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Possible Intermolecular Interaction between Quinolones and Biphenylacetic Acid Inhibits γ-Aminobutyric Acid Receptor Sites

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The combination of some new quinolone antibacterial agents with 4-biphenylacetic acid (BPAA), a metabolite of fenbufen, is known to specifically induce functional blockade of the γ-aminobutyric acid (GABA) receptors. The mechanisms of these drug interactions were further examined. Scatchard analysis of [^{3}H]muscimol binding to rat brain plasma membranes in the presence of enoxacin and BPAA revealed that a significant decrease in the number of muscimol binding sites was produced without affecting the affinity of binding to the receptors. In the presence of norfloxacin, BPAA inhibited muscimol binding the most potently of the six BPAA-related compounds tested. Fenbufen and 9,10-dihydro-γ-oxo-2-phenanthrenebutyric acid also inhibited the binding, and 4-biphenylcarboxylic acid and methyl 4-biphenylacetate inhibited it slightly, but 3-benzoylpropionic acid exhibited no competitive inhibition. Accordingly, hybrid molecules of norfloxacin and BPAA were synthesized for stereochemical analysis of these drug interactions. A hybrid with a -CONH$(CH_2)_3$- chain between norfloxacin and BPAA (flexible structure) inhibited muscimol binding, and intracisternal injection of this hybrid caused clonic convulsions in mice more potently than the combination of norfloxacin and BPAA did. In contrast, a hybrid linked by -CONH-(stretched structure) showed almost no such inhibitory effect. ^{1}H NMR analysis indicated the presence of intramolecular attraction at the quinoline ring of the hybrid exhibiting the antagonistic activity. These results suggest the possibility that quinolones and BPAA interact with the GABA receptor at nearby sites and that the binding affinity of quinolones to the GABA receptors is largely enhanced by the intermolecular interaction with BPAA.

The widespread use of new fluorinated quinolones such as ofloxacin, norfloxacin, enoxacin, and ciprofloxacin in the treatment of various bacterial infections has proven to be very effective. The effect of quinolones on the central nervous system (CNS), however, is a common adverse reaction; symptoms include headache, dizziness, and restlessness (10, 15, 17, 23). Although a variety of studies regarding the CNS effects of quinolones have already been conducted, it is difficult, at least for technical reasons, to show which nerve systems are primarily involved in these adverse reactions. While the incidences of CNS side effects are quite low, seizures have been observed more frequently in patients receiving quinolones in combination with nonsteroidal anti-inflammatory drugs such as fenbufen (5, 7, 30). This drug combination inhibits the binding of γ-aminobutyric acid (GABA) to its receptors (27, 33) and thereby abolishes GABA-induced electrophysiological changes, such as those involving chloride currents in the cerebral nerve cells (4, 16) and electroretinograms (36) of experimental animals. Therefore, the inhibition of the GABA receptors induced by quinolones would be expected to cause general CNS excitation (27, 33). However, the mechanism of synergistic binding inhibition of quinolones and 4-biphenylacetic acid (BPAA), a metabolite of fenbufen, is not fully understood (17). While BPAA sharply enhances quinolone-induced GABA receptor inhibition, BPAA itself does not inhibit GABA receptor binding at all.

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It has been shown, on the basis of a molecular modeling study, that accessible cationic and anionic sites separated by an interchange distance of about 5Å (0.5 nm) (zwitterionic form) and bulky additional binding sites (which are not essential for a specific interaction with the receptor but reinforce binding of the interaction core) are necessary to allow $GABA_A$ receptor antagonists such as gabazine, bicuculline, securinine, and R-5135 to act (25). We previously demonstrated the structure-epileptogenic activity relationship of quinolones, with special reference to their interaction with GABA receptor sites (2). The active site in the quinolone molecule responsible for the inhibition of GABA receptor binding was demonstrated to be, at least in part, the piperazine or aminopyrrolidine moieties at the 7 position of the parent molecule, which have structures similar to those of GABA receptor agonists (2, 32). Pitrazepin, a novel $GABA_A$ receptor antagonist (14), also possesses a piperazine moiety (Fig. 1). This has recently been suggested to play an important role in GABA receptor binding (9). Since the piperazine and aminopyrrolidine moieties of quinolones have a cationic nitrogen, we focused on the following question: how does combination with BPAA produce an appropriate anionic site and an antagonistic conformation for GABA receptor binding? The effects of six BPAA-related compounds on the GABA receptors were examined, and Scatchard analysis of muscimol binding was performed. On the basis of these data, a possible stacking formation between quinolones and BPAA was further analyzed by synthesizing hybrid molecules of norfloxacin and BPAA and by using ^{1}H nuclear magnetic resonance (NMR) analysis. A possible interaction between quinolones and BPAA resulting in inhibitory effects on muscimol binding to the GABA receptor sites is discussed.

