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IN VIVO PHARMACOLOGICAL CHARACTERIZATION OF AC-3933, A BENZODIAZEPINE RECEPTOR PARTIAL INVERSE AGONIST FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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Abstract—GABAergic neurons are known to inhibit neural transduction and therefore negatively affect excitatory neural circuits in the brain. We have previously reported that 5-(3-methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-1,6-naphthyridin-2(1H)-one (AC-3933), a partial inverse agonist for the benzodiazepine receptor (BzR), reverses GABAergic inhibitory effect on cholinergic neurons, and thus enhances acetylcholine release from these neurons in rat hippocampal slices. In this study, we evaluated AC-3933 potential for the treatment of Alzheimer's disease, a disorder characterized by progressive decline mainly in cholinergic function. Oral administration of AC-3933 (0.01–0.03 mg/kg) resulted in the amelioration of scopolamine-induced amnesia, as well as a shift in electroencephalogram (EEG) relative power characteristic of pro-cognitive cholinergic activators, such as donepezil. In addition, treatment with AC-3933 even at the high dose of 100 mg/kg p.o. produced no seizure or anxiety, two major adverse effects of BzR inverse agonists developed in the past. These findings indicate that AC-3933 with its low risk for side effects may be useful in the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease, benzodiazepine receptor, cognitive function, GABAA receptor, inverse agonist.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly and constitutes one of the largest health economic burdens in developed countries (Hebert et al., 2013). AD is a debilitating neurodegenerative disorder characterized by progressive functional decline in learning and memory. The histological hallmarks of AD patient brain are the appearance of senile plaques and neurofibrillary tangles, which mainly consist of amyloid-β peptide and phosphorylated Tau protein, respectively (Hardy and Allsop, 1991; Kosik, 1991). At the cellular level, reduction in synaptic density and neuronal loss are frequently observed in the brain regions important for learning and memory, particularly the cortex and hippocampus. These brain regions receive cholinergic projections from the basal forebrain complex represented by the medial septum and nucleus basalis of Meynert, among others (Schliebs and Arendt, 2011). It is therefore widely recognized that cholinergic hypofunction forms a pathological basis for learning and memory deficits in AD patients (Whitehouse et al., 1981; Coyle et al., 1983). Moreover, it is hypothesized that cholinergic hypofunction leads to abnormal excess firing in the brain, which results in increased risk for seizure and worsened neuronal survival through excitotoxicity (Hörtnagl et al., 1991). Based on these observations, various compounds with the ability to increase acetylcholine (ACh) level at the synaptic clefts by inhibiting ACh degradation and therefore boosting cholinergic neuronal transmission have been clinically evaluated, and some are currently approved for the treatment of AD. However, the clinical potential for these drugs is somewhat limited, mainly due to their mode of action on ACh signal transmission. That is, they only prolong the ''lifetime'' of ACh released from synaptic terminals without actually affecting ACh release itself. Therefore, new drugs with different mechanisms of action have long been sought-after (Raina et al., 2008).

GABAergic neurons are known to inhibit neural transduction and therefore negatively affect excitatory neural circuits in the brain (Iversen and Bloom, 1972; Sivilotti and Nistri, 1991). In the adult brain, GABAergic neurons are widely distributed in the cortex and hippocampus, two regions of the brain severely affected in AD patients (Du et al., 2001). It is therefore believed that inverse regulation of GABA receptors can lead to activation of cholinergic circuits and amelioration of the learning and memory deficits observed in AD patients.

Benzodiazepines are prototypic molecules that bind and activate the $GABA_A$ receptor, which contains the

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Abbreviations: AC-3933, 5-(3-methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-1,6-naphthyridin-2(1H)-one; ACh, acetylcholine; AD, Alzheimer's disease; ANOVA, analysis of variance; β-CCM, beta-carboline-3-carboxylate; BzR, benzodiazepine receptor; EEG, electroencephalogram; FG-7142, beta carboline-3-carboxylic acid methyl amide; SEM, standard error of the mean.

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benzodiazepine-binding subunit, i.e. the benzodiazepine receptor (BzR). To date many molecules have been found to bind and modulate BzR. We have previously reported that 5-(3-methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-1,6-naphthyridin-2(1H)-one (AC-3933), a novel BzR partial inverse agonist, enhances ACh release in response to high [K^+]-induced excitation in rat hippocampal slices. *In vivo* microdialysis revealed that AC-3933, given orally at 10 mg/kg, increases rats hippocampal ACh level (Hashimoto et al., 2014). In this study, we evaluated AC-3933 potential for treatment of AD, a disorder characterized by progressive decline in cholinergic function.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (260–300 g) and male ddY mice (16–18 g) were purchased from Japan SLC Inc. (Shizuoka, Japan) and used after an acclimation period of 5–12 days. The animals were housed in plastic cages kept in a temperature (23 ± 3 °C) and humidity (55 ± 15%) controlled room under a 12-h light–dark cycle and given food and water *ad libitum*. All experimental procedures used were approved by the Institutional Animal Care and Use Committee of Dainippon Sumitomo Pharma Co., Ltd.

