Full Paper

Antiepileptic Effects of Single and Repeated Oral Administrations of S-312-d, a Novel Calcium Channel Antagonist, on Tonic Convulsions in Spontaneously Epileptic Rats

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Abstract. We investigated the effects of single and repeated administrations of S-312-d (methyl-4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl)-thieno-[2,3-*b*]pyridine-5-carboxylate), a newly synthesized L-type Ca²⁺-channel blocker, on tonic convulsions and absence-like seizures in the spontaneously epileptic rat (SER: zi/zi, tm/tm), a genetically based animal model of human epilepsy. Single oral administrations of S-312-d dose-dependently inhibited tonic convulsions and the effects lasted for more than 2 h, although they did not attenuate the absence-like seizures. We also examined the effects of repeated administrations of S-312-d at 1 mg/kg once a day for 4 days on SER. A significant decrease in the number and total duration of tonic convulsions was observed 45 and 75 min after the first administration of the drug, respectively. The effects lasted for 24 h without changes in the background EEG or blood pressure. This inhibitory effect on the tonic convulsions was gradually strengthened by subsequent daily administrations of S-312-d and lasted for 3 days after the cessation of drug treatment. In contrast, the repeated treatment with S-312-d did not influence absence-like seizures of SER. These results suggest that S-312-d is a candidate drug that has antiepileptic effects against the convulsive seizures in human epilepsy.

Keywords: epilepsy, Ca²⁺-channel antagonist, spontaneously epileptic rat (SER), anticonvulsant

Introduction

The spontaneously epileptic rat (SER) is a double mutant (zi/zi, tm/tm) that exhibits both tonic convulsions and absence-like seizures. These convulsions and seizures are characterized by the appearance of low-voltage fast activity and 5 – 7-Hz spike-wave complexes in cortical and hippocampal EEGs, respectively. These abnormal EEG changes were observed simultaneously with the epileptic behavior after the age of 9 weeks (1, 2). A variety of conventional antiepileptic drugs (AEDs) used for the treatment of human epilepsy improve the absence-like and tonic seizures in SER

(1, 2), suggesting that SER is a useful animal model for evaluation of the short- and long-term effects of AEDs.

Abnormal calcium channel activities are considered to be involved in the seizures of SER, since a long-lasting depolarization shift with repetitive firings was induced by a single stimulus given to the mossy fibers of hippocampal CA3 pyramidal cells. This abnormal excitation of neurons was completely blocked by Ca²⁺-channel antagonists such as verapamil (3, 4), raising the possibility that such antagonists would be effective for the inhibition of seizures in the SER.

S-312-d (methyl-4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl)-thieno-[2,3-*b*]pyridine-5-carboxylate), a novel dihydrothienopyridine derivative, is a potent L-type Ca²⁺-channel blocker (5). Unlike other dihydropyri-

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dine derivative Ca^{2+} -channel antagonists, S-312-d is known to cross the blood brain barrier and to concentrate in the brain (6). In addition, S-312-d showed anti-convulsant effects on audiogenic tonic convulsions and pentylenetetrazole- or bemegride-induced clonic convulsions (7). In this study, we have examined whether S-312-d can be used as an antiepileptic, using SER as an animal model of epilepsy.

Materials and Methods

Experimental animals

All experiments were carried out according to the guidelines laid down by the animal welfare committees of the Kyoto and Hiroshima Universities. SERs were bred at the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University and kept individually in shoebox-type cages. Commercial food pellets (MF; Oriental Yeast Co., Ltd., Tokyo) and drinking water were given ad libitum. Room temperature and relative humidity were kept at $23 \pm 2^\circ\text{C}$ and 50–60%, respectively.

