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The Therapeutic Effects of Dual Orexin Receptor Antagonists on Amyloid-beta Protein-induced Cytotoxicity

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Abstract

Alzheimer's disease（AD）is the most common neurodegenerative disease of the central nervous system and is characterized by histopathological features that include amyloid β protein（Aβ）oligomerization and neurofibrillary tangles in the brain, however the cause of AD is still unknown. Studies using mice have suggested a possible for relationship between AD and sleep, specifically between orexin, a neuropeptide that regulates wakefulness, and sleep deprivation. It has been shown that brain interstitial fluid Aβ levels are significantly increased during acute sleep deprivation and orexin intracerebroventricular（icv）infusion, whereas they are decreased by administration of almorexant, a dual orexin receptor antagonist. Almorexant is considered as the therapeutic candidate for AD, however, it has not been used in clinical applications because of the toxicity. In this study, we attempted to elucidate the effects of suvorexant which has a similar mechanism in safely by assessing Aβ-induced cytotoxicity using u-138 cells. Pretreatment with 1 nM suvorexant increased the cell viability of u-138 cells treated with $A\beta_{1-42}$ compared with negative control group, although not significantly（87.2%±2.1% vs. 77.1%±11.2%; $p > 0.05$）. This suggests that suvorexant may has a direct protective effect against Aβ-induced cytotoxicity. Further assessments will be recommended.

Key words : Alzheimer's disease, Amyloid β protein（Aβ）-induced toxicity, Amyloid cascade hypothesis, Orexin, Dual orexin receptor antagonist（DORA）, Suvorexant

Introduction

Dementia is a general term for the loss of memory and other intellectual abilities serious enough to interfere with daily life. Alzheimer's disease（AD）accounts for approximately 50% of dementia cases and is the most common neurodegenerative disease of the central nervous system. According to the World Alzheimer's Disease Report 2015, published by Alzheimer's Disease International, the global population of people living with AD was about 46.8 million in 2015, but will be expected to increase to 75 million by 2030 and 131.50 million by 2050. AD is a serious disease that causes great physical and psychological distress not only to the patients but also to their relatives and caregivers. Therefore, developing a cure for AD is a very important issue.

The primary symptom of AD is a progressive decline in the cognitive function, such as memory and orientation. The main histopathological features of AD are amyloid β protein（Aβ）oligomerization and neurofibrillary tangles in the brain. An initiating factor in AD

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pathogenesis occurs when soluble, monomeric Aβ undergoes a conformational change and converts to forms such as oligomers, protofibrils, and fibrils. The accumulation of these forms of Aβ is concentration-dependent and confers toxicity[1].

Although the cause of AD remains unknown, there are several hypotheses[2)-5)]. The current mainstream opinion supports the amyloid cascade hypothesis[4)5)]. Based on this hypothesis, human amyloid precursor protein (APP) transgenic animals expressing APP and Aβ-doped cells are currently being used in experiments. Aβ, a peptide with 39 to 42 amino acids, is derived from APP undergoing sequential proteolytic processing by β- and γ-secretase. The addition of Aβ to cultured cells has been shown to be cytotoxic, and the causes of this cytotoxicity have been reported to be oxidative stress, mitochondrial dysfunction, and apoptosis[6].

A number of studies indicated the correlation between sleep and AD pathology. For example, Kang et al. showed that brain interstitial fluid (ISF) Aβ levels increased during wakefulness and decreased during sleep in APP transgenic mice[7]. They also found that the ISF Aβ levels were significantly increased during acute sleep deprivation and during orexin intracerebroventricular (icv) infusion, conversely decreasing by the icv administration of almorexant, a dual orexin receptor antagonist (DORA)[7]. DORAs are sleep-inducing agents that suppress arousal by inhibiting the binding of orexin, a wake-promoting neuropeptide, to its receptors[8]. Liguori et al. also reported that patients with moderate to severe AD had significantly higher levels of orexin in their cerebrospinal fluid (CSF) than controls[9]. Furthermore, Dietrich et al. elucidated that the oral administration of almorexant improved learning and memory functions in rat model[10]. While the usefulness is certified, almorexant is not used in clinical because of side effects including severe somnolence, fatigue, headache, and nausea[11]. Instead, suvorexant, a sleep-inducing drug harboring a similar medical efficacy with almorexant, is focused on as the new candidate for treating AD, however, the effects of this drug on AD-induced cognitive impairment and Aβ-induced cytotoxicity are still unknown.

