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N^3-Phenacyluridine, a novel hypnotic compound, interacts with the benzodiazepine receptor

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Abstract

N^3-Phenacyluridine (3-phenacyl-1-β-D-ribofuranosyluracil) has potent sedative and hypnotic activities following intracerebroventricular injection in mice. To study the mechanism of action of N^3-phenacyluridine, the interaction of this compound with the benzodiazepine receptor has been investigated. Results obtained showed that this compound inhibited specific binding of [^{3}H]flunitrazepam to synaptic membranes of bovine cortex in a concentration-dependent fashion (IC_{50} = 129 μM). Scatchard analysis of [^{3}H]flunitrazepam binding revealed that N^3-phenacyluridine interacted with the ligand at the benzodiazepine receptor binding site in a competitive manner. Ro15-1788 (8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*]1,4-benzodiazepine), a benzodiazepine receptor antagonist, also inhibited the specific binding of [^{3}H]flunitrazepam in the presence of the compound. The results suggest that the pharmacological activity of N^3-phenacyluridine may be partially mediated through the benzodiazepine receptor.

Keywords: N^3-Phenacyluridine; Sedative; Hypnotic; Central nervous system; Benzodiazepine receptor

1. Introduction

Uridine is known to possess central nervous system (CNS) depressant effects, e.g. a decrease in spontaneous activity in mice (Krooth et al., 1978) and protection against penicillin or metrazol-induced seizures (Roberts, 1973). In addition, uridine has been reported as one of the sleep-promoting substances extracted from brainstems of 24 h sleep-deprived rats (Komoda et al., 1983) and caused natural sleep following a nocturnal infusion into the rat brain (Honda et al., 1984; Inouè et al., 1984). In connection with the CNS depressant effects of uridine, Guarneri et al. (1983, 1985) showed that uridine interacted with γ-aminobutyric acid (GABA) binding site in cerebellar membranes of the rat. In 1985, we reported that N^3-benzyluridine (3-benzyl-1-β-D-ribofuranosyluracil) (Fig. 1) exhibited 28 min of sleeping time as hypnotic activity at 2.0 μmol/mouse following an intracerebroventricular (i.c.v.) injection (Yamamoto et al., 1985). The compound also interacted with GABA receptor binding (Yamamoto et al., 1990). Furthermore, we have recently found that N^3-phenacyluridine (3-phenacyl-1-β-D-ribofuranosyluracil) showed a 20-fold longer sleeping time as compared with N^3-benzyluridine in mice at the same dose (Yamamoto et al., 1994). Synergistic effect of N^3-phenacyluridine with benzodiazepine was 70-fold of the control (Yamamoto et al., 1994).

Therefore, it is important to study the effects of N^3-substituted uridine on benzodiazepine receptor binding by using N^3-phenacyluridine as a model compound.

2. Materials and methods

2.1. Materials

[*N*-methyl-^{3}H]Flunitrazepam (87.0 Ci/mmol) was purchased from NEN (Tokyo, Japan). Diazepam was obtained from Yamanouchi Seiyaku Co. (Tokyo, Japan). Ro15-1788 (8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-6-oxo-4*H*-

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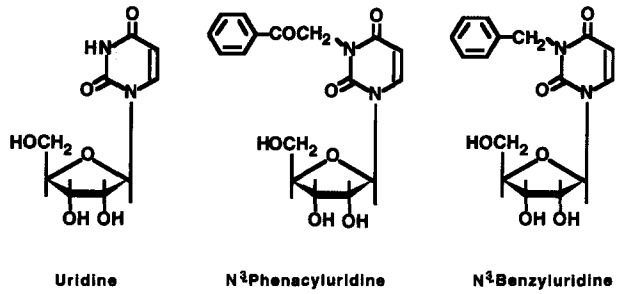

Fig. 1. Structures of uridine, N^3-phenacyluridine and N^3-benzyluridine.

imidazo[1,5*a*]1,4-benzodiazepine) was supplied by Nippon Roche Research Center (Tokyo, Japan).

N^3-Phenacyluridine was prepared according to the method previously reported (Sasaki et al., 1973; Yamamoto et al., 1994).

2.2. Tissue

Fresh bovine brain was obtained from Kanazawa-shi Meat Inspection Office (Kanazawa, Japan). Cortex dissected from the brain was frozen and stored at −70°C until needed.