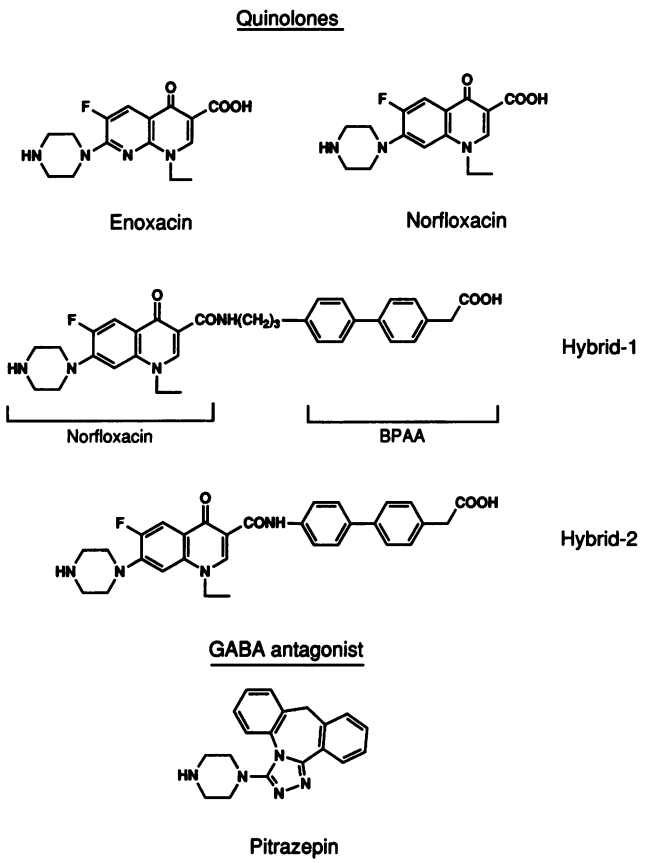

FIG. 1. Chemical structures of quinolones, norfloxacin-BPAA hybrid molecules, and a GABA antagonist, pitrazepin.

MATERIALS AND METHODS

Compounds and reagents. Norfloxacin, enoxacin, and methyl 4-biphenylacetate were synthesized at our institute. [^{3}H]Muscimol was purchased from Dupont, NEN Research Products (Boston, Mass.). All other reagents, including BPAA (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 4-biphenylcarboxylic acid (Aldrich), fenbufen (Lederle Japan, Ltd., Tokyo), 9,10-dihydro-γ-oxo-2-phenanthrenebutyric acid (Aldrich), and 3-benzoylpropionic acid (Aldrich), were commercial products of analytical grade. The chemical structures of these compounds are shown in Fig. 1 and 3. Methyl 4-biphenylacetate was diluted in dimethyl sulfoxide, and other compounds were dissolved in 0.1 N KOH for binding experiments.

Preparation of synaptic plasma membranes. Crude synaptic plasma membranes were prepared from the brains of female Sprague-Dawley rats by the method of Zukin et al. (39), as previously described (2). The whole brains were homogenized in 10 volumes of ice-cold 0.32 M sucrose and centrifuged at 1,000 × *g* for 10 min, and the supernatant was further centrifuged at 48,000 × *g* for 20 min. The resultant pellet was suspended in 0.05% Triton X-100, incubated for 30 min at 37°C (13), and washed three times in 50 mM Tris hydrochloride buffer (pH 7.1). The final suspension (2.5 mg of protein per ml) was stored at −80°C.

[^{3}H]Muscimol binding assay. The GABA receptor binding assay was performed as previously described (2). Briefly, 1-ml samples of a reaction mixture containing 100 μl of the membrane suspension, 200 μl of [^{3}H]muscimol (10 nM; specific activity, 20 Ci/mmol), 100 μl of each test compound, and the buffer were incubated at 4°C for 30 min. In the series of experiments for the Scatchard analysis (26), the final concentration of [^{3}H]muscimol was varied from 0.5 to 100 nM. The preparations were then quickly diluted by adding 10 ml of the ice-cold buffer and were filtered through glass fiber filters (GF/B; Whatman Inc., Clifton, N.J.). The filters were washed twice with an additional 5 ml of the buffer, and their radioactivities were counted with a liquid scintillation counter (LSC-903; Aloka Co., Ltd., Tokyo, Japan). Specific binding was defined as the difference between the levels of binding observed in the presence and in the absence of a large excess (1 mM) of unlabeled GABA (Tokyo Kasei Co., Tokyo, Japan). Results were expressed as the ratio of the specific binding in the presence of the test compound to that in its absence (percentage of control). For the Scatchard analysis, binding parameters were estimated by the least-squares method.