In this study, we attempted to clarify the therapeutic effect of a DORA against Aβ-induced cytotoxicity using a neural cell line.

Materials and Methods

1. Drugs

Suvorexant was acquired from Toronto Research Chemicals Inc (Toronto, Canada), and almorexant was acquired from Cayman Chemical Company (Ann Arbor, MI, USA). The drugs were dissolved in dimethyl sulfoxide (DMSO) (HAYASHI PURE CHEMICAL IND. LTD., Osaka, Japan) and diluted with phosphate-buffered saline (PBS) and F-12 medium (Wako, Osaka, Japan) to the final concentration (FC ; 1 nM).

2. Preparation of Aβ

We used $A\beta_{1-42}$ (trifluoroacetate form, Peptide Institute Inc ; Osaka, Japan). The $A\beta_{1-42}$ solution was prepared according to the method reported by Chromy et al.[12]. In brief, an Aβ-Derived Diffusible Ligand (ADDL) was prepared from solid Aβ peptide according to the method of Lambert et al.[13]. It was dissolved in DMSO to obtain a 2 mM stock solution that was further diluted to 20 μM in PBS, and incubated at 4℃ for 24 h. After incubation, the solution was centrifuged at 14,000 g for 10 minutes at 4-8 ℃. Because the soluble oligomers are contained in the supernatant, the supernatant was frozen and stored as a 20 μM reaction mixture. The FC in the medium at the time of use was 2 μM.

3. Neural cell line

We used u-138cells (ATCC® HTB-16™ ; American Type Culture Collection), which are human neuronal glioblastoma cells. They were maintained in F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin under a humidified 95% air and 5% CO_2 at 37℃.

4. Treatment of Aβ and DORA

Cultured cells were counted using a hemocytometer. Successfully cultured cells were seeded into 24-well plates at 3.0×10^4 cells per well for the MTT assay and seeded overnight prior to subsequent treatment. First, to study the cytotoxicity of the drugs, u-138 cells treated with suvorexant or almorexant or ddH_2O (negative control) for 72 h were subjected to MTT assays. The drug concentration was 1.25 nM for the first 24 h and 1 nM for the next 48 h after the addition of the medium.

Next, to determine the neuroprotective effects of drugs, u−138 cells were pretreated with suvorexant, almorexant or ddH_2O for 24 h, before the addition of 2 μM $A\beta_{1-42}$ for 48 h prior to the MTT assays. The drug concentration was 1.25 nM for the first 24 h and 1 nM for the next 48 h after the addition of $A\beta_{1-42}$ and the medium（Fig. 1）.

5. The cell viability assay

For the MTT assay, u−138 cells were first treated with 5 mg/ml MTT for 4 h at 37℃. After removing the media, 100 μL DMSO（≥99%）was added to dissolve the formazan crystals formed. The absorbance was measured at 570 nm using a microplate reader（BIO−RAD iMark Microplate Reader；Bio−Rad Laboratories, CA, USA）[14]. Controls consisted of cells treated with 2 μM $A\beta_{1-42}$ after pretreatment with ddH_2O（negative control, $A\beta+/ddH_2O$）or almorexant（positive control, $A\beta+/$Alm）, and cells untreated with $A\beta_{1-42}$ after pretreatment with ddH_2O（normal negative control, $A\beta-/ddH_2O$）. The cell viability was described and compared as a relative value when the value of $A\beta-/ddH_2O$ was set at 100%. The experiment was repeated three times with three independent samples.

6. Statistical analyses

Results are presented as the mean±standard deviation. Statistical analyses were conducted using an analysis of variance（ANOVA）and the Tukey−Kramer HSD test, with $P<0.05$ considered statistically significant.

