mutant Notch3 shows a greater propensity to form aggregates, and these aggregates resist degradation, leading to accumulation in the endoplasmic reticulum (ER). In this study, we searched for low-molecular compounds that decrease the amount of mutant Notch3 aggregates. Using a cell-based system, we found that degradation of preformed mutant aggregates was enhanced by treatment with either 4,5-dianilinophthalimide (DAPH) or staurosporine aglycone (SA), both of which inhibit amyloid β (Aβ) fibrillization. Regarding other low-molecular compounds interacting with Aβ fibrils, thioflavin T (ThT) also enhanced the clearance of mutant Notch3. These findings suggest that DAPH, SA, and ThT are potent reagents to dissociate the preformed aggregates of mutant Notch3 by disruption of intermolecular contacts of misfolded proteins. Our study may provide the basis for the development of a pharmacological therapy for CADASIL.

1. Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary small vessel disease causing recurrent subcortical ischemic strokes and vascular dementia [1–3]. The pathological hallmarks of the disorder are degeneration of vascular smooth muscle cells (VSMCs) and the abnormal accumulation of granular osmiophilic material (GOM) [4,5]. CADASIL is caused by missense mutations and small deletions in *NOTCH3* [6,7], and the extracellular domain of Notch3 is a constituent of GOM [8]. Although the formation of GOM is considered to be involved in the disease process, the molecular mechanisms by which Notch3 mutations lead to vascular degeneration remain unclear [9–16]. Recently, we have shown that mutant Notch3 is more prone to form aggregates that are accumulated in the endoplasmic reticulum (ER) and is considerably resistant to ER-associated degradation (ERAD) than wild-type Notch3 [17]. These findings indicate that the cytotoxic effects of mutant Notch3 may be related to the formation and accumulation of mutant aggregates in the ER.

In the present study, we searched for low-molecular compounds that efficiently degrade the preformed aggregates of mutant Notch3. Using tetracycline (Tet)-on inducible stable cell lines, we found that the degradation of preformed mutant aggregates was facilitated by treatment with 4,5-dianilinophthalimide (DAPH) and staurosporine aglycone (SA), which inhibit amyloid β (Aβ) fibrillization [18,19]. Furthermore, thioflavin T (ThT), which interacts with Aβ fibrils and senile plaques [20], also accelerated the clearance of mutant Notch3 aggregates. These findings may invigorate hope for a pharmacological therapy of CADASIL patients.

2. Materials and methods

2.1. Low-molecular compounds

4,5-Dianilinophthalimide (DAPH), staurosporine aglycone (SA), and trehalose were purchased from Sigma. Tauroursodeoxycholic

Abbreviations: CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; ER, endoplasmic reticulum; DAPH, 4,5-dianilinophthalimide; SA, staurosporine aglycone; Aβ, amyloid β; ThT, thioflavin T; VSMCs, vascular smooth muscle cells; GOM, granular osmiophilic material; ERAD, ER-associated degradation; Tet, tetracycline; TUDCA, tauroursodeoxycholic acid; 4PBA, 4-phenylbutyric acid; ThS, thioflavin S; PIB, Pittsburgh compound-B; HEK, human embryonic kidney.

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acid (TUDCA) was from Calbiochem, and thioflavin T (ThT), curcumin, and 4-phenylbutyric acid (4PBA) were from Wako. Thioflavin S (ThS) was from ICN Biomedicals. Tetrahydrocurcumin and resveratrol were kind gifts from Dr. Wakako Maruyama (National Center for Geriatrics and Gerontology). Pittsburgh compound-B (PIB), that is, 2-(4′-methylaminophenyl)-6-hydroxybenzothiazole [21], was a gift from Dr. Seiji Iwasa (Toyohashi University of Technology).

2.2. Stable cell lines

We previously established stable human embryonic kidney (HEK) 293 cell lines in which the expression of Notch3 can be induced using the Tet-on regulatory system (T-Rex system; Invitrogen) [17]. Several cell lines expressing either wild-type or mutant [arginine 133 to cysteine (p.R133C) or cysteine 185 to arginine (p.C185R)] human Notch3 were obtained [17]. The stable cell lines were maintained in DMEM containing 10% fetal bovine serum, 200 μg/ml Zeocin (Invitrogen), and 10 μg/ml blasticidin-S (Invitrogen).