Synthesis of hybrid molecules of norfloxacin and BPAA. The carboxyl groups at the 3 position of norfloxacin and the *para* position of the phenyl ring of BPAA were linked by introducing a -CONH$(CH_2)_3$- (hybrid 1) or -CONH- (hybrid 2) chain (Fig. 1). The piperazine moiety of norfloxacin and the carboxyl group of BPAA were preserved in the hybrid molecules. Precursors of each hybrid have *t*-butoxycarbonyl and ethyl groups at cationic amine and anionic carboxylate moieties of the hybrid molecules, respectively. The inhibitory effects of these hybrid compounds on the $GABA_A$ receptor were examined by the same [^{3}H]muscimol binding assay.

Intracisternal injection of hybrid molecules into mice. Epileptogenic activity of hybrid molecules in mice was examined by intracisternal injection. Norfloxacin, BPAA, and hybrid molecules were dissolved in 0.1 N NaOH, and the solutions were adjusted to a pH of about 10. For combination of these compounds, injection solution was made by mixing the drug solutions together. Ten microliters of the drug solutions was intracisternally injected into 3-week-old male ddY mice by the method described previously (35). The incidence of clonic convulsion and the subsequent death of each mouse were recorded for 2 h.

Measurement of ^{1}H NMR spectra of hybrid molecules. Finally, possible intramolecular attractions of the hybrid molecules were examined by utilizing ^{1}H NMR analysis. Hydrogen peaks in the ^{1}H NMR (400 MHz) spectra of the hybrids and their precursors were compared. These compounds were dissolved in dimethyl-d_6 sulfoxide.

RESULTS

Scatchard analysis of [^{3}H]muscimol binding to GABA receptor sites. Binding sites of [^{3}H]muscimol can be mainly characterized with regard to a dissociation constant (K_d), indicating a binding affinity to GABA receptor sites, and a maximum number of binding sites by the Scatchard analysis. To examine whether combinations of quinolones and BPAA modulate the GABA receptor sites, we studied the effects of drug combination on the Scatchard plots. When concentrations of the [^{3}H]muscimol in the binding assay were varied from 0.5 to 100 nM, the amount of specific binding reached a plateau, and the amount of nonspecific binding remained within 16% of the amount of total binding (data not shown). The Scatchard plots of data obtained from one of the experiments are shown in Fig. 2. The analysis of the binding data from controls and from the combination of enoxacin (10^{-6} M) and BPAA (10^{-5} M) revealed linear plots with K_d values of 52.1 ± 3.1 nM and 73.5 ± 7.2 nM (mean ± standard error; n = 3), respectively. Thus, the synaptic membranes prepared from rat brain tissue were characterized as having a single class

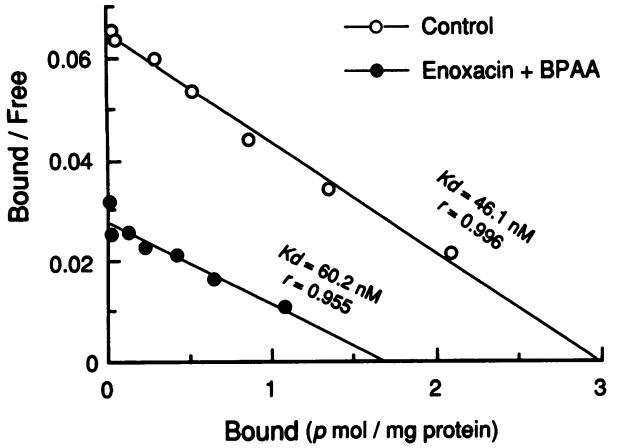

FIG. 2. Scatchard plots of [^{3}H]muscimol binding to rat brain plasma membranes. This graph represents one of the three independent experiments performed in the presence or absence of BPAA (10^{-5} M) and enoxacin (10^{-6} M). Each point is the average of values from triplicate assays.