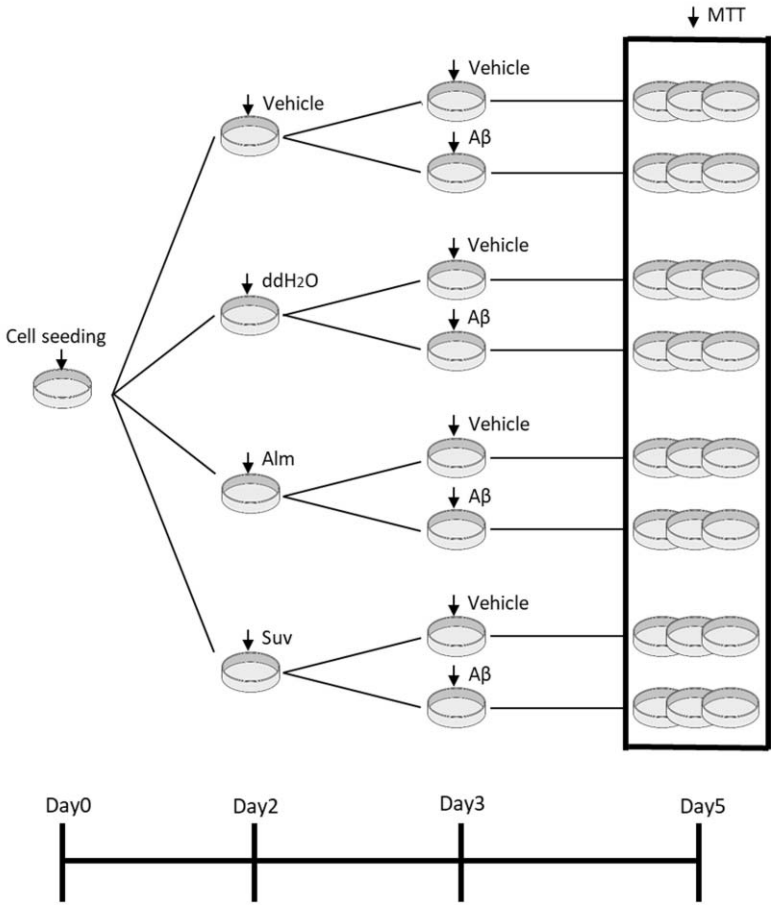

Figure 1. The experimental procedure, from cell seeding to the MTT assay. In MTT assay, we prepared 3 wells for each of the 8 conditions in a single experiment and calculated the average for each condition. Furthermore, the experiment was repeated three times on different days, and the averages of the three experiments were calculated and compared. In short, nine wells were prepared for one condition. Alm：almorexant, Suv：suvorexant.

Results

1. The administration of DORA did not impair cellular viability

Our assessments about cellular viability revealed that administration of almorexant or suvorexant did not affect the cellular viability of u-138 cells (107.6%±5.4%：almorexant；109.4%±3.6%：suvorexant；$p>0.05$ comparing with negative controls).

2. The administration of DORA prevented the cytotoxic effects of Aβ

Treatment with $A\beta_{1-42}$ significantly reduced the viability of u-138 cells pretreated with ddH_2O comparing with the Aβ-untreated group (77.1%±11.2% vs. 100%；$p<0.05$). In contrast, pretreatment with almorexant significantly improved the cell viability of u-138 cells treated with $A\beta_{1-42}$ (92.4%±4.4%；$p<0.05$). On the other hand, pretreatment with suvorexant also increased the cell viability of u-138 cells treated with $A\beta_{1-42}$, while there was no significant difference comparing with negative control group (87.2%±2.1%；$p>0.05$) (Fig. 3).