2.3. Western blot analysis

Cells (1.2×10^6) were seeded in six-well plates, treated with 2 μg/ml tetracycline for 24 h, and then cultured in a fresh medium without tetracycline. For treatment of low-molecular compounds, the cells were incubated in the medium containing one of these compounds for 2 days. At indicated times, the cells were harvested and lysed in solution A containing 1% Triton X-100, 0.1 M Tris–HCl (pH 7.4), 0.15 M NaCl, and a protease inhibitor cocktail (Boehringer Mannheim). Lysates (30 μg/lane) were separated on a 7–10% SDS–polyacrylamide gel, and the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked in TBST [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20] containing 5% nonfat milk and probed using the following the primary and secondary antibodies: a rabbit polyclonal anti-human Notch3 antibody (AbN2, 1 μg/ml) [14,17] and a mouse monoclonal anti-GAPDH antibody (Sigma, 1:2000); a HRP-conjugated goat anti-rabbit antibody (BD Biosciences, 1:3000) and a HRP-conjugated sheep anti-mouse antibody (GE Healthcare, 1:2000). Immunoreactive proteins were detected using Western Lightning chemiluminescence reagents (Perkin Elmer). Protein concentration was determined by micro-BCA assay (Pierce).

2.4. Immunocytochemistry

Cells were cultured in 35-mm Petri dishes coated with poly-L-lysine and fixed in 4% paraformaldehyde in PBS at 4 °C for 10 min. After treatment with 0.2% Triton X-100 for 10 min, cells were blocked with PBS containing 3% fetal bovine serum for 30 min and incubated for 1 h with an anti-Notch3 antibody (AbN2, 1 μg/ml) at room temperature. Cells were washed three times with PBS and incubated for 1 h with a Rhodamine Red-labeled goat anti-rabbit antibody (Molecular Probes) at a 1:1000 dilution in PBS. After three washes in PBS, cells were examined under a light microscope (Olympus). Cells with aggregates were quantified manually by counting cell numbers in phase contrast and immunofluorescence microscopy images and scored as the percentage of the total number of cells.

2.5. Statistical analysis

Data are presented as means ± standard deviation (S.D.). Statistical analysis was performed using unpaired *t*-test (two-tailed) or one-way ANOVA with Dunnett's multiple comparison post hoc test (PRISM version 5.0a; GraphPad Software, La Jolla, CA, USA). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effects of low-molecular compounds on clearance of mutant Notch3

We established stable HEK293 cell lines in which expression of Notch3 was inducible using the Tet-on regulatory system and showed by pulse-chase analysis and Western blot analysis that the aggregates of mutant Notch3 resisted degradation and accumulated in the ER [17]. Because the expression of Notch3 could be induced and silenced by adding and removing tetracycline, respectively, the degradation rate of Notch3 is easily determined by Western blot analysis. As shown in Fig. 1A, wild-type Notch3 rapidly disappeared within 1 day after silencing its expression, whereas considerable amounts of mutant Notch3 were still detected after 2 days.

Using these cell lines, we searched for low-molecular compounds that facilitated the degradation of the mutant Notch3 aggregates. Previous studies have shown that the chemical