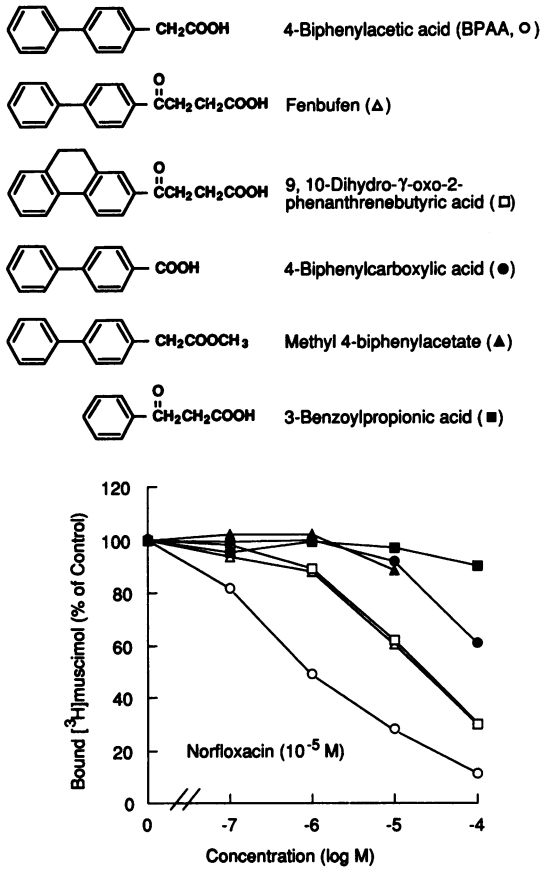

FIG. 3. Displacement of [^{3}H]muscimol binding to rat brain plasma membranes by BPAA-related compounds in the presence of norfloxacin. Each point is the average of values from triplicate assays.

of low-affinity binding sites. While the drug combination reduced the binding affinity of muscimol by approximately 40%, these changes in the K_d values were not significant. In contrast, the maximum number of binding sites for the controls was estimated to be 3.07 ± 0.13 pmol/mg of protein and that for the combination was estimated to be 1.78 ± 0.08 pmol/mg of protein (a 72% decrease compared with the control value; $P < 0.01$) on the basis of three separate experiments. The K_d values did not change upon the addition of BPAA alone (data not shown). Therefore, the combination of enoxacin and BPAA had a minimal effect on the binding affinity of muscimol for the GABA receptor sites, but it caused a significant reduction of the number of binding sites.

Effect of BPAA-related compounds on [^{3}H]muscimol binding to GABA receptor sites. The structure-activity relationship of BPAA with respect to GABA receptor inhibition was next examined by the [^{3}H]muscimol binding assay. A higher concentration of norfloxacin (10^{-5} M) was used to enhance the inhibitory effects of BPAA-related compounds. For the six compounds tested, BPAA inhibited muscimol binding most potently at concentrations of 10^{-7} M or more (Fig. 3). Fenbufen and 9,10-dihydro-γ-oxo-2-phenanthrenebutyric acid, which has a rigid planar biphenyl ring, inhibited the binding to very similar degrees in a dose-dependent manner. Compared with these compounds, 4-biphenylcarboxylic acid, which has a linear axis in its chemical structure, and methyl 4-biphenylacetate, which is an ester form of BPAA, were found to exhibit weaker inhibitory activities. Meanwhile, in the case of 3-benzoylpropionic acid, a desphenyl form of fenbufen, no competitive inhibition was observed even at the highest concentration, 10^{-4} M. Therefore, the inhibitory activities of BPAA and fenbufen were decreased by introducing methyl ester into the carboxyl group and by converting the BPAA structure to a linear chemical structure, respectively. In addition, the planar biphenyl form of fenbufen was found to be necessary to produce the interaction with norfloxacin.

Synthesis of norfloxacin-BPAA hybrid molecules and their inhibitory activity on [^{3}H]muscimol binding. The results of these two experiments together with our previous findings (2) suggest that a piperazine moiety of the quinolones and a carboxyl group of BPAA play important roles in the synergism of GABA receptor inhibition and that the intermolecular interactions between quinolones and BPAA may play a part in the inhibitory effects. We thought that in such cases the two moieties would bind to GABA receptor sites and therefore synthesize norfloxacin-BPAA hybrid molecules in which the chemical structures essential for both drugs to exhibit the synergistic inhibitory effect would be preserved (Fig. 1). Hybrid 1 is designed to be capable of a parallel relationship between the quinolone and biphenyl rings (flexible structure). Hybrid 2 cannot form such a folded structure; instead, it forms a stretched structure. While BPAA alone did not inhibit [^{3}H]muscimol binding, norfloxacin alone and norfloxacin in combination with equal molecular concentrations of BPAA inhibited the binding at 10^{-5} M and above and at 10^{-6} M and above, respectively (Fig. 4), as previously reported (2). Meanwhile, hybrid 1 itself sharply inhibited muscimol binding at concentrations as low as 10^{-7} M and exhibited stronger inhibitory effects than the combination of norfloxacin and BPAA. Further combination of hybrid 1 with norfloxacin or BPAA did not enhance inhibition of the binding at all. In contrast, hybrid 2 alone did not inhibit the binding even at 10^{-5} M. The binding inhibition induced by hybrid 2 in combination with norfloxacin or BPAA was less than one-tenth of that induced by the combination of norfloxacin and BPAA. In other words, hybrid 2 did not enhance the inhibitory effects of norfloxacin on muscimol binding.