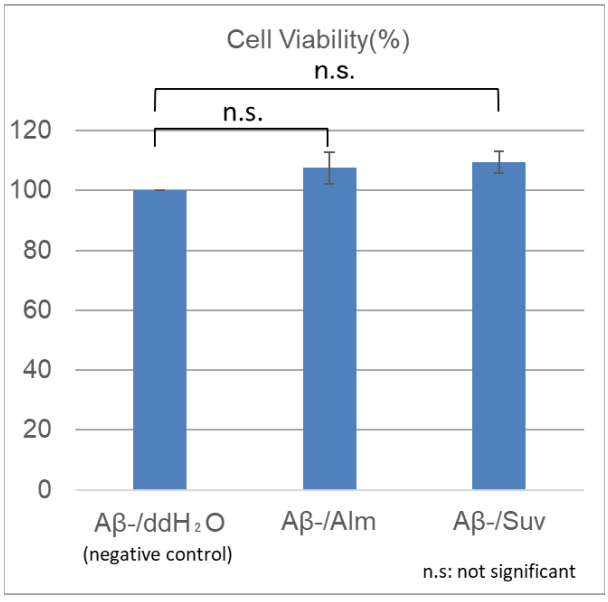

Figure 2. Cytotoxicity of almorexant and suvorexant in u-138 cells. Aβ- indicates cells were not treated with Aβ after pretreatment with the indicated drug of ddH_2O, almorexant (Alm) or suvorexant (Suv). No drug-induced cytotoxicity was observed. N=9, data shown as mean±SD.

Discussion

DORAs are sleep-inducing agents that suppress arousal by inhibiting the binding of orexin, a wake promoting neuropeptide, to its receptors. Suvorexant is one of the DORAs with a molecular weight of 450.92 and a molecular formula of $C_{23}H_{23}ClN_6O_2$. Suvorexant suppresses wakefulness by inhibiting the binding of orexin A, which is the wake promoting neuropeptide, to the orexin 1 and 2 receptors (OX1R and OX2R, respectively), and the binding of orexin B to the OX2R[8]. Both receptors involve with suppression of REM sleep, and OX2R mainly contributes to the stabilization of arousal by orexin[15)16)].

DORA has also been reported to have effects on the AD pathology, particularly Aβ. For example, studies using rodents revealed that DORA treatment reduced Aβ pathology[7)] and improved the cognitive function[9)]. Furthermore, other group elucidated that reduction of Aβ was observed in an orexin knockout mouse model[17)]. It is considered that the mechanism of these effects composes of both a direct effect of inhibition of orexin[9)] and an indirect effect of increased sleep time caused by reduced orexin receptor activity[17)].

Our experiments revealed that administration of 1 nM almorexant or suvorexant did not impair the viability of u-138 cells. This result suggests that neither drug is cytotoxic at the concentration. We also elucidated that

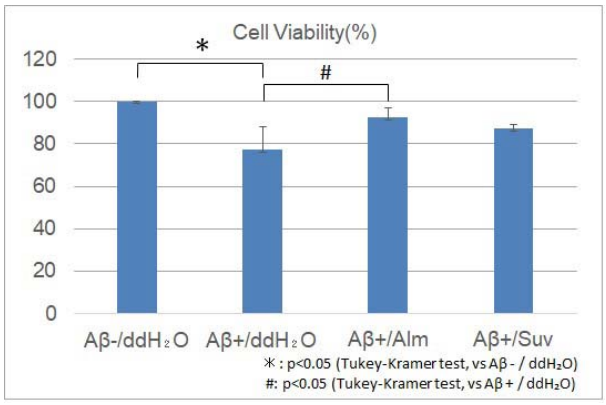

Figure 3. Cytoprotective effects of almorexant and suvorexant on u-138 cells treated with $A\beta_{1-42}$. Aβ-/ddH_2O indicates pretreatment with ddH_2O followed by no Aβ treatment, Aβ+/ddH_2O indicates pretreatment with ddH_2O followed by Aβ treatment, Aβ+/Alm indicates pretreatment with almorexant followed by Aβ treatment, and Aβ+/Suv indicates pretreatment with suvorexant followed by Aβ treatment. N=9, data shown as mean±SD.

treatment with 2 μM Aβ_{1-42} significantly reduced the viability of u−138 cells compared with the untreated group, suggesting that Aβ has significant cytotoxicity, indicated by previous studies[6]. Furthermore, we successfully showed that pretreatment of 1 nM almorexant significantly increased the cell viability of u−138 cells treated with Aβ_{1-42}. Pretreatment of 1 nM suvorexant also increased the cell viability of u−138 cells treated with Aβ_{1-42}, but could not show the significant difference. Almorexant is more effective in improving cell viability, however, we consider suvorexant may possibly exhibit a certain degree of effect. We estimate the reason why the therapeutic effect of suvorexant was mild depends on the method of administration. One possibility is to use by single administration. It may be better to use by repeated administration in future clinical trial.

