Biochemical Pharmacology, Vol. 58, pp. 617–621, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00129-X

Interaction of Anisatin with Rat Brain γ-Aminobutyric Acid$_A$ Receptors: Allosteric Modulation by Competitive Antagonists

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ABSTRACT. Anisatin, a toxic sesquiterpene isolated from the Japanese star anise (*Illicium anisatum* L.), competitively inhibited the specific binding of [^{3}H]4′-ethynyl-4-*n*-propylbicycloorthobenzoate ([^{3}H]EBOB), a non-competitive antagonist of γ-aminobutyric acid (GABA)$_A$ receptors, to rat brain membranes with an IC_{50} value of 0.43 μM. R 5135, a competitive GABA antagonist, decreased the potency of anisatin in inhibiting [^{3}H]EBOB binding in a negatively cooperative manner. Two other competitive antagonists, SR 95531 (gabazine) and (−)-bicuculline methiodide, had similar effects. On the other hand, R 5135 exerted little influence on the potencies of the other non-competitive antagonists tested: EBOB, picrotoxinin, isopropylbicyclophosphate, and dieldrin. Thus, anisatin was clearly different from the other non-competitive antagonists in responding to the action of competitive antagonists on GABA$_A$ receptors. These findings suggest that the binding region of anisatin might overlap with that of the other non-competitive antagonists, but that anisatin must interact with other specific region(s). BIOCHEM PHARMACOL **58**;4:617–621, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. γ-aminobutyric acid; GABA receptor; anisatin; non-competitive antagonist; competitive antagonist; allosteric modulation

GABA$_A$‖ receptors are hetero-pentameric proteins that form GABA-gated Cl^- channels in the synaptic membrane and mediate synaptic inhibition in the mammalian brain [1]. GABA$_A$ receptors are also the site of action of a variety of medicinal compounds such as barbiturates, benzodiazepines, etc., as well as agonists and antagonists [2]. Most of these compounds modulate GABA$_A$ receptor functions, producing physiological, pharmacological, or toxicological effects.

Anisatin (Fig. 1) is a toxic sesquiterpene isolated from the seeds and carpels of the Japanese star anise (*Illicium anisatum* L.) [3, 4]. Anisatin and related terpenoids also exhibit analgesic and sedative effects at low dosage [5]. Previous mode-of-action studies have indicated that this terpene antagonizes the action of GABA in the crayfish neuromuscular junction and the frog spinal cord [6, 7]. Using patch-clamp techniques with rat dorsal root ganglion neurons, we have recently demonstrated that anisatin blocks GABA-gated Cl^- channels in the open state and decreases the probability of channel opening.** In binding assays, anisatin was shown to competitively inhibit specific binding of [^{3}H]EBOB, a non-competitive GABA antagonist, to rat brain membranes.**

Thus, both electrophysiological and biochemical observations indicate that anisatin acts as a non-competitive antagonist of GABA$_A$ receptors. However, anisatin must at least have a different mode of interaction from those of other non-competitive antagonists, because it bears a skeleton that is quite different from those of other terpenes such as PTX and picrodendrins that act as non-competitive antagonists [8]. Therefore, we examined the binding properties of anisatin in rat brain GABA$_A$ receptors, particularly in terms of allosteric modulation; based on this examination, we report here that the interacting region of anisatin is allosterically linked to the binding site of competitive antagonists. As a result, it can be assumed that although the binding sites are identical, the interacting regions of anisatin and of other non-competitive antagonists may differ.

MATERIALS AND METHODS

Chemicals

Anisatin was isolated from the fruits of *I. anisatum*. R 5135 and SR 95531 (gabazine) were gifts from Dr. Peter Hunt

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‖ *Abbreviations:* GABA, γ-aminobutyric acid; GABA$_A$ receptor, γ-aminobutyric acid type A receptor; EBOB, 4′-ethynyl-4-*n*-propylbicycloorthobenzoate or 1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo [2.2.2]octane; PTX, picrotoxinin; BMI, (−)-bicuculline methiodide; IPPO, isopropylbicyclophosphate or 4-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-oxide; and TBPS, *t*-butylbicyclophosphorothionate or 4-*t*-butyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide.