EEG recordings and the experimental paradigm

Six male and 7 female SERs were used for the single dose experiment and 3 male and 3 female SERs were used for the repeated dose experiment. SERs aged 9 weeks were used in this study. Under sodium pentobarbital anesthesia (30 mg/kg, i.p.), EEG electrodes, a silver ball-tipped electrode and an enamel-coated stainless-steel electrode, were chronically implanted into the left frontal cortex and left hippocampus as previously described (1, 2). An indifferent electrode was placed on the frontal cranium. Animals were allowed to recover in the cages for at least 7 days after the implantation of the electrodes. Then, each animal was placed in a sound-attenuated box ($40 \times 40 \times 40$ cm) with a small window for behavioral observation. EEGs were recorded with a pen writing polygraph (RM-6200; Nihon Kohden, Tokyo) and stored on a diskette through MacLab/8 (AD Instruments, Castle Hill, Australia). The frequency of EEGs was analyzed using a trend monitor (OEE-7102, Nihon Kohden). Our previous study demonstrated that tonic convulsions and absence-like seizures were characterized by the appearance of low-voltage fast activity and 5–7-Hz spike-wave-like complexes in EEGs, respectively (2). We also revealed that the changes in the EEG during absence-like or tonic seizures corresponded to the respective abnormal epileptic behavior (2). Therefore, the frequency and duration of tonic convulsions were evaluated from those of the low-voltage fast activity on the EEG. Also, the frequency and duration of absence-like seizures was

evaluated from those of 5–7-Hz spike-wave-like complexes. When a 5–7-Hz spike-wave-like complex lasted for over 1 s, it was regarded as an absence-like seizure. When the time interval between two spike-wave-like complexes was less than 1 s, the complex was regarded as a single seizure. A blowing stimulus was applied to back of each animal every 5 min to induce an aroused state and a consistent tonic seizure.

For the EEG recording and drug application, animals were transferred into the recording box at 9:00 a.m. After a 30-min habituation, a control cortical and hippocampal EEG was recorded for 30 min (9:30–10:00 a.m.). For the single dose experiment, S-312-d at a dose of 1, 5, or 10 mg/kg was orally given to the animal at 10:00–10:15 a.m. and the EEG was continuously recorded for 120 min. We allowed a one-week washout period at least when a second dose was to be given to the same animal. For the repeated dose experiment, S-312-d was orally administered into each animal once a day at 10:00–10:15 a.m. for 4 days. On the first day, EEGs were recorded 4 times per day (10:30–12:00, 13:00–13:30, 15:00–15:30, and 18:00–18:30) after the administration. On the second and third days, EEGs were recorded twice a day, before and after the drug administration (9:30–10:00 and 10:30–12:00 a.m., respectively). On the fourth day, the recording paradigm was the same as that on the first day. After the final administration of the drug, EEGs were additionally recorded twice a day (9:30–10:00 and 17:00–17:30) for 5 days.

Data analyses

Data represent the mean $\pm$ S.E.M. of the number (times) and duration (s) of tonic convulsions or absence-like seizures during the 30-min observation period. The statistical significance of the difference between the values before and after the drug administration was determined using Student's paired *t*-test. If the *P* value was less than 0.05, results were considered to be significant.

Drug

S-312-d (methyl-4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl)-thieno-[2,3-*b*]pyridine-5-carboxylate) was dissolved in 0.5 ml polyethylene glycol/100 g body weight and administered orally to the animal at a dose of 1–10 mg/kg (Fig. 1).

Results

Effects of a single dose of S-312-d on tonic convulsions

Before the drug administration, the animals showed tonic convulsions 3–6 times with a total duration of

Fig. 1. Structural formula of S-312-d.

38–156 s during the 30-min observation period. The tonic convulsions were dose-dependently inhibited by the oral administration of S-312-d at 1, 5, and 10 mg/kg (Figs. 2 and 3). This inhibitory effect was also seen during the final 30-min observation period (90–120 min after the administration) (Fig. 3). During this period, the number of tonic convulsions (times) in animals treated with 1, 5, and 10 mg/kg of S-312-5 ($n = 8, 5$, and 5 , respectively) was significantly reduced to 2.3 ± 0.8 , 1.6 ± 0.5 , and 0.8 ± 0.8 from 4.8 ± 0.3 , 5.4 ± 0.2 , and 5.0 ± 0.4 , the values before the drug administration, respectively ($P < 0.05$, Fig. 3A). Similarly, the total duration of the convulsion seizure in SERs treated with 10 mg/kg of S-312-d was significantly decreased (Fig. 3B). During the last 30-min period, a complete blockade of seizures was seen in 2 of 11, 1 of 5, and 5 of 7 animals treated with 1, 5, and 10 mg/kg of S-312-d, respectively.

