

Identification of approved drugs that inhibit the binding of amyloid β oligomers to ephrin type-B receptor 2

Koichiro Suzuki^{1,2}, Takahiro Aimi¹, Tomoaki Ishihara¹ and Tohru Mizushima³

1 Division of Drug Discovery and Development, Faculty of Pharmacy, Keio University, Minato-ku, Tokyo, Japan

2 Research Fellow of Japan Society for the Promotion of Science, Chiyoda-ku, Tokyo, Japan

3 LTT Bio-Pharma Co., Ltd, Minato-ku, Tokyo, Japan

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Correspondence

T. Ishihara, Division of Drug Discovery and Development, Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan
Fax/Tel: +81 3 5400 2624
E-mail: ishihara-tm@pha.keio.ac.jp

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Ephrin type-B receptor 2 (EphB2) is a member of the receptor tyrosine kinase family and plays an important role in learning and memory functions. In patients with Alzheimer's disease (AD) and in mouse models of AD, a reduction in the hippocampal EphB2 level is observed. It was recently reported that normalization of the EphB2 level in the dentate gyrus rescues memory function in a mouse model of AD, suggesting that drugs that restore EphB2 levels may be beneficial in the treatment of AD. Amyloid β (A β) oligomers, which are believed to be key molecules involved in the pathogenesis of AD, induce EphB2 degradation through their direct binding to EphB2. Thus, compounds that inhibit the binding of A β oligomers to EphB2 may be beneficial. Here, we screened for such compounds from drugs already approved for clinical use in humans. Utilizing a cell-free screening assay, we determined that dihydroergotamine mesilate, bromocriptine mesilate, cepharanthine, and levonorgestrel inhibited the binding of A β oligomers to EphB2 but not to cellular prion protein, another endogenous receptor for A β oligomers. Additionally, these four compounds did not affect the binding between EphB2 and ephrinB2, an endogenous ligand for EphB2, suggesting that the compounds selectively inhibited the binding of A β oligomers to EphB2. This is the first identification of compounds that selectively inhibit the binding of A β oligomers to EphB2. These results suggest that these four compounds may be safe and effective drugs for treatment of AD.

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common cause of dementia. Because the world's population is aging, the number of AD patients has been increasing; however, there are currently no disease-modifying treatments for AD. Thus, development of disease-modifying drugs is important.

One hallmark pathological feature of AD is senile plaques, which are composed of amyloid β peptide

(A β). Monomeric A β easily self-assembles to form soluble oligomers, protofibrils, and fibrils. A correlation between the amount of soluble A β oligomers and cognitive impairment in AD patients has been reported [1]. In addition, soluble A β oligomers inhibit long-term potentiation (LTP) [2,3] which is involved in learning and memory functions [4]. Therefore, A β oligomers are believed to play an important role in the learning and memory dysfunction observed in patients with AD.

Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AD, Alzheimer's disease; A β , amyloid β peptide; BRO, bromocriptine mesilate; BSA, bovine serum albumin; CEP, cepharanthine; DIH, dihydroergotamine mesilate; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; EphB2, ephrin type-B receptor 2; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HRP, horseradish peroxidase; LCA, lithocholic acid; LEV, levonorgestrel; LTP, long-term potentiation; PBS, phosphate-buffered saline; PrP^C, cellular prion protein; SD, standard deviation.

Some endogenous receptors for A β oligomers, such as ephrin type-B receptor 2 (EphB2) and cellular prion protein (PrP^C), appear to be involved in the inhibitory effects of A β oligomers on LTP [5–7]. EphB2, a member of the receptor tyrosine kinase family [8], is a receptor for ephrinB ligands (B1, B2, and B3) and ephrinA5 [9] and functions in synaptic plasticity and synapse formation [10–12]. Mice lacking EphB2 exhibit LTP impairment and memory dysfunction [11,12]. Reduced EphB2 levels are observed in postmortem hippocampal tissue from patients with AD [13] and in mouse models of AD [5,13,14]. A β oligomers directly bind to EphB2 and induce its internalization and proteasome-mediated degradation [5]. In addition, artificial expression of EphB2 in the dentate gyrus rescues LTP and memory function in a mouse model of AD [5]. These results suggest that inhibiting A β oligomer binding to EphB2 may be therapeutically beneficial in the treatment of AD.