2.3. Membrane preparation

Crude synaptic membranes from bovine brain were prepared according to the method of Zukin et al. (1974) with slight modification. Bovine cortex was homogenized with a Polytron homogenizer in 10 volumes of ice-cold 0.32 M sucrose for 1 min, followed by centrifugation at $1000 \times g$ for 10 min. The pellet was discarded and the supernatant was centrifuged at $20\,000 \times g$ for 20 min. The resulting pellet was homogenized for 15 s in 40 volumes of ice-cold distilled water, then dispersed with a Polytron homogenizer. After centrifugation of the suspension at $8000 \times g$ for 20 min, the supernatant with soft buffy upper layer of the pellet was carefully collected and the supernatant fraction was combined. This fraction was then centrifuged at $48\,000 \times g$ for 20 min, and the pellet was homogenized for 15 s in 40 volumes of 50 mM Tris-citrate buffer (pH 7.1) followed by centrifugation at $48\,000 \times g$ for 20 min. The pellet was homogenized for 15 s in 10 volumes of the buffer and then stored at −70°C for at least 24 h. Furthermore, washing procedure was performed twice to remove endogenous inhibitors and then stored at −70°C for at least 24 h.

On the day of binding assay, the frozen membrane fraction was thawed and suspended in 40 volumes of 50 mM Tris-citrate buffer and incubated at 25°C for 30 min and centrifuged at $48\,000 \times g$ for 20 min. The pellet was then homogenized in enough volume of the buffer to produce a protein concentration of about 0.25 mg/ml. Protein determination was performed by the method of Lowry et al. (1951).

2.4. Benzodiazepine receptor binding assay

[^{3}H]Flunitrazepam binding assay was carried out as previously described by Yamamoto et al. (1992). The binding assay was carried out in 1.0 ml of 50 mM Tris-citrate buffer (pH 7.1), containing 0.2–0.3 mg protein of synaptic membrane. The synaptic membranes were incubated with 0.5 nM [^{3}H]flunitrazepam in the presence or absence of N^3-phenacyluridine and/or Ro15-1788. The incubation conditions were set at 4°C for 1 h. Specific binding of [^{3}H]flunitrazepam was defined as radioactivity displaced by 10 μM flunitrazepam. The incubation mixture was filtered through Whatman GF/B filters using a Cell Harvester (Brandel Model M-24) and the radioactivity was counted by liquid scintillation counter.

For Scatchard analysis, the concentrations of [^{3}H]-flunitrazepam were varied between 0.1–5.0 nM. [^{3}H]Flunitrazepam was incubated with synaptic membranes in the presence or absence of N^3-phenacyluridine. Routinely, 5 concentrations of [^{3}H]flunitrazepam were used to determine the kinetics. Nonspecific binding obtained in the presence of 10 μM of flunitrazepam was subtracted from the total binding to determine the specific binding.

IC_{50} of N^3-phenacyluridine on [^{3}H]flunitrazepam binding was determined by the method of Litchfield and Wilcoxon (1949).

2.5. Statistical analysis

Statistical significance of difference was calculated using one way analysis of variance.

3. Results

The effects of N^3-phenacyluridine on benzodiazepine receptor binding was examined using ligand binding assay. Fig. 2 shows an inhibitory effect of N^3-phenacyluridine on the specific binding of [^{3}H]flunitrazepam to bovine synaptic membrane. N^3-Phenacyluridine concentration dependently displaced [^{3}H]flunitrazepam binding and its IC_{50} (concentration that inhibits binding by 50%) of 129 μM was obtained (Fig. 2). N^3-Phenacyluridine, 1 mM, completely inhibited the [^{3}H]flunitrazepam binding. Kinetic analyses of the effect of N^3-phenacyluridine (10 or 50 μM) on [^{3}H]flunitrazepam binding were performed, and the representative Scatchard analyses are shown in Fig. 3. Scatchard analyses showed a significant increase in the dissociation constant of specific binding of [^{3}H]-flunitrazepam to the synaptic membranes, whereas the maximal number of binding sites (B_{max}) did not change (Table 1). Apparent affinity constants (K_D) of [^{3}H]-flunitrazepam binding in the presence of N^3-phenacyluridine at 50 μM were significantly increased as compared with that of the control. In contrast, the apparent Bmax was unchanged. Fig. 4 shows inhibitory effects of Ro15-

