Sedative–hypnotic effect of YZG-330 and its effect on chloride influx in mouse brain cortical cells

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Abstract This study was to examine the sedative–hypnotic effect of YZG-330 and its influence on Cl⁻ influx in mouse cortical cells. In a sleep time-prolongation test in which mice were administered a threshold dosage of sodium pentobarbital (ip), YZG-330 (0.125, 0.5 and 2 mg/kg, po) prolonged the sleep time by 25% (P<0.05), 64% (P<0.01) and 506% (P<0.001), respectively. Thereafter, treatment with YZG-330 permitted mice that had woken up after the threshold dose of sodium pentobarbital (ip) to fall asleep again. A Cl⁻-sensitive fluorescent probe, N-(ethoxycarbonylmethyl)-methoxyquinolinium bromide (MQAE), was used to determine the effect of YZG-330 on Cl⁻ influx. YZG-330 (0.3, 0.6 and 1.5 mM) increased Cl⁻ influx in mouse cortical cells in a concentration-dependent manner. These data suggest that YZG-330 has a hypnotic effect in mice, and the effect may be related to an increase in Cl⁻ influx in cortical cells.

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1. Introduction

Chronic insomnia affects a significant proportion of the population. It has an appreciable impact on the quality of life, and can even lead to accidents. Studies have shown that many γ-aminobutyric acid (GABA)-ergic neurons in the central nervous system (CNS) are related to sleep. GABA is an important neurotransmitter in the mammalian CNS, and has been shown to play an important role in the modulation of sleep. GABA can enhance inhibitory synaptic transmission through GABAA receptors. This receptor has multiple non-identical binding sites, some of which bind hypnotic drugs; indeed, three “generations” of hypnotics have been based on GABAA receptor-mediated inhibitory processes, and studies have demonstrated that the GABAA receptor has important roles in the modulation of barbiturate-induced sleep.

Gastrodia elata is a perennial herb. The dry tuber is called Rhizoma gastrodiae, and is a commonly used Chinese herbal medicine for the treatment of headaches, dizziness, vertigo and convulsive illnesses (e.g., epilepsy, tetanus). Studies have shown that N\(^6\)-(4-hydroxybenzyl) adenine riboside extracted from Gastrodia elata can prevent the apoptosis of PC12 cells in a concentration-dependent manner. We previously reported that this substance causes sedation and can enhance sodium pentobarbital-induced sleep. YZG-330 (Fig. 1) is a derivative of N\(^6\)-(4-hydroxybenzyl) adenine riboside and is a new compound. In this work we evaluated the sedative–hypnotic effect of YZG-330 and its action on Cl\(^−\) channels. We also propose a possible mechanism of action.

2. Materials and methods

2.1. Animals

Mice (ICR, male) were obtained from Vital River Laboratories (Beijing, China) and were housed in acrylic cages (45 \(\times\) 60 \(\times\) 25 cm) with water and food available ad libitum under a 12-h light–dark cycle. The study protocol was approved by the Ethics Committee of the Chinese Academy of Medical Sciences (Beijing, China).

2.2. Reagents

YZG-330 (purity > 99%, HPLC) was synthesized by Professor Jiangong Shi, Department of Phytochemistry of the Institute of Materia Medica (Beijing, China). N-(6-methoxyquinolyl)-acet-oethyl ester (MQAE) was obtained from Invitrogen (Carlsbad, CA, USA). Nigericin and tributyltin were purchased from Sigma (St Louis, MO, USA). Sodium pentobarbital was obtained from Serva (Heidelberg, Germany). Injectable diazepam was purchased from Tianjin Pharmaceuticals (Tianjin, China); diazepam tablets were obtained from Beijing YiMin Pharmaceutical (Beijing, China).

2.3. Instruments

96-well enzyme assay plates were obtained from Costar (USA) and read on a microplate reader from BioTek, SynergyMX, USA.

2.4. Sodium pentobarbital-induced sleep studies

Sodium pentobarbital was diluted in distilled water. Diazepam was suspended in 0.5% CMC-Na solution. YZG-330 was dissolved in distilled water with 1% Tween 80. All experiments were carried out between 1:00–5:00 pm. Eight mice were used in each treatment group. Diazepam (2 mg/kg) and YZG-330 (0.125, 0.5 and 2 mg/kg) were administered orally to animals 25 min before the injection of sodium pentobarbital (40 mg/kg, ip). After the injection of sodium pentobarbital, each mouse was observed for the onset of sleep. Animals were positioned on their backs and their loss of the righting reflex marked the onset of sleep. Mice were observed continuously, and the time of waking (characterized by regaining the righting reflex) was noted. The sleep time was defined as the time taken for the animal to regain spontaneous movements.