The number of drugs reaching the marketplace each year is decreasing, mainly due to unexpected adverse effects being revealed at advanced stages of clinical trials. To overcome this problem, we took advantage of a recent strategy for drug discovery and development that focuses on examining drugs already approved for use in humans (i.e., drug repositioning). In this strategy, compounds with clinically beneficial pharmacological activity are screened from a library of approved drugs already in clinical use to be developed for new indications. One major advantage of this strategy is a decreased risk for unexpected adverse effects in humans because the safety of these drugs has already been well characterized in humans [15].

In the present study, from a library of approved drugs already in clinical use, we screened for compounds that inhibited the binding of A β oligomers to EphB2. We identified dihydroergotamine mesilate (DIH), bromocriptine mesilate (BRO), cepharanthine (CEP), and levonorgestrel (LEV). Although these compounds inhibited the binding of A β oligomers to EphB2, they did not inhibit the binding of an endogenous ligand, ephrinB2, to EphB2. This is the first time that compounds that selectively inhibited the binding of A β oligomers to EphB2 have been identified.

Materials and methods

Materials

Biotin-conjugated A β 42 (biotin-A β) was obtained from AnaSpec Inc. (Fremont, CA, USA). EphB2 receptor ectodomains fused to the Fc portions of human IgG (EphB2-Fc) and biotinylated ephrinB2-Fc were purchased from

R&D systems Inc. (Minneapolis, MN, USA). His-tagged human PrP^C was obtained from Merck Co. (Darmstadt, Germany). Bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MI). Horseradish peroxidase-conjugated streptavidin (HRP-streptavidin) was obtained from GE Healthcare UK Ltd. (Little Chalfont, UK). The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). The antibodies against A β (6E10) and PrP^C (ICSM35) were purchased from Covance Inc. (Emeryville, CA, USA) and D-Gen Co. (London, UK), respectively. Phenol red-free Dulbecco's modified Eagle medium (DMEM) and 1,1,1,3,3,3-hexafluoro-2 propanol (HFIP) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The library of drugs approved for clinical use was obtained from our laboratory stock [16].

A β monomer and oligomer preparation

A β monomers and oligomers were prepared as described previously [16]. Biotin-A β was dissolved in HFIP and incubated at room temperature for 1 h and then on ice for 10 min. The HFIP was evaporated and the peptides were dissolved in DMSO for a final concentration of 10 mM. The peptides were diluted in phenol red-free DMEM to give a final concentration of 100 μ M and subjected to sonication (AIWA Co., Tokyo, Japan) for 10 min.

For oligomer preparation, the A β solution described above was incubated at 37 °C for 16 h and then centrifuged at 20 400 *g* for 15 min. The supernatant was frozen in liquid nitrogen and stored at –80 °C until use.

For monomer preparation, the sample was centrifuged and stored in a manner similar to that for oligomer preparation, without the incubation at 37 °C for 16 h.

Characterization of A β oligomers by immunoblotting

To characterize A β oligomers, the supernatant was mixed with equal amounts of sample buffer and boiled at 98 °C for 5 min. Samples were applied to polyacrylamide sodium dodecyl sulfate gels and subjected to electrophoresis, after which proteins were immunoblotted with 6E10.

Cell-free binding assay

The cell-free binding assay was performed as described previously [16]. To evaluate binding between A β and EphB2, EphB2-Fc was dissolved in phosphate-buffered saline (PBS) to a final concentration of 0.5 μ g·mL^{–1} and applied to a 96-well ELISA plate and left at 4 °C overnight. Each well was washed with PBS containing 0.05% Tween 80 (T-PBS) and blocked with 2% BSA at room temperature for 1 h. Then, each well was washed with T-PBS and incubated with both

biotin-A β (200 nM) and each tested drug in DMEM at room temperature for 1 h. Each well was then washed with T-PBS and incubated with HRP-streptavidin in PBS (1 : 2000 dilution) at room temperature for 30 min. Each well was washed with T-PBS and incubated with ABTS. Absorbance at 412 nm was measured using a plate reader (Infinite M1000; TECAN Group Ltd., Mannedorf, Switzerland). Biotin-A β binding to EphB2 was calculated as follows:

OD_{Blank}: absorbance of well without both EphB2 and each tested drug

OD_{Control}: absorbance of well with EphB2 but without any tested drug

OD_{Drug}: absorbance of well with both EphB2 and each tested drug

$$\text{A}\beta \text{ binding (OD}_{412}) = \text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}}$$

$$\text{A}\beta \text{ binding (\%)} = \frac{\text{OD}_{\text{Drug}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}}} \times 100$$

As a quality parameter for this assay, Z'-factor [17] was calculated as follows:

μ_{Control} : mean of OD_{Control}

σ_{Control} : standard deviation of OD_{Control}

μ_{Blank} : mean of OD_{Blank}

σ_{Blank} : standard deviation of OD_{Blank}

$$Z' = 1 - \frac{3 \times \sigma_{\text{Control}} + 3 \times \sigma_{\text{Blank}}}{|\mu_{\text{Control}} - \mu_{\text{Blank}}|}$$

To evaluate binding between A β and PrP^C, EphB2-Fc was replaced with His-tagged human PrP^C.

To evaluate binding between ephrinB2 and EphB2, biotin-A β in DMEM was replaced with biotinylated ephrinB2-Fc in PBS (500 ng·mL⁻¹).

Chemical library and screening

Each drug in the library was dissolved in water or DMSO, depending on the drug. The concentration of each drug in the library was 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 100 mM, 2 mg·mL⁻¹, 10 mg·mL⁻¹, or 100 mg·mL⁻¹, depending on the drug. Each drug was diluted 1000 times for the screening (final concentration, 1 μ M, 5 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M, 2 μ g·mL⁻¹, 10 μ g·mL⁻¹, or