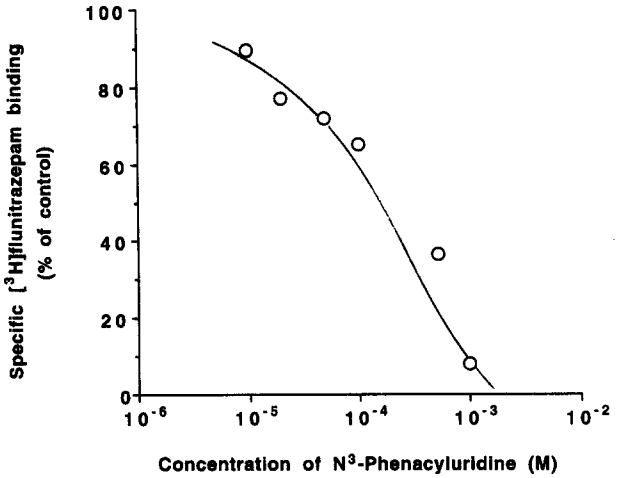

Fig. 2. Effect of N^3-phenacyluridine on specific [^{3}H]flunitrazepam binding to bovine synaptic membranes. Membranes were incubated at 4°C for 60 min with 0.5 nM [^{3}H]flunitrazepam and increasing concentrations of N^3-phenacyluridine (10^{-5}–10^{-3} M). Nonspecific binding was obtained in the presence of 10 μM flunitrazepam, and specific binding was expressed as percentage of control binding.

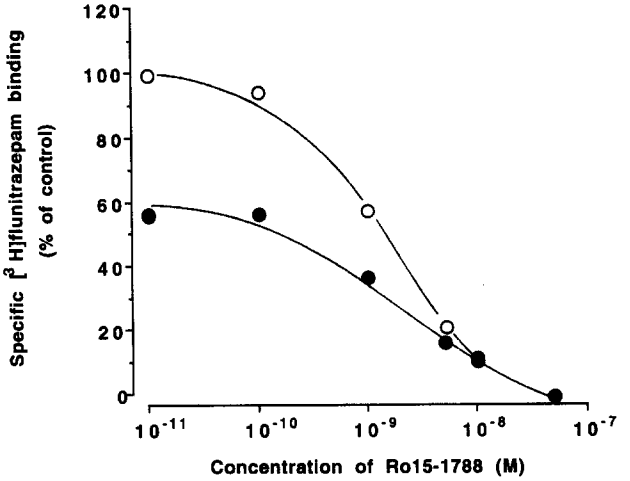

Fig. 4. Inhibitory effect of N^3-phenacyluridine on specific [^{3}H]-flunitrazepam binding to bovine synaptic membranes. Membranes were incubated at 4°C for 60 min with 0.5 nM [^{3}H]flunitrazepam and increasing concentrations of Ro15-1788 (10^{-7}–10^{-11} M), in the absence (○) and presence (●) of 250 μM N^3-phenacyluridine. Nonspecific binding was obtained in the presence of 10 μM flunitrazepam, and specific binding was expressed as percentage of control binding.

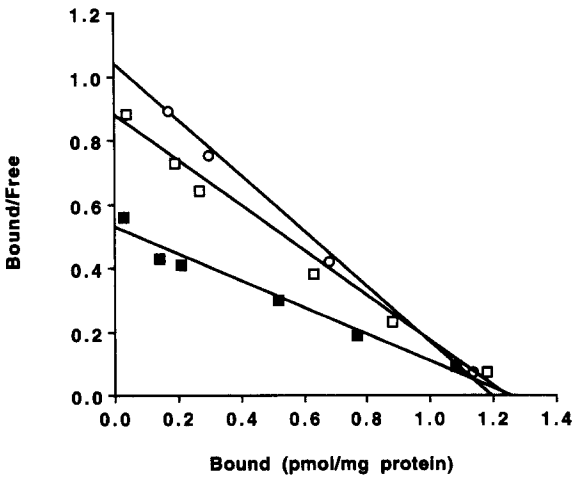

Fig. 3. Representative Scatchard plots of [^{3}H]flunitrazepam binding in the absence (control: ○) or presence of N^3-phenacyluridine (10 μM, □, 50 μM: ■) in the frontal cortex of bovine brain. The binding assay was performed as described in Materials and methods.

Table 1
Effect of N^3-phenacyluridine on [^{3}H]flunitrazepam binding

Compound	Concentration	K_D (nM)	B_{max} (pmol/mg protein)	n
Control		1.27 ± 0.22	1.10 ± 0.06	5
N^3-Phenacyl-uridine	10 μM	1.69 ± 0.24	1.30 ± 0.08	3
	50 μM	3.17 ± 0.49[a]	1.19 ± 0.09	3

Each value represents mean ± S.E.M. [a] $P < 0.01$ for difference from the control value.