Norfloxacin-BPAA hybrid molecule induces epileptogenic activity in mice. We examined whether the synthesized hybrid 1 molecule has epileptogenic activity in mice. When 10 nmol

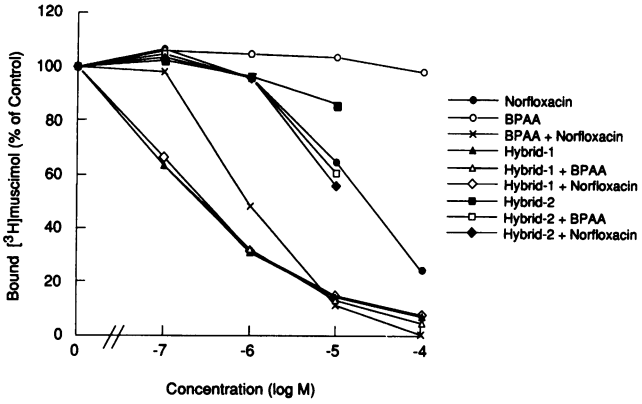

FIG. 4. Displacement of [^{3}H]muscimol binding to rat brain plasma membranes by norfloxacin-BPAA hybrid molecules in the presence of norfloxacin and BPAA. Each point is the average of values from triplicate assays.

each of norfloxacin and BPAA dissolved in the same solution were administered intracisternally, clonic convulsions were observed at approximately 1 min after the injection (Table 1). Injection of hybrid 1 caused clonic convulsions at 7 min after the injection and subsequent death at a dose as low as 0.1 nmol. The time to onset with hybrid 1 was slower than that with the combination of norfloxacin and BPAA, probably suggesting slower tissue penetration rates for hybrid 1. While we could not test 10 nmol of hybrid 2 because of its low solubility, 1 nmol of hybrid 2 did not induce any convulsions under the same conditions. Norfloxacin or BPAA alone (10 nmol) did not induce clonic convulsions. The epileptogenic activity of norfloxacin and the hybrid molecules thus is closely related to their inhibitory activity against GABA receptor binding as shown in Fig. 4.

Norfloxacin-BPAA hybrid molecule presents chemical shift of ^{1}H at quinoline moiety. We finally measured the ^{1}H NMR spectra of the norfloxacin-BPAA molecules and the precursor compounds (Fig. 5). The precursors of hybrid 1 and hybrid 2 have bulky *t*-butoxycarbonyl and ethyl groups at crucial positions of the norfloxacin and BPAA moieties for $GABA_A$ receptor inhibition. These precursors therefore do not inhibit muscimol binding (data not shown). The assignable peaks of Ha, Hb, and Hc in the spectra of the norfloxacin moiety of hybrid 1 appeared in a higher field than the corresponding peaks in those of hybrid 2. The shifted values of Ha, Hb, and Hc ($\Delta\delta$) were 0.34 ppm, 0.25 ppm, and 0.25 ppm, respectively. In contrast, a minimal to slight shift of the hydrogen peaks between precursors for hybrid 1 and precursors for hybrid 2 was observed. While the total peak values for hydrogens on the BPAA moiety of hybrid 1 were significantly smaller than those for hydrogens on the BPAA moiety of hybrid 2, we could not assign the peak corresponding to each structure (data not shown). These results thus suggest that the norfloxacin moiety of hybrid 1 has an intramolecular attraction greater than that of hybrid 2.

TABLE 1. Epileptogenic activity of norfloxacin-BPAA hybrid molecules in mice

Treatment	Dose (nmol)[a]	Result[b]		
		Clonic convulsion		Death (no. of mice)[c]
		No. of mice[c]	Onset time (min)[d]	
Norfloxacin	10	0		0
BPAA	10	0		0
Norfloxacin + BPAA	1 + 1	0		0
	10 + 10	4	1.3 ± 0.3	0
Hybrid 1	0.01	0		0
	0.1	4	7.0 ± 2.8	2
	1	4	2.8 ± 0.5	4
Hybrid 2	1	0		0

[a] For combination experiments compounds were prepared separately and mixed with injection solution (10 μl).
[b] Groups of four mice received compounds intracisternally, and epileptogenic activity in each mouse was recorded for 2 h.
[c] Number of mice of the four receiving the compound(s) that developed the response.
[d] Data are means ± standard errors.