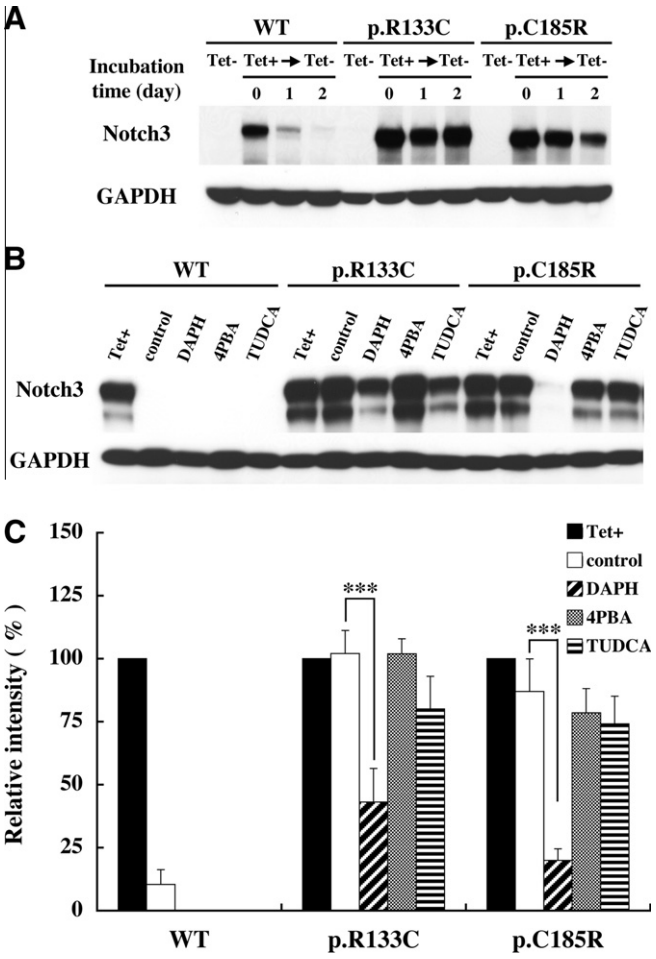

Fig. 1. Effects of low-molecular compounds on degradation of mutant Notch3. (A) Cells were treated with (Tet+) and without (Tet−) 2 μg/ml tetracycline for 24 h and then incubated in the standard medium. The cells were harvested at the indicated times (0–2 days) after the medium change and subjected to Western blot analysis. A representative Western blot is shown. (B) After treatment with 2 μg/ml tetracycline for 24 h (Tet+), cells were incubated in the standard medium with 10 μM DAPH, 20 mM 4PBA or 20 mM TUDCA and without a chemical compound (control) for 2 days. A representative Western blot is shown. (C) Densitometry of the Western blot (B) were performed to estimate relative amounts of wild-type Notch3 and mutant Notch3. Results represent means ± S.D. of data from five experiments and are shown as the percentage of Tet+. ***, $P < 0.001$ relative to untreated cells (control) for each cell line.

chaperones 4PBA and TUDCA modulate the stability of the mutant proteins and inhibit the formation of aggregates [22–24]. In addition, DAPH has been reported to inhibit and reverse the formation of Aβ42 fibrils [18,19]. Therefore, we investigated the effects of these compounds on the clearance of mutant Notch3 aggregates. Stable cells were treated with these compounds for 2 days after stopping the expression of Notch3. As shown in Fig. 1B, treatment with DAPH markedly decreased the amount of mutant Notch3, indicating that this compound effectively enhanced the degradation of mutant Notch3. The amount of mutant Notch3 slightly decreased in cells treated with either 4PBA or TUDCA, although the effect varied among different cell lines expressing mutant Notch3. Quantitative analysis of data showed that only treatment with DAPH resulted in a significant decrease in the amount of mutant Notch3 (Fig. 1C).

3.2. DAPH enhances degradation of mutant Notch3

We determined the effect of DAPH on the clearance of the mutant Notch3 aggregates by immunocytochemical analysis of stable cells using an anti-Notch3 antibody (AbN2). As shown in Fig. 2A, treatment with DAPH significantly decreased the number of cells containing mutant aggregates. Quantitative immunocytochemical analysis revealed that the percentage of cells with mutant Notch3 aggregates to total cells decreased from 35% to 18% for cells expressing the p.R133C mutant and from 33% to 9% for cells expressing the p.C185R mutant following DAPH treatment (Fig. 2B). These results indicate that DAPH enhances the degradation of preformed aggregates of mutant Notch3.