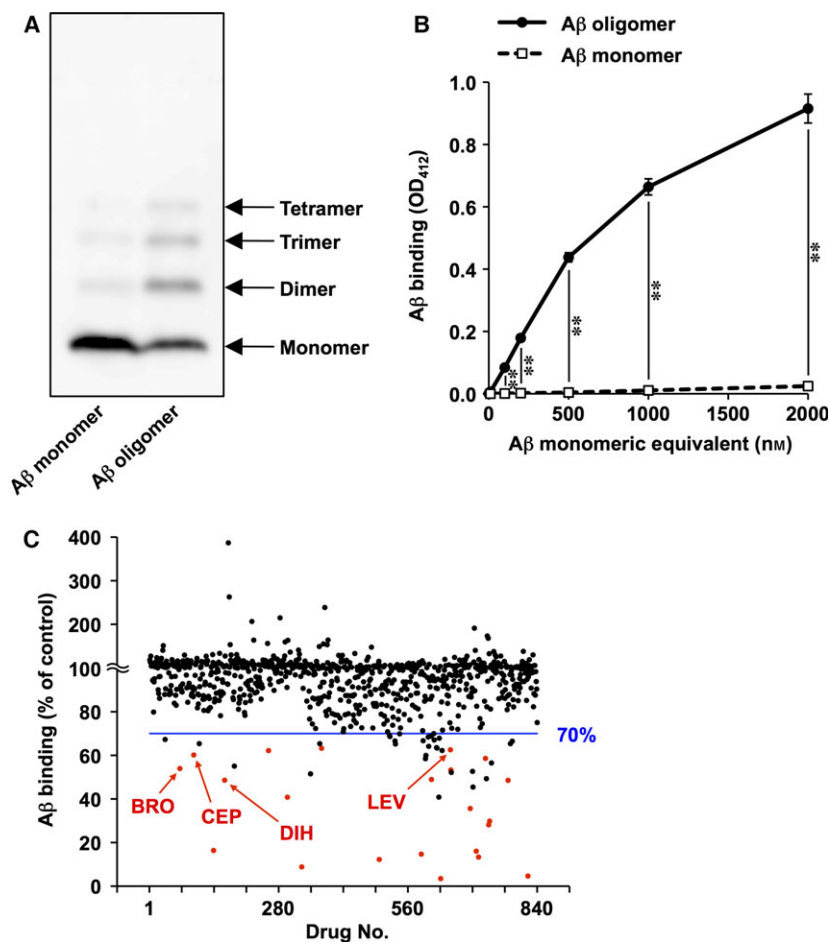


Fig. 1. Screening of approved drugs for inhibitors of A β oligomer binding to EphB2. A β monomers and oligomers were prepared. (A) Both monomeric and oligomeric forms of biotin-A β were analyzed by immunoblotting. (B) The abilities of the monomeric and oligomeric forms of biotin-A β to bind to EphB2 were compared using a cell-free binding assay. Values are mean \pm SD ($n = 3$). ** $P < 0.01$. (C) Each of 840 approved drugs was added simultaneously with A β oligomers to EphB2-coated wells, and the amount of bound biotin-A β was determined. Values are means ($n = 2$, duplicate wells per each drug in the same plate). Red dots indicate the 22 compounds that are listed in Table 1. Arrows indicate dihydroergotamine mesilate (DIH), bromocriptine mesilate (BRO), cepharanthine (CEP), and levonorgestrel (LEV).

100 $\mu\text{g}\cdot\text{mL}^{-1}$). We confirmed that 0.1% of water or DMSO did not affect this assay. Screening was performed in duplicate wells per each drug in the same reader plate.

Statistical analysis

All values are expressed as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's test was used to evaluate differences among three or more groups. Differences were considered statistically significant at *P* values less than 0.05.

Results and Discussion

Screening of approved drugs for compounds that inhibit the binding of A β oligomers to EphB2

To screen for inhibitors of A β oligomer binding to EphB2, we used a cell-free binding assay. In this assay, biotin-A β was applied to EphB2-coated wells, and

after washing, the amount of bound biotin-A β was determined using HRP-streptavidin. A β oligomers were prepared by incubation at 37 °C for 16 h, and we confirmed the formation of A β oligomers by immunoblotting (Fig. 1A).

We first compared the ability of A β monomers and oligomers to bind EphB2. We found that A β oligomers bound to EphB2 more efficiently than did the monomers (Fig. 1B). This result was consistent with a previous report [5] and suggested that this assay was appropriate for screening compounds that inhibited the binding of A β oligomers to EphB2.

From a library of 840 drugs approved for clinical use, we screened for compounds that inhibited the binding of A β oligomers to EphB2. Each compound was applied to the cell-free binding assay simultaneously with A β oligomers, and the amount of bound biotin-A β was determined (Fig. 1C). As an assay quality parameter, we calculated *Z'*-factor to be 0.89,

Table 1. Inhibitory effect of each compound on the binding of A β oligomers to EphB2 or PrP^C.

Compound	A β binding (% of control)			
	EphB2			PrP ^C
	Screening	Reproducibility		
Montelukast sodium	3.5	0.0	6.0 \pm 0.4	0.4 \pm 0.8
Pirarubicin hydrochloride	4.6	19.7 \pm 1.8	33.1 \pm 3.6	55.8 \pm 9.3
Minocycline hydrochloride	8.8	3.9 \pm 1.4	4.7 \pm 2.1	4.2 \pm 5.9
Tosufloxacin tosilate	12.2	11.2 \pm 0.7	19.1 \pm 2.6	42.2 \pm 1.7
Diclazuril	13.3	11.3 \pm 1.7	20.8 \pm 5.1	12.4 \pm 4.8
Suramin sodium	14.7	16.4 \pm 1.9	21.2 \pm 0.6	8.6 \pm 4.0
Miltefosine	16.0	50.1 \pm 1.9	53.0 \pm 4.4	43.4 \pm 2.3
Cytochrome <i>c</i>	16.4	27.1 \pm 1.9	32.0 \pm 2.0	53.4 \pm 2.9
L-thyroxine	28.1	53.8 \pm 7.0	60.1 \pm 3.7	61.0 \pm 9.3
Abamectin	29.8	40.1 \pm 2.1	45.4 \pm 5.7	35.4 \pm 12.5
Nystatin	35.6	33.2 \pm 4.7	57.5 \pm 0.8	41.6 \pm 5.0
Lysozyme hydrochloride	40.8	48.7 \pm 3.8	60.6 \pm 2.0	48.3 \pm 1.2
Bexarotene	48.5	32.7 \pm 1.7	46.2 \pm 1.2	23.3 \pm 5.9
Dihydroergotamine mesilate	48.6	62.7 \pm 6.7	65.3 \pm 5.6	82.7 \pm 5.8
Retinoic acid	48.9	39.5 \pm 13.9	62.8 \pm 2.3	27.8 \pm 1.5
Mifepristone	53.3	49.1 \pm 8.8	55.7 \pm 2.2	20.7 \pm 2.0
Bromocriptine mesilate	53.9	57.9 \pm 6.3	69.5 \pm 5.8	99.4 \pm 9.3
Toltrazuril	58.6	55.8 \pm 1.6	61.2 \pm 8.7	35.9 \pm 4.5
Cepharanthine	60.2	50.1 \pm 4.0	66.3 \pm 7.4	113.4 \pm 4.5
Indigocarmine	62.2	55.9 \pm 2.4	64.1 \pm 8.5	47.4 \pm 4.2
Levonorgestrel	62.6	64.1 \pm 2.0	65.2 \pm 4.2	95.6 \pm 6.3
Oxytetracycline hydrochloride	63.2	53.7 \pm 3.2	65.7 \pm 4.8	58.0 \pm 3.7