Effects of a single dose of S-312-d on absence-like seizures

In contrast to the tonic convulsions, the number and duration of absence-like seizures were not significantly affected by S-312-d at 1 mg/kg (Fig. 4: A and B). The higher doses of S-312-d (5 and 10 mg/kg) tended to prolong the total duration of the absence-like seizures, although these effects were not dose-dependent or significant (Fig. 4: A and B).

Effects of repeated administration of S-312-d on tonic convulsions

Before the drug treatment, the mean number and total duration of tonic convulsions during the 30-min observation periods were 4.6 ± 0.5 times ($n = 5$) and 155.8 ± 17.5 s ($n = 5$), respectively (Fig. 5). After 1 mg/kg of S-312-d was administered orally, inhibitory effects of the drug on the tonic convulsions appeared within the first day. S-312-d significantly decreased the number and total duration of tonic convulsions

during the first (15–45 min after the administration) and second (45–75 min after the administration) observation periods, respectively. During the period 90–120 min after the administration, the number and total duration of tonic convulsions were significantly ($P < 0.05$) decreased to 0.6 ± 0.2 times and 45.0 ± 21.4 s from the control levels as described above, respectively. A significant S-312-d-induced inhibition of tonic convulsions was still observed 24 h after the administration. The tonic convulsions were further inhibited by subsequent and additional administrations of S-312-d on the second, third, and fourth days. The maximum inhibitory effect was observed 30–60 min after the administration on the fourth day. During the 30-min observation period, the number and total duration of convulsion was reduced to 0.4 ± 0.2 times and 10.2 ± 10.2 s, respectively. The inhibitory effects of repeated administration of S-312-d were still detected 3 days after the final application on the fourth day (Fig. 5) and disappeared 4–6 days after the cessation of the drug treatment (data not shown).

Effects of repeated administration of S-312-d on absence-like seizures

Before the drug administration, the mean number (times) and total duration (s) of absence-like seizures during the 30-min observation periods were 20.0 ± 8.5 ($n = 4$) and 59.6 ± 33.3 ($n = 6$), respectively (Fig. 6). In contrast to the tonic convulsions, the absence-like seizures were not significantly affected by S-312-d (Fig. 6). The background EEG was not changed even after the fourth administration of S-312-d (data not shown).

Discussion

Our previous electrophysiological studies on SER have demonstrated that the application of a single stimulus to the hippocampal mossy fibers readily induces a long-lasting depolarization shift concomitantly with repetitive firing in the CA3 pyramidal cells. This abnormal excitability of SER neurons became prominent with aging, in parallel with the frequent occurrence of epileptic seizures, and was completely blocked by the Ca^{2+} -channel antagonist verapamil (3, 4). Our patch clamp study using acutely dissociated hippocampal cells has also shown that the threshold for opening the L-type Ca^{2+} channels was lower in SERs than control littermates (zi/zi , $+/-$) (8). These findings raise the possibility that the abnormal functioning of Ca^{2+} channels, especially L-type Ca^{2+} channels, causes the epileptic seizure of SER. Regarding the involvement of such an abnormality in epilepsy, epileptic seizures in

Fig. 2. The effects of a single administration of S-312-d on the epileptic seizures in SER. Representative data are shown in the figures. A: Inhibitory effects of S-312-d (1 mg/kg, p.o.) on tonic convulsions. These are EEGs that were recorded from the cortex (Cx) and hippocampus (Hpc) 30 min after the drug application. Arrows indicate the timing of the blowing stimulus on the back of the animal. The solid line under the EEG represents the period of tonic convulsions with low-voltage fast activity. A single administration of S-312-d obviously inhibited the appearance of low-voltage fast activity. B: Effects of S-312-d (1 mg/kg, p.o.) on absence-like seizures. These EEGs were recorded from the cortex (Cx) and hippocampus (Hpc) 30 min after the drug application. An interrupted line under the EEG represents an absence-like seizure with a 5–7-Hz spike-wave-like complex. A single administration of S-312-d did not affect the appearance of 5–7-Hz spike-wave-like complexes.

kindled animals and pentylenetetrazol-induced seizures have been reported to be related to the abnormal activation of Ca^{2+} channels (9–11). In addition, the

abnormal excitability seen in neurons obtained from the epileptic focus of patients is suggested to be at least partly due to an abnormality of Ca^{2+} channels (12–16).