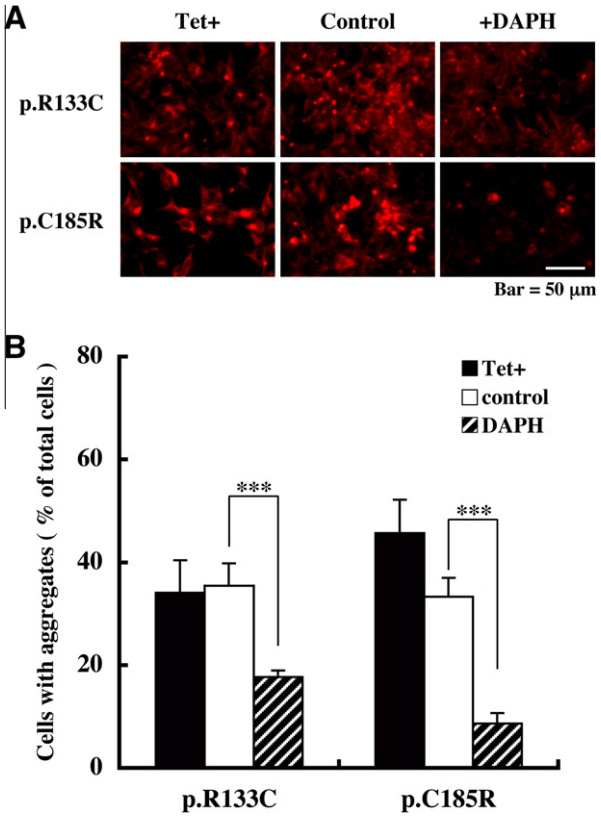

Fig. 2. Effect of DAPH on degradation of preformed mutant aggregates. Cells were treated with 2 μg/ml tetracycline for 24 h (Tet+) and then incubated in the standard medium with 10 μM DAPH or without DAPH (control) for 2 days. (A) Cells were fixed and stained with an anti-Notch3 antibody. Representative photographs are shown. Scale bar, 50 μm. (B) Cells with aggregates were quantified manually by counting cell number 0 day (Tet+) and 2 days after treatment of DAPH. Results represent means ± S.D. of data from five independent images and are shown as the percentage of total number of cells. $^{***}P < 0.001$ relative to untreated cells (control) of each cell line.

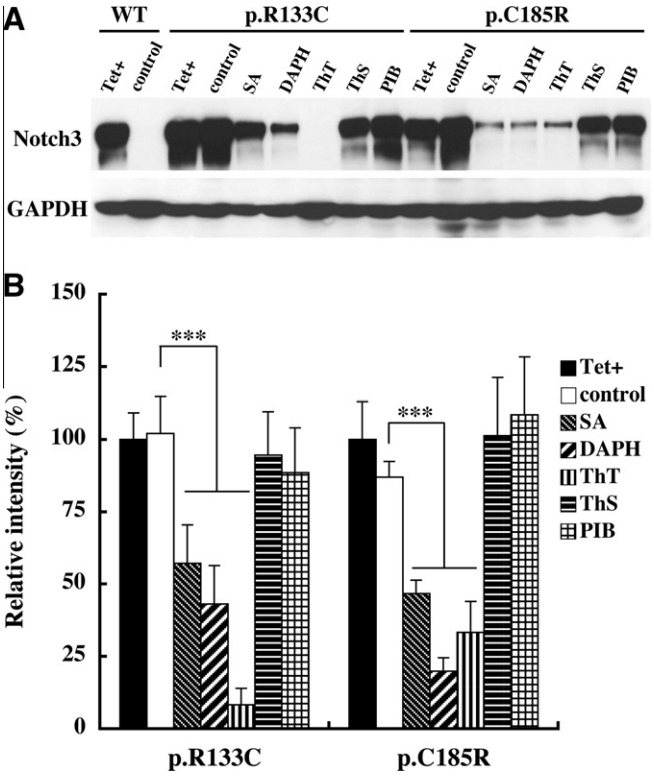

Fig. 3. Effects of Aβ-binding compounds on degradation of mutant Notch3. (A) Cells were treated with 2 μg/ml tetracycline for 24 h (Tet+) and then incubated in the standard medium with 20 μM SA, 10 μM DAPH, 2 μM ThT, 40 μM ThS, or 40 μM PIB and without a chemical compound (control) for 2 days. Cells were lysed and subjected to Western blot analysis. A representative Western blot is shown. (B) Densitometry of the Western blot was performed to estimate relative amounts of mutant Notch3. Results represent means ± S.D. (DAPH $n = 6$, others $n = 4$) and are shown as the percentage of Tet+. $^{***}P < 0.001$ relative to untreated cells (control) for each cell line.

3.3. Identification of other low-molecular compounds as enhancers of mutant Notch3 degradation

Because DAPH has been reported to inhibit Aβ42 fibrillization [18,19], we determined the effects of other compounds that interact with either Aβ fibrils or amyloid deposits (Fig. 3A and B). SA, an analog of DAPH, was less effective in degrading mutant Notch3 aggregates than DAPH. ThT and ThS, both of which bind the β-sheet structure of amyloid deposits [20], showed their contrasting effects on the degradation of mutant Notch3, the former was more potent than DAPH for the p.R133C mutant, but the latter did not decrease the amount of mutant Notch3. PIB, an amyloid-imaging probe of PET [21], did not degrade mutant Notch3. As shown in Table 1, we also analyzed several compounds including antioxidants (tetrahydrocurcumin and resveratrol), an autophagy inducer (trehalose) [25] and an inhibitor of Aβ aggregation (curcumin) [26]. However, these compounds did not decrease the amount of mutant Notch3, suggesting that the degradation of mutant Notch3 was facilitated by a limited type of inhibitor of Aβ fibrillization.

4. Discussion

Using stable HEK293 cell lines with inducible expression of mutant Notch3, we have shown that the degradation of preformed

Table 1
Summary of effects of low-molecular compounds on degradation of Notch3 aggregates.