Received 16 November 1998; accepted 17 February 1999.

** Ikeda T, Ozoe Y, Okuyama E, Nagata K, Honda H, Shono T and Narahashi T, *Br J Pharmacol*, in press.

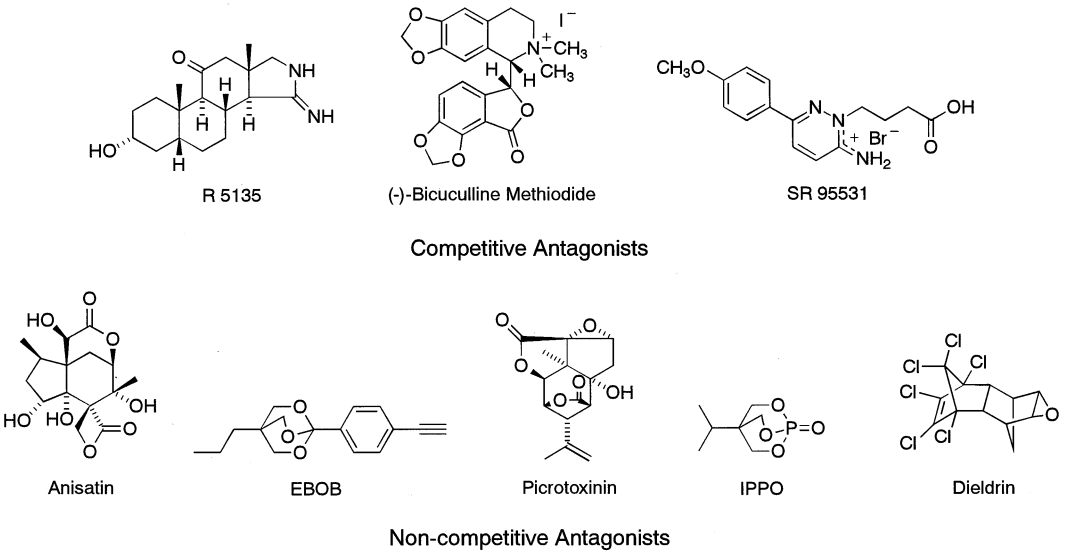

FIG. 1. Structures of competitive and non-competitive antagonists of $GABA_A$ receptors. The absolute stereochemistry of anisatin was recently established by total synthesis [23].

(Roussel Uclaf) and Dr. Jean-Pierre Chambon (Sanofi Recherche), respectively. BMI and PTX were purchased from Sigma–Aldrich Japan K.K. Dieldrin and GABA were purchased from Wako Pure Chemical Industries, Ltd. EBOB and IPPO were synthesized in our laboratory at Shimane University. [^{3}H]EBOB (38.0 Ci/mmol) was purchased from NEN™ Life Science Products, Inc.

Binding Studies

[^{3}H]EBOB binding studies were performed using rat brain P_2 membranes as described previously [8]. Briefly, a homogenate of whole rat brains in 1 mM EDTA was centrifuged at 1000 *g* for 10 min, and the supernatant at 25,000 *g* for 30 min. A suspension of the resulting pellets in 1 mM EDTA was dialyzed three times against 2 L of distilled, deionized water for 2 hr. Centrifugation of the inner solution at 25,000 *g* for 30 min gave brain membranes, which were stored at −80° until use. In binding experiments, anisatin was incubated at 37° for 90 min with membranes (125 μg protein), 0.5 nM [^{3}H]EBOB, and other indicated compounds in 1.0 mL of 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. After incubation, the mixtures were filtered through GF/B filters (Whatman International Ltd.) and were rapidly rinsed twice with 5 mL of the ice-cold buffer, using a Brandel M-24 cell harvester (Biomedical Research and Development Laboratories, Inc.). The radioactivity of [^{3}H]EBOB that specifically bound to membranes on the filters was measured with a liquid scintillation counter. Non-specific binding was determined in the presence of 5 μM unlabeled EBOB. Each experiment was performed in duplicate and repeated two or three times. IC_{50} values were determined by the Probit method. Concentration–inhibition curves were fitted to the logistic equation.