Fig. 3. Dose-dependent effects of a single administrations of S-312-d (square: 1 mg/kg, $n = 8$; circle: 5 mg/kg, $n = 5$; triangle: 10 mg/kg, $n = 4$) on the tonic convulsion in SER. A: Effects of S-312-d on the occurrence of tonic convulsion. A blowing stimulus was applied to the back of the animal every 5 min. The numbers of tonic convulsions during the indicated periods are shown as the mean $\pm$ S.E.M. S-312-d significantly inhibited the occurrence of tonic convulsions in a dose-dependent manner. $*P < 0.05$, as compared with the value before the administration. B: Effects of S-312-d on the total duration of tonic convulsions. The duration of tonic convulsions during the indicated periods is shown as the mean $\pm$ S.E.M.. S-312-d at the dose of 10 mg/kg significantly reduced the total duration of tonic convulsions. S-312-d at 1 and 5 mg/kg tended to decrease the total duration of tonic convulsions. $*P < 0.05$, as compared with the value before the administration.

Fig. 4. Effects of a single administration of S-312-d (square: 1 mg/kg, $n = 8$; circle: 5 mg/kg, $n = 3$; triangle: 10 mg/kg, $n = 5$) on absence-like seizures in SER. A: Effects of a single administration of S-312-d on the occurrence of absence-like seizures. The numbers of absence-like seizures during the indicated periods are shown as the mean $\pm$ S.E.M. S-312-d did not affect the number of absence-like seizures. B: Effects of a single administration of S-312-d on the total duration of absence-like seizures. The total duration of absence-like seizures during the indicated periods is shown as the mean $\pm$ S.E.M. S-312-d tended to increase the total duration of absence-like seizures although the effect was not significant.

Fig. 5. Effects of repeated administration of S-312-d (1 mg/kg, p.o.) on tonic convulsion in SER. S-312-d was administered daily for 4 days at 10:00 a.m. Data represent the number (A) and total duration (B) of tonic convulsions during the indicated observation periods (means $\pm$ S.E.M., $n = 5$). Repeated administration of S-312-d (1 mg/kg, p.o.) had significant inhibitory effects on both the number (A) and total duration (B) of tonic convulsions. This inhibitory effect was augmented by repeated administration of S-312-d (Drug administration) and retained for 3 days after the cessation of the drug application (Recovery). * $P < 0.05$, as compared with the value before the administration.

Furthermore, a variety of mutations in the Ca^{2+} -channel subunit gene have been found in human or mouse hereditary epileptic disease (17–19). These findings support that the abnormal functioning of the Ca^{2+} channel is, at least in part, involved in the generation of epileptic seizures.

To our knowledge, very few reports have revealed that dihydropyridine Ca^{2+} -channel antagonists is effective in ameliorating epileptic disorders. Paczynski et al. reported that nimodipine attenuated kainic acid-induced seizures in rats (20). To address whether L-type Ca^{2+} -channel antagonists are candidate antiepileptics, we examined the effects of S-312-d, a novel dihydrothienopyridine derivative Ca^{2+} -channel antagonist, on tonic convulsions and absence-like seizures of SER. As expected, single and repeated administrations of this drug clearly attenuated the tonic convulsions in SER,

consistent with our previous data showing that L-type Ca^{2+} blockers improve the abnormal excitability of CA3 neurons (3, 4). This is also in agreement with the finding that S-312-d had anticonvulsive effects on audiogenic tonic and drug-induced clonic convulsions (7). In contrast, this drug had no effect on absence-like seizures of SERs. We could not clarify the reason for this discrepancy; however, it suggests that an abnormality of Ca^{2+} channels is involved in the pathogenesis of tonic convulsions, but not that of absence-like seizures.

In our previous study, oral administration of S-312-d at a dose of 1 mg/kg did not lower the blood pressure of SERs, although hypotension (10–20 mmHg) developed at the doses of more than 5 mg/kg (21), indicating that S-312-d can improve epileptic seizures without influencing blood pressure. In addition, a single

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