Chemical reagents

AC-3933, donepezil hydrochloride, and beta carboline-3-carboxylic acid methyl amide (FG-7142) were synthesized in our laboratories. Scopolamine hydrobromide, flumazenil and flunitrazepam were obtained from Sigma–Aldrich Japan (Tokyo, Japan), and methyl beta-carboline-3-carboxylate (β-CCM) was obtained from Research Biochemicals International (Natick, MA, USA). [^{3}H] Ro15-1788 was purchased from Perkin Elmer Japan (Kanagawa, Japan).

For oral administration, AC-3933, donepezil, and FG-7142 were suspended in 0.5% tragacanth gum solution. For intravenous administration of AC-3933 and β-CCM, compounds were dissolved in 0.2 N-HCl at a concentration of 5 mg/ml and then diluted with saline, and animals in the vehicle-treated group were given 0.08 N-HCl saline instead of test-drugs. [^{3}H] Ro15-1788 was diluted in saline and administered by intravenous injection. Scopolamine was dissolved in saline and administered by subcutaneous injection. Flumazenil or flunitrazepam were dissolved in 0.4% Tween80 in saline or saline, respectively, and administered intraperitoneally.

Assessment of the effects of AC-3933 in amnesia rat and mouse model

To evaluate the effects of AC-3933 on learning and memory, we used the Y-maze test in rats and the novel object recognition test in mice.

The Y-maze test in rats is based on reduced cholinergic transmission induced by scopolamine, a muscarinic ACh receptor blocker, and thus faithfully mimics cholinergic hypofunction observed in the brains of AD patients. Rats spatial working memory performance was assessed by recording spontaneous alternation behavior in the Y-maze test, essentially as described by Itoh et al. (1993). Briefly, AC-3933, donepezil or the vehicle was orally administrated to rats 90 min before the test, followed by subcutaneous injection of scopolamine 30 min before the test. Where indicated, flumazenil was also administered 30 min before the test. During the test session, each rat was placed at the end of an arm of the maze and allowed to move freely for 8 min. The sequence of arm entries was recorded, and alternation behavior was defined as entry into all three arms on consecutive occasions. Percent alternation behavior for each rat was calculated by the following formula: {actual alternation/(total arm entries − 2)} × 100. Data are expressed as the mean ± standard error of the mean (SEM).

In the novel object recognition test, mice were used to evaluate cognition, particularly recognition memory. This test consists of two trials (T1 and T2) separated by the inter trial interval of 5 h. Mice were orally administered vehicle or AC-3933 60 min before T1, and allowed to explore two identical objects for 5 min. In T2, one of the two familiar objects was replaced by a novel object, and the time spent in exploring the familiar object and the novel object was recorded for 5 min. Exploration of an object was defined as sniffing and/or touching with the nose. Discrimination index for each mouse was calculated by the following formula: Discrimination index = (Exploration time for novel object − Exploration time for familiar object)/(Exploration time for novel object + Exploration time for familiar object).

Evaluation of the effects of AC-3933 on cortical electroencephalogram (EEG) recording in rats

As BzR inverse agonists are known to affect cortical EEG (Ongini et al., 1983; Massotti, 1985), we investigated the effects of AC-3933 on cortical EEG recording in rats and compared them to those of donepezil, an acetylocholinesterase inhibitor. The screw electrodes were supradurally implanted under anesthesia with sodium pentobarbital (50 mg/kg, i.p.) over the right frontal cortex (AP, +3.5 mm; ML, +3.0 mm), ipsilateral occipital cortex (AP, −8.0 mm; ML, +4.0 mm), and midline over the cerebellum as a ground electrode according to the Stereotaxic Coordinates of Paxinos and Watson (1982). After surgery, the rats were allowed two weeks for recovery before being used for EEG recording. EEG signals were transmitted through a flexible cable to a mercury slip-ring system connected to a bioelectric amplifier unit (1253A, NEC San-ei Instruments, Ltd., Tokyo, Japan) for amplification at a time constant of 0.1 s and a low pass filter setting of 100 Hz, followed by A/D conversion (ADM-1698BPC, Micro Science, Tokyo, Japan). EEG recordings were performed for 6 h each day on two consecutive days. On the first day, animals were administered the vehicle. On the second day, they were administered AC-3933 or donepezil 1 h after the beginning of the session. Two softwares, Turbo Pascal for human interface and BollandC++ with a Turbo Assembler, were used for