Among 52 compounds that inhibited more than 30% of the A β oligomer binding to EphB2 in the screening, 22 compounds that inhibited more than 30% of the A β oligomer binding to EphB2 in additional two independent experiments are listed. The effects of these 22 compounds on the binding of A β oligomers to EphB2 in the screening and in the additional two independent experiments (reproducibility) and on the binding of A β oligomers to PrP^C were shown. The four compounds that inhibited less than 30% of the A β oligomer binding to PrP^C are highlighted in gray. Values are mean (*n* = 2, 'Screening' column) or mean \pm SD (*n* = 3). The concentration of nystatin or retinoic acid was 50 μM in the screening or 100 μM in other experiments. The concentration of cytochrome *c* or lysozyme hydrochloride was 10 $\mu\text{g}\cdot\text{mL}^{-1}$ in all experiments. The concentration of other all compounds was 100 μM in all experiments.

supporting the quality of this assay. Among these 840 compounds, 52 compounds inhibited more than 30% of the A β oligomer binding to EphB2. We examined

Table 2. Compounds that enhance the binding of A β oligomers to EphB2.

Compound	A β binding (% of control)
Domiphen bromide	386.8
Dopamine hydrochloride	262.8
Pentamidine isethionate	238.6
Levodopa	214.9
Fluvoxamine maleate	206.6

Five compounds that increased the A β oligomer binding to EphB2 to more than 200% in the screening are listed. Values are mean ($n = 2$). The concentration of drugs was 100 μM .

the effect of these compounds on the binding in additional two independent experiments. We selected 22 compounds that inhibited more than 30% of the A β oligomer binding to EphB2 in both experiments (Table 1, red dots in Fig. 1C).

Through this screening procedure, we also found five compounds that enhanced the binding of A β oligomers to EphB2 to more than 200% (Table 2).

To select compounds that selectively inhibited the A β oligomer binding to EphB2, we next examined the effects of these 22 compounds on the binding of A β oligomers to PrP^C. We found four compounds (DIH, BRO, CEP, and LEV; Fig. 2A) that inhibited less than 30% of the A β oligomer binding to PrP^C (Table 1). As shown in Fig. 2B, each of these four compounds inhibited the binding of A β oligomers to EphB2 in a concentration-dependent manner. Additionally, under