Compound	^{1}H-Chemical shift (δ ppm)		
	Ha	Hb	Hc
3 (Hybrid-2)	9.10	8.13	7.30
1 (Hybrid-1)	8.76	7.88	7.05
Δδ	0.34	0.25	0.25
4 (Hybrid-2 precursor)	8.95	7.98	7.19
2 (Hybrid-1 precursor)	8.78	7.91	7.13
Δδ	0.17	0.07	0.06

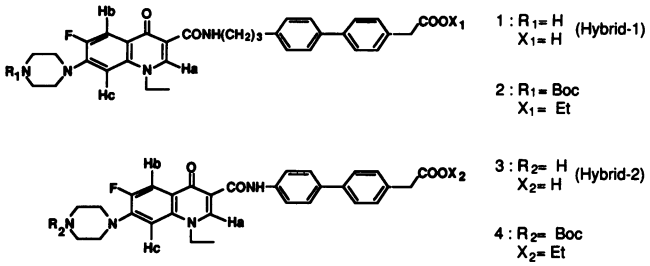

FIG. 5. ^{1}H NMR spectral data for norfloxacin-BPAA hybrid molecules and the related precursor compounds. Δδ, values of the peak spectrum shifted between two hybrid molecules. Compounds were dissolved in dimethyl sulfoxide. Boc, *t*-butoxycarbonyl; Et, ethyl.

DISCUSSION

The $GABA_A$ receptor-mediated drug interaction of quinolones and BPAA is not fully understood, despite the vast number of recent studies (2–4, 11, 12, 16, 17, 19, 27, 31, 33, 37). While BPAA itself does not bind to the $GABA_A$ receptor, it dramatically enhances norfloxacin-induced inhibition of muscimol binding. More than 50% of the binding was inhibited at the combined concentration of 10^{-6} M each (Fig. 4), which is the achievable concentration in the brains of mice developing clonic convulsions (34, 37). There are at least three possible mechanisms by which the synergistic binding inhibition of the $GABA_A$ receptor occurs. The first possibility is an independent action of both drugs. BPAA may, for instance, impair the structure of muscimol, and quinolone binds to the $GABA_A$ receptor more easily. The second possibility is that BPAA binds to its own receptor sites to alter the $GABA_A$ receptor conformation, which becomes more suitable for quinolone (31). Each compound, in this case, binds to different affinity sites of the same receptor molecule and/or associated molecules. The third possibility is that an association complex of the two drugs becomes a potent inhibitor without influencing the receptor characteristics. The first and second possibilities were examined by Scatchard analysis of muscimol binding in the

presence or absence of enoxacin and BPAA, which indicated the existence of a single population of receptor sites. The apparent K_d for muscimol, which represents the binding affinity, was similar to that previously reported (8, 18). Combination of enoxacin and BPAA inhibited the muscimol binding without significant changes in the binding affinity of muscimol to GABA receptor sites. While Scatchard analysis is not the only way to characterize the binding sites, the fact that enoxacin and BPAA did not affect K_d values of muscimol binding may indicate that BPAA does not alter the receptor conformation of the low-affinity sites to make them more appropriate for enoxacin, which has a piperazine moiety structurally related to a GABA receptor agonist, isonipecotic acid (22).

In order to assess the third possibility, we need to better understand the structure-activity relationships of the stereochemistry of $GABA_A$ receptor antagonists, which were recently reviewed (25). Molecular modeling studies indicate that antagonistic structure requires accessible cationic and anionic sites separated by an intercharge distance of about 5 Å (0.5 nm). These zwitterionic sites are also essential for potent $GABA_A$ receptor agonists such as muscimol and 4,5,6,7-tetrahydroisoxazolo (5,4-c) pyridin-3-ol (6, 21). Antagonistic structure also requires bulky *additional* binding sites, which are not essential for a specific interaction with the receptors but reinforce binding of the interaction core. These bulky additional binding sites of antagonists may inhibit serial events which occur following the binding of GABA to its receptors. $GABA_A$ receptor antagonists, including pitrazepin, gabazine, bicuculline, and securinine, can be characterized sufficiently by these two structural requirements (25). The molecular structure of pitrazepin (Fig. 1) has been determined by X-ray diffraction. It was suggested that the triazole ring and the secondary amine of the piperazine ring might play the same role as the anionic carboxylate and the cationic ammonium group in the GABA molecule (9). We previously indicated that an unsubstituted piperazine ring of quinolones is necessary for the inhibition of $GABA_A$ receptor binding (2). The piperazine ring of quinolones therefore appears to serve as the cationic site of the antagonistic structure.