RESULTS

Effects of Anisatin on Specific [^{3}H]EBOB Binding to Rat Brain Membranes

Anisatin inhibited [^{3}H]EBOB binding to $GABA_A$ receptors in rat brain membranes with an IC_{50} value of 0.43 μM (Table 1). In comparison with other terpenoid GABA antagonists, anisatin was almost equipotent to PTX (IC_{50} = 0.62 μM), but was approximately 27-fold less potent than picrodendrin Q (IC_{50} = 16 nM), which is the most potent among terpenoid GABA antagonists [8]. A Scatchard analysis of the inhibition has revealed that anisatin is a competitive inhibitor of [^{3}H]EBOB binding, i.e. a non-competitive antagonist of $GABA_A$ receptors.*

Effects of R 5135 on Anisatin Inhibition of [^{3}H]EBOB Binding

R 5135 [9] (Fig. 1), a competitive GABA antagonist, was tested for its effect on the potency of anisatin in inhibiting specific [^{3}H]EBOB binding to rat brain membranes. Figure 2 shows that R 5135 produced a rightward shift of the concentration–inhibition curve of anisatin in a negatively cooperative manner [10], with the slope of the Schild plot less than unity. The IC_{50} value of anisatin significantly increased with increasing concentrations of R 5135 (Table 1). However, the increase appeared to occur in two stages (from 0 to 1 nM and from 10 to 100 nM) in the R 5135 concentration range of 0–1 μM. The potency of anisatin was decreased approximately sixfold by 0.1–1 μM R 5135.

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TABLE 1. Effects of GABA and competitive antagonists on the potency of anisatin in inhibiting specific [^{3}H]EBOB binding to rat brain membranes

Compound	Conc. (nM)	IC_{50} (μM) of anisatin	95% CL	Hill coeff. n_H
None	—	0.43	0.34–0.55	0.85
R 5135	1	1.34	1.08–1.66	1.09
R 5135	10	1.12	0.89–1.41	0.82
R 5135	100	2.53	2.01–3.18	0.89
R 5135	1000	2.60	2.08–3.24	0.96
SR 95531	100	1.05	0.85–1.29	1.03
BMI	10000	1.89	1.54–2.31	1.11
GABA	300	0.59	0.49–0.72	1.14
GABA	4000	0.46	0.36–0.59	0.83

Rat brain membranes were incubated with 0.5 nM [^{3}H]EBOB and anisatin in the absence and presence of GABA or competitive antagonists in 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. IC_{50} values of anisatin with 95% confidence limits (CL) were obtained from mean values of two or three experiments, each done in duplicate.

Effects of SR 95531 and BMI on Anisatin Inhibition of [^{3}H]EBOB Binding

Two other competitive GABA antagonists (Fig. 1), SR 95531 [11] and BMI [12, 13], were examined to determine whether they exhibit effects similar to those of R 5135. Both competitive antagonists shifted anisatin's inhibition curve to the right (Fig. 3), indicating that the shift was caused by a mechanism common to competitive antagonists. SR 95531 (0.1 μM) and BMI (10 μM) significantly increased the IC_{50} value of anisatin (Table 1). The potency of anisatin was reduced two- and fourfold by SR 95531 and BMI, respectively. The rank order of potency (R 5135 > SR 95531 > BMI) was the same as that of their potencies as GABA antagonists [11, 14].

Effects of R 5135 on the Potency of Non-competitive GABA Antagonists in Inhibiting [^{3}H]EBOB Binding

R 5135 was examined to determine whether it shifts the concentration–inhibition curves of other various non-competitive antagonists (Fig. 1), EBOB, IPPO, PTX, and dieldrin [15]. R 5135 did not affect the potency of unlabeled EBOB in inhibiting [^{3}H]EBOB binding (Table 2), although R 5135 produced only small increases (6–13% at 1 nM–10 μM) in specific [^{3}H]EBOB binding (data not shown). The potencies of the other non-competitive antagonists tested were also little affected by R 5135. These findings indicate that the mechanism underlying the rightward shift is specific to anisatin.