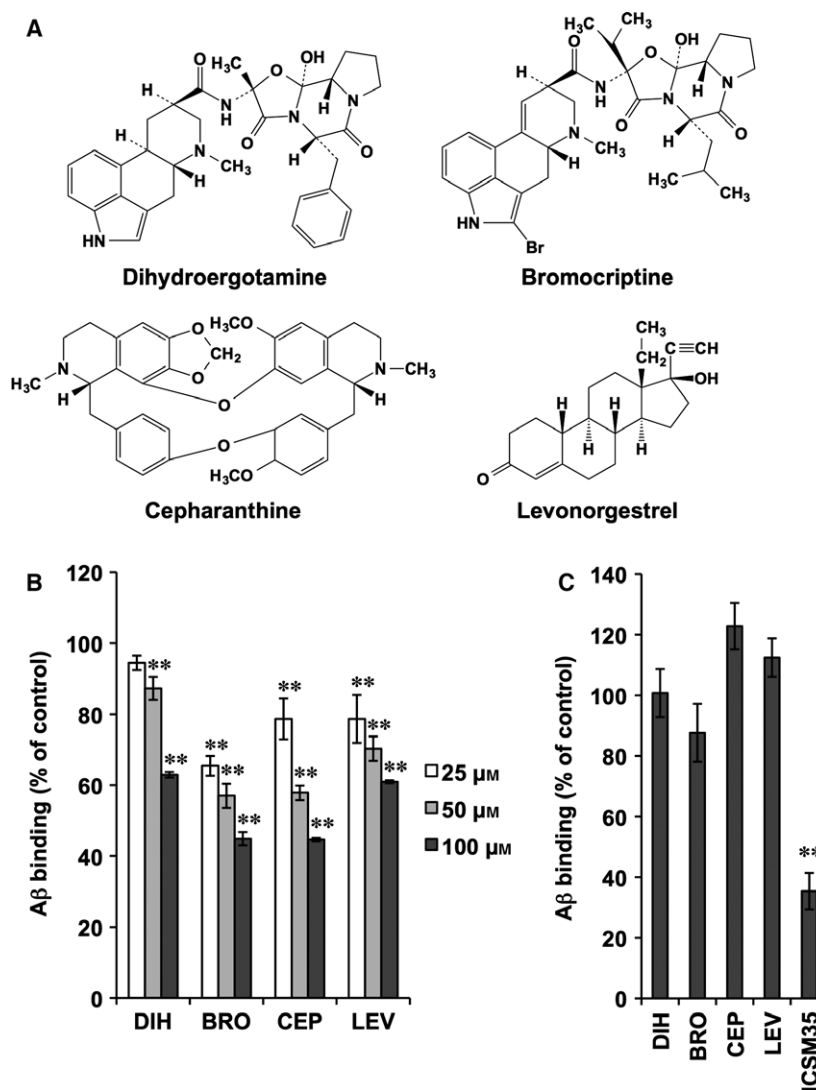


Fig. 2. Selective inhibition of the binding of A β oligomer to EphB2 by selected compounds. (A) Structures of the four selected compounds are shown. (B) The indicated concentration of each compound was added simultaneously with A β oligomers to EphB2-coated wells, and the amount of bound biotin-A β was determined. (C) Each compound (100 μM) or ICSM35 (2 $\mu\text{g}\cdot\text{mL}^{-1}$) was added simultaneously with A β oligomer to PrP^C-coated wells, and the amount of bound biotin-A β was determined. Values represent the mean \pm SD ($n = 3$). ** $P < 0.01$. Basically similar results were obtained in another independent experiment.

conditions in which an antibody against PrP^C (ICSM35) [7] inhibited the binding of A β oligomers to PrP^C, none of these four compounds inhibited the binding (Fig. 2C). These results suggested that these four compounds selectively inhibited the binding of A β oligomers to EphB2.

Effects of selected compounds on the binding of ephrinB2 to EphB2

The results in Fig. 2 suggested that the four selected compounds interacted with EphB2 rather than with A β oligomers to inhibit their binding. Thus, it is possible that these compounds may inhibit the binding between EphB2 and its endogenous ligands, which may cause adverse clinical effects. Thus, we examined the effects of these four compounds on the binding of one of the endogenous ligands, ephrinB2, to EphB2. As shown in Fig. 3, none of these four compounds inhibited the binding of ephrinB2 to EphB2. We confirmed the inhibitory effect of lithocholic acid, which interferes with Eph–ephrin interactions [18], on the binding of ephrinB2 to EphB2 (Fig. 3).