A remaining point of interest in GABA receptor inhibition is how the anionic site and the additional binding sites of the antagonistic structure are produced by the combination of quinolones and BPAA. To examine the structure-activity relationships of BPAA, the inhibitory activities of BPAA-related compounds, including fenbufen, 9,10-dihydro-γ-oxo-2-phenanthrenebutyric acid, methyl 4-biphenylacetate, 4-biphenylcarboxylic acid, and 3-benzoylpropionic acid, against muscimol binding were compared. The results revealed that a free carboxyl group and not a phenyl ring but a planar biphenyl ring were structures essential for BPAA to exhibit interaction with norfloxacin. These results present the possibility that the carboxyl group of BPAA may serve as the anionic site for GABA receptor interaction. If this is the case, BPAA has to come close enough to the quinolone molecule to produce the zwitterionic core with a 5-Å (0.5-nm) intercharge distance from the ammonium group of quinolones in order to bind to the $GABA_A$ receptors.

To explore this unique possibility we synthesized norfloxacin-BPAA hybrid molecules by linking the carboxyl group of norfloxacin and the phenyl ring of BPAA. Hybrid 1, with its flexible structure between the two components, inhibited receptor binding and caused convulsions in mice more potently than the combination of norfloxacin and BPAA. The 50% inhibitory concentration of hybrid 1 for muscimol binding is as low as 0.3 μM, which is comparable to that of pitrazepin (25). In contrast, hybrid 2, which has a stretched structure between the norfloxacin and BPAA moieties, completely lost the inhibitory effect on GABA receptor binding. The epileptogenic activity of hybrid 1 was more than 100 times stronger than that of hybrid 2, suggesting that separation of the carboxyl group from the piperazine group in the hybrid 2 molecule produces a structure inactive for GABA receptor binding. In addition, physicochemical examination using ^1H NMR analysis revealed that hydrogens in the quinoline ring of the hybrid 1 molecule but not those in that of the hybrid 2 molecule interact with intramolecular moieties. When a proton approaches the face of a benzene ring, the proton peak in the NMR spectrum appears in a higher field (20). Although with only two compounds, hybrid 1 and hybrid 2, it is impossible to make many conclusions, these results suggest the possibility that the carboxyl group of the BPAA moiety in hybrid 1 approaches the piperazine ring to serve as the anionic site for GABA receptor interaction. This intramolecular interaction may also be responsible for the dramatically enhanced inhibition of muscimol binding induced by norfloxacin in combination with BPAA and for the occurrence of clonic convulsions, mediated through the third mechanism. If the first and second mechanisms mentioned above, in which quinolones and BPAA interact with independently distant binding sites, are primarily operating, hybrid 1 and hybrid 2 may exhibit similar inhibitory effects because presumably active moieties, i.e., the 7 position of norfloxacin and the carboxylate of BPAA, are equally conserved in each molecule. However, we cannot exclude the possibility that the different inhibitory effects observed for the two hybrids are due to the different lengths of the bridges connecting the norfloxacin and BPAA moieties rather than to flexibility in the molecules.

Even if these speculations about the role of BPAA are correct, we still do not know why the existence of the loop structure of hybrid 1 becomes possible or whether the combination of norfloxacin and BPAA can also have a coupled structure similar to that of hybrid 1 at the GABA receptor sites. While it is nothing more than a hypothesis, one possible answer to these questions, based on the results of the present study, might be a stacked aromatic ring formation with a folded conformation between the norfloxacin and BPAA moieties in the hybrid 1 molecule (Fig. 6). The planar heteroaromatic ring in quinolones and the biphenyl ring of BPAA may reach a parallel conformation by means of the π-π interaction. If so, the secondary amine of the piperazine ring in quinolones and the carboxyl group in BPAA approach to positions close enough to enable them to produce an accessible interaction core with the $GABA_A$ receptor and serve as cationic and anionic sites, respectively. These sites resemble isonipecotic acid, a GABA receptor agonist, in their intercharge distance (Fig. 6). The loop of the molecule, with its stacked aromatic rings, would act as a bulky additional binding site reinforcing the receptor binding. Support for the feasibility of such a stacking hypothesis is provided by other studies, which showed that compounds with a folded conformation of stacked aromatic rings were energetically stable (24). While this drug interaction model entails a lot of additional experiments, such as NOE ^1H NMR and X-ray analysis, data from the present experiments as well as data from previous reports on other nonsteroidal anti-inflammatory drugs (28, 37) will support this hypothesis. Introduction of a methyl group into the piperazine ring of the quinolone, especially at the secondary amine of the ring, as in the case of ofloxacin, reduces the inhibition of GABA receptor binding because of increased steric hindrance of the cationic site (2). A planar biphenyl ring (which occurs in BPAA and naproxen) and structures similar to it (possibly