Effects of GABA on Anisatin Inhibition of [^{3}H]EBOB Binding

GABA allosterically inhibits [^{3}H]EBOB binding in rat brain membranes, with an IC_{50} value of 1.1 μM [16]. However, GABA failed to affect the potency of anisatin in inhibiting [^{3}H]EBOB binding when tested at 0.3 and 4.0 μM, GABA concentrations causing 25 and 75% inhibitions of [^{3}H]EBOB binding, respectively [16] (Fig. 4).

DISCUSSION

A diverse group of compounds (see Fig. 1 for examples) have been reported to date to act at the non-competitive

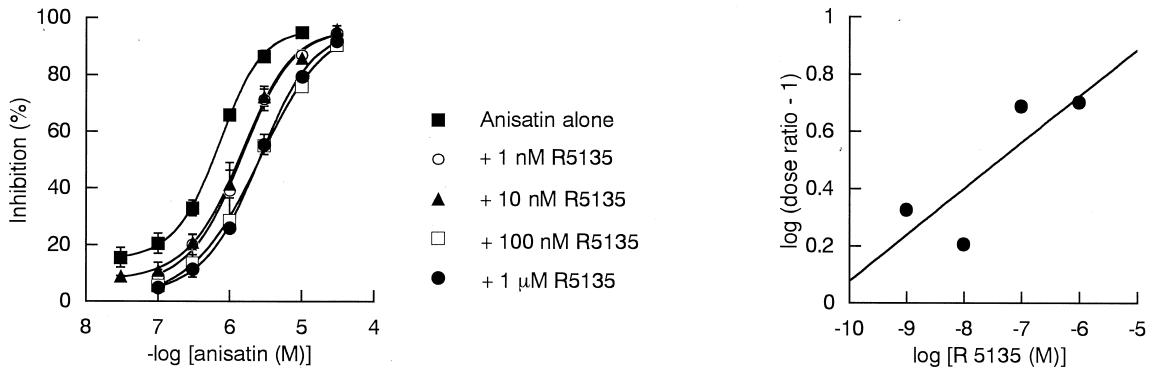

FIG. 2. Effects of R 5135 on the potency of anisatin in inhibiting specific [^{3}H]EBOB binding to rat brain membranes. Rat brain membranes were incubated with 0.5 nM [^{3}H]EBOB and anisatin in the absence and presence of R 5135 in 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. Left: anisatin concentration–inhibition curves in the absence and presence of R 5135. Right: a Schild plot with a least squares regression line. Data are means ± SD of two or three experiments, each done in duplicate.

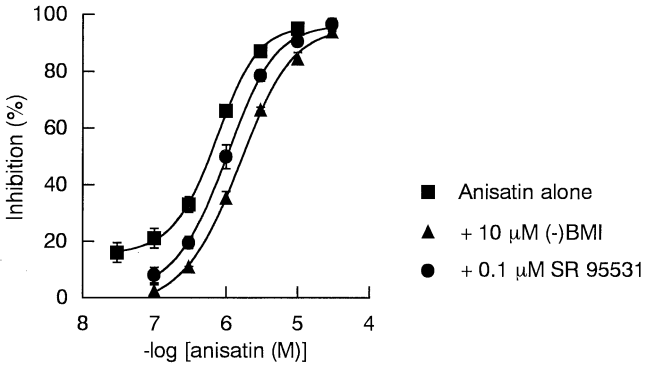

FIG. 3. Effects of SR 95531 and BMI on the potency of anisatin in inhibiting specific [^{3}H]EBOB binding to rat brain membranes. Rat brain membranes were incubated with 0.5 nM [^{3}H]EBOB and anisatin in the absence and presence of competitive antagonists in 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. Data are means ± SD of two experiments, each done in duplicate.

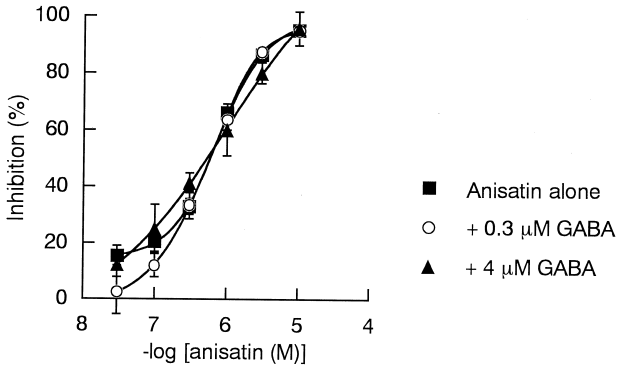

FIG. 4. Effects of GABA on the potency of anisatin in inhibiting specific [^{3}H]EBOB binding to rat brain membranes. Rat brain membranes were incubated with 0.5 nM [^{3}H]EBOB and anisatin in the absence and presence of GABA in 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. Data are means ± SD of two or three experiments, each done in duplicate.