The problem of DORA in increased cytotoxicity due to elevated blood levels of the drug should be also overcome. Regarding suvorexant, the cytotoxicity is considered to be very low at biologically active concentrations, and is already used as a sleep inducer in clinical. In this point of view, we consider that suvorexant will be the suitable choice for treating AD.

Our results suggest that both drugs have a direct protective effect on Aβ induced toxicity *in vitro*. Of note, these findings also suggest the possibility that the cell activity, i.e. the resistance of u−138 cells to Aβ is increased by treatment with both drugs. However, in our examination, neither drug significantly changed the viability of u−138 cells compared with the untreated group. Therefore, we consider both drugs to also have a direct protective effect on Aβ protein−induced toxicity *in vitro*.

We also confirmed that almorexant has a direct protective effect on Aβ−induced toxicity *in vitro*. This is consistent with previous studies that confirmed the anti−Aβ and cognitive−improving effects of almorexant in animal studies[7)9)]. Therefore, we feel that our cell biology experiments have provided *in vitro* evidence supporting these two previously reported findings. Furthermore, the results of our experiments may answer the question of whether the effects of DORAs on Aβ are direct or indirect. The observation that DODA reduced Aβ toxicity in cell cultures was independent of sleep. The direct effects of DORA by inhibiting orexin receptors may therefor also help improve the cognitive function in rodents. However, this does not exclude the possibility that the improved cognitive function in rodents may be an indirect effect of an increased sleep duration.

The protective effects of DORAs on the cytotoxicity of Aβ were observed in the present study, suggesting that detoxifying effects on the neurotoxicity of Aβ can be expected. The most likely mechanism underlying the protective effect of DORA on the cytotoxicity of Aβ is the orexin receptor antagonism shared by both, but the details of this effect remain unclear. In addition, these views are based to solely on the amyloid cascade hypothesis. Therefore, if we assume that Aβ aggregation is a consequence rather than a cause of AD, even if DORAs reduce Aβ−induced toxicity *in vitro*, it would not necessarily improve the cognitive function *in vivo*. We must thus determine whether or not suvorexant reduces Aβ−induced toxicity and ISF Aβ levels *in vivo* similarly to almorexant and whether or not it improves the cognitive function in mice and humans.

Regarding limitation, this study was an *in vitro* cell biology experiment and did not directly investigate the effect of suvorexant on improving the cognitive function in AD patients or preventing AD. Therefore, for clinical applications, we need to confirm the effects on the actual cognitive function and safety through *in vivo* animal experiments and clinical trials on humans. Furthermore, these conclusions are based on the amyloid cascade hypothesis that Aβ aggregation and tau protein hyperphosphorylation are the main causes of AD. However, many substances have been found to have no effect on improving the cognitive function in clinical trials, despite reducing the Aβ production and toxicity *in vitro* or *in vivo*. This presents us with the fundamental question of whether Aβ aggregation and tau protein hyperphosphorylation are truly the cause of AD or merely phenomena that result from AD caused by other mechanisms. Therefore, the amyloid cascade hypothesis should be further tested. Furthermore, the protective effect of these two DORAs on Aβ observed in the present experiment was assumed to be due to their effects on orexin signaling, but the molecular mechanism underlying this effect remains unclear. Further research is thus needed to confirm this point.

Conclusion

In this study, we confirmed that both almorexant and suvorexant might have a protective effect on Aβ−induced toxicity. The results of this study suggest that

suvorexant may be a safe and effective treatment or prophylactic agent for AD. Further studies for elucidating the mechanism about cytoprotective effect of DORA will be needed.

Conflicts of interest

There are no conflicts of interest in this study.