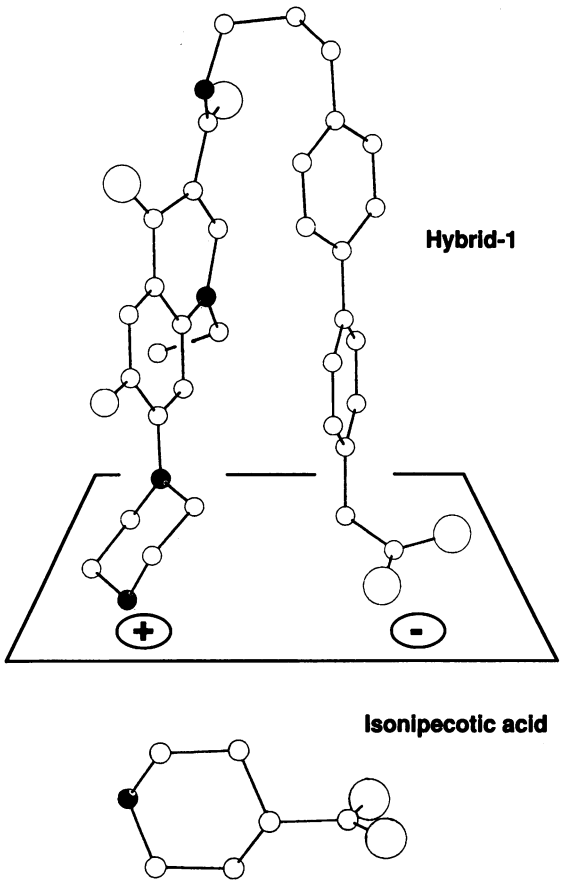

FIG. 6. Presumed possible conformation of the norfloxacin-BPAA hybrid-1 molecule at the $GABA_A$ receptor site. The secondary amine of the piperazine ring and the carboxyl group of the BPAA moiety may serve as cationic and anionic sites, respectively. The planar quinoline ring and the biphenyl ring may adopt parallel conformation, by means of the π-π interaction. As a result, the cationic and anionic sites approach to positions close enough to enable them to produce the accessible interaction core with the $GABA_A$ receptor. These sites resemble isonipecotic acid in their intercharge distance. The looped upper part, with its stacked aromatic rings, is thought to act as a additional binding site that reinforces the receptor binding (25).

provided by fenoprofen and indomethacin) attached with an acetic acid group might be proper structures for stacking formation, and esterization of the carboxyl group of BPAA is critical for the inhibitory effects. BPAA, which enhances the binding inhibition most, may provide better conformation than fenbufen or 4-biphenylcarboxylic acid for the production of the anionic site. It is known that introduction of a bulky aromatic group in the 1 position of the quinolone molecule causes a decrease in GABA receptor inhibition. Tosfloxacin, which has a difluorophenyl moiety at the 1 position of the quinoline ring, exhibits much weaker binding inhibition than norfloxacin, enoxacin, and ciprofloxacin, which have an ethylene or a cyclopropane moiety at the same position (2, 19). The size of this moiety would sterically hinder the stacking formation and/or the interaction with the additional binding site.

The torsion angle between the quinoline ring and the piperazine ring with an extended chair form in ofloxacin was found to be approximately 53 degrees by X-ray analysis (38). It is, however, unclear whether the piperazine ring of quinolones would rotate to the planar conformation for the quinoline ring, resulting in the formation of the stable association between quinolones and BPAA. Further physicochemical experiments should eventually enable us both to solve this problem and to determine whether quinolones and BPAA can form stacked associates at GABAergic synaptic junctions.

Possible regulation of quinolone neurotoxicity by modulation of other neurotransmitter systems such as the glutamate receptors was not addressed in our studies. Adverse reactions induced by quinolones themselves are not explained by our hypothesis. In addition, while combinations of quinolones and BPAA do not seem to directly interact with the glutamate receptors biochemically or electrophysiologically (11, 29), intrathecal injection of glutamate antagonists rather than GABA agonists protects mice against convulsions developed by combination of quinolones and BPAA (1). Therefore, it is likely that synergistic inhibition of the $GABA_A$ receptor occurs directly by means of the two-drug combination and that subsequent convulsions may develop through the involvement of a variety of neuron networks. In conclusion, the present results suggest that the inhibitory activity of quinolones on the GABA receptor sites, in the presence of BPAA, may be due to the intermolecular interaction with the BPAA molecule.

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