2.5. Sodium pentobarbital-treated re-onset sleep in mice

Experiments were carried out as described above. At the moment when an animal awoke, diazepam (2 mg/kg) or YZG-330 (0.125, 0.5 and 2 mg/kg) was administered orally. Animals were observed continuously, and the number of mice that lost their righting reflex was noted. The percentage of re-onset sleep was calculated using the following formula:

\[
\text{Percentage of re-onset sleep} = \frac{\text{number of animals falling asleep again/total number of animals}}{100}\%.
\]

2.6. Preparation of mouse cortical cells

Mice were killed by decapitation without the induction of anesthesia. The brain cortex was dissected rapidly, separating gray matter from white matter on ice. The gray matter was minced and homogenized in a chilled glass homogenizer. The homogenate was transferred to a 50 mL centrifuge tube and the homogenizer was rinsed with 30 mL ice-cold cortical cell preparation buffer [in mM: NaCl (118.5), KCl (4.7), MgSO\(_4\) (1.18), CaCl\(_2\) (1.0), HEPES (20), Tris (9), r-glucose (10)] and added to the homogenate, which was then centrifuged at 1000 \(\times\) g for 5 min at 4 °C. The supernatant was discarded and the re-diluted homogenate was poured onto a pre-wetted nylon filter unit (40 \(\mu\)m). The filtrate was centrifuged at 300 \(\times\) g for 5 min at 4 °C and the supernatant was discarded.

2.7. Measurement of Cl\(^−\) influx

MQAE is a fluorescent indicator for Cl\(^−\). Its fluorescence is quenched upon interaction with Cl\(^−\). We incubated cortical cells with MQAE and detected the fluorescence of MQAE. When Cl\(^−\) fluxes into cells, the fluorescence of MQAE is partly quenched. The amount of fluorescent quenching and concentration of Cl\(^−\) in the cell have a concentration–response relationship. Hence, we can use MQAE to detect the Cl\(^−\) influx.

![Chemical structure of YZG-330](image)
Cells were resuspended in 10 mM MQAE dissolved in chloride-containing buffer [in mM: HEPES (10), d-glucose (10), MgSO4 (1), K2HPO4 (2.4) KH2PO4 (0.6), CaSO4 (1) and NaCl (130)] and loaded for 1 h at 37 °C. After loading, the suspension was centrifuged at 300 × g for 5 min at 4 °C. The supernatant was discarded. The pellet was resuspended gently with chloride-containing buffer and centrifuged at 300 × g for 5 min at 4 °C; this step was repeated three times. The supernatant was discarded. The pellet was resuspended gently with chloride-containing buffer with or without compounds. Repeat measurements of fluorescence were initiated immediately using a fluorescence microplate reader (excitation, 360 nm; emission, 460 nm). Three wells were used for each group. The initial fluorescence was Fl and the final fluorescence Ft. Taking the control group (Ftcon−Ftcon) as a maximum value of 100%, the effect of each drug treatment was calculated using the formula: (Fsam−Ftcon)/(Ftcon−Ftcon) × 100%

2.8. Statistical analyses

Data were expressed as mean ± SEM and analyzed by the Student’s t-test. Statistical analyses were carried out using SPSS ver13.0 (SPSS, Chicago, IL, USA). P < 0.05 was considered significant. For the re-onset sleep test, Fisher’s exact test was used to compare the rate of re-sleep between the control and each of the other groups. P < 0.05 was considered significant.

3. Results

3.1. Influence of YZG-330 on sodium pentobarbital-induced sleep

After sodium pentobarbital (40 mg/kg, ip) was administered, the sleep latency and sleeping time of the control group was 4.5 ± 0.6 min and 47.0 ± 3.7 min, respectively (Fig. 2). Diazepam (2 mg/kg, po) was administered 25 min before the injection of sodium pentobarbital and significantly shortened sleep latency by 38% (P < 0.05). YZG-330 (0.125, 0.5 and 2 mg/kg, po) was administered 25 min before sodium pentobarbital and shortened sleep latency by 6%, 20% and 12%, respectively (P > 0.05) (Fig. 2A), and prolonged the duration of loss of the righting reflex by 25% (P > 0.05), 64% (P < 0.01) and 506% (P < 0.001) respectively (Fig. 2B), comparing with the control group.

3.2. Influence of YZG-330 on re-sleep in sodium pentobarbital-treated mice

The prevalence of re-onset sleep in the mice from the vehicle group was 0% (Table 1). Diazepam (2 mg/kg, po) could induce re-onset sleep in 87% of mice (P < 0.01). YZG-330 (0.125, 0.5 and 2 mg/kg, po) could induce the mice to re-sleep, and the prevalence was 25%, 50% and 63% (P < 0.05) respectively. YZG-330 could increase the rate of re-onset sleep in a concentration-dependent manner in sodium pentobarbital-treated mice.

3.3. Influence of YZG-330 on Cl− influx in mouse cortical cells

Each well had about 1.6 × 10^5 mouse cortical cells. In the process of fluorescence reading, a slight increase in the fluorescence of the control group was detected. At the end of each experiment nigericin and tributyltin were added to obtain minimum fluorescence. Addition of sodium pentobarbital (20 mM) caused a markedly stepwise decrease in the fluorescence of MQAE within 20 min. (Fig. 3), with an efficacy of 71%.