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References

1) Selkoe DJ：Cell biology of protein misfolding：the examples of Alzheimer's and Parkinson's diseases. Nat Cell Biol 6：1104-1054, 2004.
2) Khan MB, Khan MM, Khan A, Ahmed ME, Ishrat T, Tabassum R, Vaibhav K, Ahmad A, Islam F：Naringenin ameliorates Alzheimer's disease (AD)-type neurodegeneration with cognitive impairment (AD-TNDCI) caused by the intracerebroventricular-streptozotocin in rat model. Neurochem Int 61：1081-1093, 2012.
3) Swerdlow RH：Pathogenesis of Alzheimer's disease. Clin Interv Aging 2：347-359, 2007.
4) Behl C, Davis JB, Lesley R, Schubert D：Hydrogen peroxide mediates amyloid β protein toxicity. Cell 77：817-827, 1994.
5) Liang H, Zhang Y, Shi X, Wei T, Lou J：Role of Notch-1 signaling pathway in PC12 cell apoptosis induced by amyloid beta-peptide (25-35). Neural Regener Res 9：1297-1302, 2014.
6) Zhao J, Zhang G, Li M, Luo Q, Leng Y, Liu X：Neuro-protective effects of aloperine in an Alzheimer's disease cellular model. Biomed Pharmacother 108：137-143, 2018.
7) Kang JE, Lim MM, Bateman RJ, Lee JJ, Smyth LP, Cirrito JR, Fujiki N, Nishino S, Holtzman DM：Amyloid-β Dynamics Are Regulated by Orexin and the Sleep-Wake Cycle. Science 326：1005-1007, 2009.
8) Brisbare-Roch C, Dingemanse J, Koberstein R, Hoever P, Aissaoui H, Flores S, Mueller C, Nayler O, van Gerven J, de Haas SL, Hess P, Qiu C, Buchmann S, Scherz M, Weller T, Fischli W, Clozel M, Jenck F：Promotion of sleep by targeting the orexin system in rats, dogs and humans. Nat Med 13：150-155, 2007.
9) Liguori C, Romigi A, Nuccetelli M, Zannino S, Sancesario G, Martorana A, Albanese M, Mercuri NB, Izzi F, Bernardini S, Nitti A, Sancesario GM, Sica F, Marciani MG, Placidi F：Orexinergic System Dysregulation, Sleep Impairment, and Cognitive Decline in Alzheimer Disease. JAMA Neurol 71：1498-1505, 2014.
10) Dietrich H, Jenck F：Intact learning and memory in rats following treatment with the dual orexin receptor antagonist almorexant. Psychopharmacology (Berl) 212：145-154, 2010.
11) Hoever P, Hay J, Rad M, Cavallaro M, van Gerven JM, Dingemanse J：Tolerability, pharmacokinetics, and pharmacodynamics of single-dose almorexant, an orexin receptor antagonist, in healthy elderly subjects. J Clin Psychopharmacol 33：363-370, 2013.
12) Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P, Finch CE, Krafft GA, Klein WL：Self-assembly of Abeta (1-42) into globular neurotoxins. Biochemistry 42：12749-12760, 2003.
13) Lambert MP, Viola KL, Chromy BA, Chang L, Morgan TE, Yu J, Venton DL, Krafft GA, Finch CE, Klein WL：Vaccination with soluble Abeta oligomers generates toxicity-neutralizing antibodies. J Neurochem 79：595-605, 2001.
14) Shadab M, Sook YG, Yong HH, Chee LH, Wong S, Choudhury H：In vitro neuroprotective effects of naringenin nanoemulsion against β-amyloid toxicity through the regulation of amyloidogenesis and tau phosphorylation. Int J Biol Macromol 118：1211-1219, 2018.
15) Sakurai T：The neural circuit of orexin (hypocretin)：maintaining sleep and wakefulness. Nat Rev Neurosci 8：171-181, 2007.
16) Mieda M, Hasegawa E, Kisanuki YY, Sinton CM, Yanagisawa M, Sakurai T：Differential roles of orexin receptor-1 and -2 in the regulation of non-REM and REM sleep. J Neurosci 31：6518-6526, 2011.

17）Roh JH, Jiang H, Finn MB, Stewart FR, Mahan TE, Cirrito JR, Heda A, Snider BJ, Li M, Yanagisawa M, de Lecea L, Holtzman DM：Potential role of orexin and sleep modulation in the pathogenesis of Alzheimer's disease. J Exp Med 211：2487–2496, 2014.

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「The authors declare no conflict of interest.」