antagonist-binding site of $GABA_A$ receptors to inhibit GABA-gated Cl^- currents. Three-dimensional quantitative structure–activity relationship studies have revealed that most of these non-competitive antagonists possess some common physico-chemical properties and structural aspects to allow for interaction with the same site [17]. However, the unique structure of anisatin, which has also been demonstrated to act as a non-competitive antagonist [6, 7], led us to speculate that the interaction site of anisatin might not be the same as that of other non-competitive antagonists. In this respect, it is interesting to consider the observations of Squires *et al.* [18] that R 5135 (tested at 10 nM) reverses the inhibitory effect of anisatin (tested at 100 nM) on the specific binding of [^{35}S]TBPS, a non-competitive antagonist, to rat brain membranes, while it has either no effect on or potentiates the inhibitory effects of other non-competitive antagonists. In the present study, we extended this experiment, showing that: (i) R 5135 decreases the potency of anisatin in inhibiting [^{3}H]EBOB binding in a negatively cooperative manner; (ii) other competitive antagonists tested also have effects similar to those of R 5135; (iii) R 5135 exerts little effect on the potencies of other non-competitive antagonists; and (iv) GABA does not affect the potency of anisatin. Thus, anisatin is clearly different from other non-competitive antagonists in responding to the actions of competitive antagonists on $GABA_A$ receptors.

TABLE 2. Effects of R 5135 on the potencies of non-competitive antagonists in inhibiting specific [^{3}H]EBOB binding to rat brain membranes

Compound	IC_{50} (nM) of non-competitive antagonist	95% CL	Hill coeff. n_H
EBOB	2.47	1.98–3.08	0.97
EBOB + R 5135	2.61	2.11–3.24	1.05
IPPO	488	383–622	0.82
IPPO + R 5135	313	240–408	0.73
PTX	398	319–496	1.00
PTX + R 5135	609	492–753	1.00
Dieldrin	77.5	60.3–99.8	0.76
Dieldrin + R 5135	70.9	54.9–91.6	0.72

Rat brain membranes were incubated with 0.5 nM [^{3}H]EBOB and various non-competitive antagonists in the absence and presence of 10 nM R 5135 in 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride. IC_{50} values of non-competitive antagonists with 95% confidence limits (CL) were obtained from mean values of two experimetns, each done in duplicate.

It has recently been suggested that bicuculline and SR 95531 act as allosteric inhibitors of the opening of $GABA_A$ receptor channels [19]. In recombinant $GABA_A$ receptors containing α1, β2, and γ2L subunits, these compounds inhibit Cl^- currents induced not only by GABA, but also by alphaxalone and pentobarbital, which bind to sites distinct from the GABA-binding site. These results indicate that the occupation of the GABA-binding site by competitive antagonists does not merely inhibit GABA binding but also induces conformational changes in $GABA_A$ receptors to inhibit the action of alphaxalone and pentobarbital. Consistent with this view, the binding of competitive antagonists probably induces conformational changes in the binding site of non-competitive antagonists as well, so that the affinity of anisatin is decreased while the affinities of other non-competitive antagonists remain unchanged. Meanwhile, GABA also induces conformational changes in $GABA_A$ receptors [18], but this change does not affect the affinity of anisatin.

Although the binding site of non-competitive antagonists has not yet been identified, it is thought to exist within GABA-gated Cl^- channels [20–22]. The binding region of anisatin might overlap with those of other non-competitive antagonists, but anisatin must interact with other specific region(s). Detailed studies of this interaction of anisatin with recombinant or brain $GABA_A$ receptors may help to determine whether the property of anisatin revealed in the present study is related to the analgesic or sedative effects of anisatin [5].

We thank Hirotsugu Tachibana for his technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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