Compound	Conc.	Effect	Function
4,5-Dianilinophthalimide (DAPH)	(10 μM)	+++	Inhibitor of Aβ fibrillization
Staurosporine aglycone (SA)	(20 μM)	++	DAPH analog and apoptosis inducer
Thioflavin T (ThT)	(2 μM)	+++	Bind to Aβ and senile plaques
Thioflavin S (ThS)	(40 μM)	–	Bind to Aβ and senile plaques
Curcumin	(30 μM)	–	Inhibitor of Aβ aggregation
PIB	(40 μM)	–	Imaging probe for senile plaques
4-Phenylbutyric acid (4PBA)	(20 mM)	–	Chemical chaperone
Tauroursodeoxycholic acid (TUDCA)	(20 mM)	–	Chemical chaperone
Trehalose	(20 mM)	–	Autophagy inducer
Tetrahydrocurcumin	(20 μM)	–	Antioxidant
Resveratrol	(20 μM)	–	Antioxidant

The effects of chemical compounds were determined as described in Fig. 1C and Fig. 3B. Triple plus (+++) and double plus (++) signs indicate the strong and mild elimination effects on mutant aggregates, respectively. Minus (–) signs represent the insignificant degradation effect on mutant aggregates.

mutant Notch3 aggregates is enhanced by treatment with low-molecular compounds, namely, DAPH and SA, which are novel reagents preventing fibril formation and the neurotoxicity of Aβ42 peptides [18,19]. However, the exact mechanisms of clearing mutant Notch3 aggregates by the inhibitors of Aβ fibrillization are unclear. Previous study using a cell-free system had indicated that DAPH and its analogs change the β-sheet conformation of amyloid fibrils by inhibiting and disrupting the formation of intermolecular contacts, although SA was less potent in inhibiting Aβ fibrillization than DAPH [19]. Because DAPH shows no effect on fiber assembly or disassembly of Tau, α-synuclein and Ure2 [19], the compounds may interact with a specific conformational element of Aβ42 fibrils. Thus, it is likely that DAPH might also recognize a common structure in mutant Notch3 aggregates and disrupt intermolecular contacts of mutant Notch3, resulting in the dissociation and degradation of the aggregates. In addition, ThT showed the ability to degrade aggregates of mutant Notch3. Interestingly, the clearance of mutant Notch3 aggregates was enhanced by the treatment with ThT but not ThS, although both compounds have been known to interact with β-sheet-containing Aβ fibrils and are used as a fluorescence probe to analyze β-sheet formation [20]. Thus, ThT but not ThS may interact with the common element in mutant aggregates that is recognized by DAPH. On the other hand, our previous study has indicated that mutant Notch3 is more prone to form aggregates compared with wild-type Notch3, and mutant aggregates impairs cell proliferation [17]. Therefore, we examined whether DAPH could promote cell proliferation. However, DAPH mildly attenuates cell growth. For this reason, we could not evaluate the exact effect of DAPH on cell proliferation by enhancing the degradation of mutant aggregates in the present study.

Several low-molecular compounds have also been reported to reverse the misfolding of mutant membrane and secretory proteins. 4PBA and TUDCA are chemical chaperones that stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins [23,24,27]. Trehalose is an autophagy enhancer and inhibits the formation of aggregates and attenuates the toxicity of mutant huntingtin and α-synuclein [25,28]. However, these chemical chaperones or compounds had little effect on the clearance of mutant Notch3, suggesting that the aggregate formation of mutant Notch3 is not reduced by altering the conditions of the ER or inducing autophagy.

Our studies also have shown that the Tet-on inducible cell lines are a useful cellular system for screening the reagents to dissociate the preformed aggregates of mutant Notch3. One of the advantages of this cellular system is that the effects of compounds can be tested under physiological conditions. It has been considered that low-molecular compounds being active in the cell-free system are often inactivate in vivo, because they are not taken up into cells, they interact nonspecifically with other proteins, or they are metabolized into inactive forms. In addition, the efficacy of many compounds is simply determined by quantitative Western blot analysis. Therefore, this cellular system represents an additional tool for studying the processes occurring during the formation of mutant Notch3 aggregates and therapeutic reagents affecting such processes.

In conclusion, using the Tet-on inducible stable cell lines, we screened for low-molecular compounds that efficiently degrade the preformed aggregates of mutant Notch3. By this method, we found that the degradation of preformed mutant aggregates was enhanced by treatment with either DAPH or SA, which inhibits Aβ fibrillization. Our study may be useful for the development of a pharmacological therapy for CADASIL.

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