Recently, some compounds were reported to inhibit the binding of A β to its receptors. For example, Deane *et al.* [19] identified FPS-ZM1 as an inhibitor for the binding of A β to the receptor for advanced glycation end products, and Risse *et al.* identified Chicago Sky Blue 6B as an inhibitor for the binding of A β to PrP^C [20]. Furthermore, Fu *et al.* reported that rhynchophylline binds to EphA4 and stimulated LTP in the presence of A β oligomer [21].

As described above, A β oligomers directly bind to EphB2 and induce its degradation, resulting in LTP impairment and memory dysfunction [5]. Because artificial induction of EphB2 expression in the dentate gyrus rescues LTP and memory function in a mouse model of AD [5], induction of EphB2 expression is a potential drug target for treatment of AD. However, EphB2 is also involved in the development of cancer [22,23] and may stimulate cancer progression. Thus, inhibiting the binding between A β oligomers and EphB2 is a better target for developing drugs aimed at AD treatment. Here, we found 22 compounds with such activity. Among them, 18 compounds also inhibited the binding of A β oligomers to PrP^C, whereas four compounds did not. Although we focused on the latter group for development of AD therapeutics, the former group is also interesting. In addition to EphB2, other receptors for A β oligomers, such as PrP^C [6,7,24], leukocyte immunoglobulin-like receptor B2 [25], and IgG Fc γ receptor II-b [26], are involved in the inhibitory effect on LTP and in neurotoxicity.

Therefore, compounds that inhibit the binding of A β oligomers to various receptors may have beneficial effects for treatment of patients with AD. Indeed, among the 18 compounds identified here, montelukast [27], minocycline [28,29], and bexarotene [30] reportedly have beneficial effects in animal models of AD.

The four compounds we identified that selectively inhibited the binding of A β oligomers to EphB2 (DIH, BRO, CEP, and LEV) are approved for use in humans as treatments for migraine, Parkinson's disease, and leucopenia, as well as for use as a contraceptive, respectively. Among these compounds, DIH, BRO, and CEP penetrate the blood–brain barrier [31–33]. Thus, our results suggest that these compounds, especially DIH, BRO, and CEP, may be safe and effective drugs for treatment of AD. On the other hand, these compounds required relatively high concentration to show their inhibitory effect on the binding of A β oligomers to EphB2. Therefore, we also consider the drug modification, using these approved medicines as leads.

As described above, the compounds identified in this study are expected to ameliorate A β oligomer-induced LTP impairment. EphB2 overexpression suppresses A β oligomer-induced neurotoxicity in cultured hippocampal neurons [34], and activation of EphB2 attenuates tau phosphorylation in a mouse model of AD [35]. Because both A β oligomer-induced neurotoxicity and

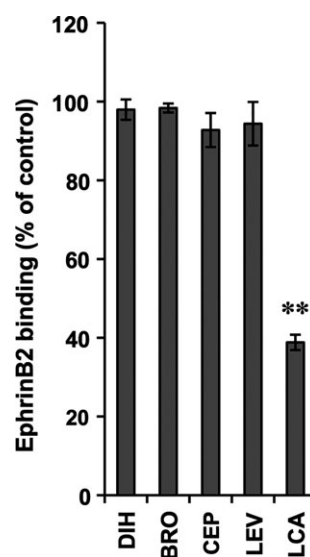


Fig. 3. Effects of selected compounds on the binding of ephrinB2 to EphB2. Each compound (100 μ M) was added simultaneously with biotinylated ephrinB2-Fc to EphB2-coated wells, and the amount of bound biotinylated ephrinB2-Fc was determined. Values represent the mean \pm SD ($n = 3$). ** $P < 0.01$. LCA, lithocholic acid. Basically similar results were obtained in another independent experiment.

tau phosphorylation are important factors in AD pathology, the four compounds identified here may also prove useful for AD treatment through these mechanisms.

In conclusion, we screened for compounds that selectively inhibit the binding of A β oligomers to EphB2 from a library of approved drugs already in clinical use, and identified dihydroergotamine mesilate, bromocriptine mesilate, cepharanthine, and levonorgestrel. We propose further analysis of these compounds as candidates for AD treatment.

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Author contribution

KS, TI, and TM conceived and designed the project; KS and TA acquired the data; KS analyzed and interpreted the data; and KS, TI, and TM wrote the paper.

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