Both diazepam and YZG-330 decreased MQAE fluorescence significantly in a concentration-dependent manner. The EC50 of diazepam was 0.6 mM (Fig. 4), while the EC50 of YZG-330 was 1.5 mM (Fig. 5).

4. Discussion

In the present study we used sodium pentobarbital-induced sleep to evaluate the hypnotic effect of YZG-330. The results showed that YZG-330 could prolong the duration of sleep in sodium pentobarbital-treated mice in a dose-dependent manner, at 2 mg/kg, more effectively than diazepam.

Three generations of hypnotic agents are based on GABA_A receptor-mediated inhibitory processes. The GABA_A receptor is a GABA/benzodiazepine receptor–Cl− channel complex. This receptor belongs to the cysteine ring-ligand gating channel superfamily. Drugs may act on the GABA_A receptor at several sites to induce

![Figure 2](image-url)  
Figure 2  Effect of YZG-330 on the onset and duration of sleep in sodium pentobarbital-treated mice. (A) Sleep latency and (B) sleep time after the administration of diazepam and YZG-330. The data are expressed as mean ± SEM, n=8. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group.
The excitability of neurons results in sedative hypnotic effects: these compounds were found to increase Cl\(^{-}\) influx, hyperpolarizing the cell membrane and reducing the excitability of neurons\(^{5,6}\), resulting in sedative–hypnotic actions.

Oh et al. used cerebellar granule neurons loaded with the Cl\(^{-}\) fluorescence probe MQAE to detect compounds with sedative and hypnotic effects: these compounds were found to increase Cl\(^{-}\) flux in a dose-dependent manner\(^{1,7,8}\). During sleep, the excitability of the brain cortex is inhibited\(^{9}\). Thus, the cortical cells of mice could be used to examine the effects of compounds on Cl\(^{-}\) flux, thereby imitating the mechanism of action of the compounds in vivo. We incubated mouse cortical cells with MQAE. MQAE can enter cells by permeation, allowing us to detect the fluorescence of MQAE at an excitation wavelength 360 nm and emission wavelength of 450 nm. When a compound that opens Cl\(^{-}\) channels is presented, Cl\(^{-}\) influx resulted, quenching the fluorescence of MQAE. The amount of fluorescence quenching and the concentration of Cl\(^{-}\) in the cell had a concentration–response relationship. Hence, we used MQAE to measure the Cl\(^{-}\) influx. During fluorescence measurements, the cells settled gradually, so a slight increase in the fluorescence of the control group was detected when reading from the bottom of the plate. Nigericin is an antiporter of K\(^{+}\) and H\(^{+}\). Tributyltin is an OH\(^{-}\)/Cl\(^{-}\) antiporter that equalizes the Cl\(^{-}\) gradient included in the buffers. The Cl\(^{-}\) concentration outside the cell is much higher than that in the cell under normal physiological conditions. When nigericin and tributyltin were added, the Cl\(^{-}\) concentration within and outside the cell is quickly equalized, and the fluorescence is at its lowest. We assume that when nigericin and tributyltin are administered, it represents a maximal effect (100%).

The experimental results revealed that when mouse cortical cells were loaded with the Cl\(^{-}\) fluorescence probe MQAE, addition of sodium pentobarbital caused a continuous decrease in fluorescence compared with the control group. This result was repeatable, so using Cl\(^{-}\) fluorescence probe to detect Cl\(^{-}\) influx was achievable. Diazepam could also induce Cl\(^{-}\) influx after administration, reaching a maximal effect at 20 min after administration, with an EC\(_{50}\) of 0.6 mM. YZG-330 markedly and immediately reduced fluorescence with an EC\(_{50}\) of 1.5 mM.

Diazepam is the second generation of hypnotics, believed to act at GABA\(_{A}\) receptor–chloride channel complex to increase the opening of the Cl\(^{-}\) channel, resulting in its sedative–hypnotic effect. YZG-330 has similar effects in both in vivo and in vitro tests.

In our experiments, the dose of YZG-330 used in vivo was 2 mg/kg, however, the concentration of YZG-330 used in vitro was at the millimolar level. This apparent discrepancy in effective concentrations reflects the very different methods of measurement of effectiveness; fluorescence was detected in cell culture with very

### Table 1: Effect of YZG-330 on the re-onset of sleep in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Number of mice falling asleep/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2</td>
<td>7/8***</td>
</tr>
<tr>
<td>YZG-330</td>
<td>0.125</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4/8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5/8*</td>
</tr>
</tbody>
</table>

\*\(P<0.05\); ***\(P<0.001\) vs. the vehicle group.
low sensitivity, and the abundance of GABA<sub>A</sub> receptors in adult mouse cortical neurons is low. We also determined the effect of YZG-330 on Cl<sup>-</sup> influx in cerebellar granule cells, where micromolar levels of the compound can lead to the influx of Cl<sup>-</sup>; the cortical cell density we used is about one thousand times that of the cerebellar granule cell.

In conclusion, YZG-330 has a sedative–hypnotic effect in mice, and can cause rapid Cl<sup>-</sup> influx in mouse cortical cells. This may be one of its mechanisms of action.

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**References**