1788, a benzodiazepine receptor antagonist, on specific binding of [^{3}H]flunitrazepam in the presence or absence of N^3-phenacyluridine (250 μM). Ro15-1788 also decreased the specific binding of [^{3}H]flunitrazepam in a concentration-dependent manner. Addition of 250 μM N^3-phenacyluridine caused 50% inhibition of specific binding of [^{3}H]flunitrazepam.

4. Discussion

In our previous study, we found for the first time that one of the uridine derivatives, N^3-benzyluridine, exerted hypnotic action in mice following i.c.v. administration (Yamamoto et al., 1985). Further experiments have been conducted to determine structure-activity relationships of *N*-substituted uridine and thymidine in the CNS. Mice were used to test more potent compounds or structural requirements for the hypnotic activity of pyrimidine nucleosides (Yamamoto et al., 1986, 1987a,b; Kimura et al., 1991, 1993). Our results demonstrated that N^3-phenacyluridine is a more potent hypnotic compound than N^3-benzyluridine. The interaction of N^3-phenacyluridine with CNS depressants was also examined using barbiturate and benzodiazepine, since uridine and its related compounds are known to enhance drug-induced narcosis and motor incoordination (Yamamoto et al., 1986, 1987b). N^3-Phenacyluridine significantly prolonged the pentobarbital-induced sleep (by about 6-fold) following i.c.v. injection as compared to the control, while N^3-phenacyluridine injected with diazepam resulted in about 70-fold increase over the control (Yamamoto et al., 1994). It is noted that the degree of synergistic effects of N^3-phenacyluridine

was markedly different between the pentobarbital- and the diazepam-induced sleep. Moreover, the potentiation of diazepam-induced sleep by N^3-phenacyluridine was the strongest among the compounds tested previously. The potentiation of diazepam-induced sleeping time by N^3-benzyluridine was 3-fold as compared to the control, even though the injection dose was same as that of N^3-phenacyluridine. These results indicated that a benzodiazepine receptor may be involved in the mechanism of action of N^3-phenacyluridine. It is well known that the benzodiazepine binding site is coupled to GABA binding site, chloride channel, picrotoxin binding site and barbiturate active site to form a $GABA_A$ receptor in the brain (Olsen, 1981). Certain barbiturates affect the benzodiazepine and GABA bindings (Olsen, 1981; Willow and Johnston, 1981). Pentobarbital is known to increase [^{3}H]diazepam binding to synaptic membranes. Therefore, we assumed that uridine derivatives affect the GABA-benzodiazepine receptor-chloride channel complex similarly to that of barbiturates because of the structural similarity. N^3-Phenacyluridine, however, decreased [^{3}H]flunitrazepam binding to the synaptic membranes, namely, the effect of N^3-phenacyluridine on the benzodiazepine receptor could be distinguished from that of barbiturates. The interaction of N^3-phenacyluridine with the benzodiazepine receptor was in a competitive manner. The Scatchard analysis revealed that the affinity of flunitrazepam for the benzodiazepine receptor binding site are affected by N^3-phenacyluridine, while the apparent number of the binding sites remains unchanged. Asano and Spector (1979) reported that inosine and hypoxanthine but not uracil and cytosine were identified as endogenous ligands for the benzodiazepine receptor. We successfully found that the pyrimidine derivative also interacted with the benzodiazepine receptor. The results suggest that N^3-phenacyluridine may act on the benzodiazepine receptor site as an agonist.

Since N^3-phenacyluridine interacted with the benzodiazepine receptor, N^3-phenacyluridine may affect the $GABA_A$ receptor and the chloride ionophore through the benzodiazepine binding site. Interaction of uridine with GABA binding sites in cerebellar membranes of rats was reported by Guarneri et al. (1983, Guarneri et al., 1985) and Yamamoto et al. (1990). They have reported that the ability of uridine to compete with GABA at its binding sites needed much higher concentration of uridine (1 mM). The Scatchard plot of GABA binding with 1 mM N^3-benzyluridine showed an increase in the dissociation constant without a change in the B_{max}, indicating a competitive inhibition of the GABA binding by 1 mM N^3-benzyluridine. Therefore, the $GABA_A$ receptor may also be modulated by N^3-phenacyluridine.

The present study suggests that N^3-phenacyluridine interacts with the benzodiazepine receptor, and indicates that this compound is a useful model compound to examine the mechanism of sleep in the CNS. Effects of N^3-phenacyluridine on the $GABA_A$ receptor and chloride channel are now under investigation.

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