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Effect of the anti-inflammatory agent fenbufen on the quinolone-induced inhibition of γ-aminobutyric acid binding to rat brain membranes *in vitro*

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Quinolonecarboxylic acid derivatives (quinolones*), used in the oral treatments of urinary, biliary, intestinal and pulmonary tract infections, are known to elicit epileptogenic neurotoxic effects, such as headache, dizziness, nausea, vomiting and paroxysmal epilepsy. The mechanism of these quinolone-induced side-effects has not been established. Squires and Saederup [1] previously reported that two quinolones, i.e. norfloxacin and pipemidic acid, reverse the inhibitory effect of γ-aminobutyric acid (GABA) on the binding of [^{35}S]butylbicyclophosphorothionate (TBPS) to rat brain membranes, suggesting that these quinolones are GABA antagonists. Moreover, we have demonstrated recently [2] that several quinolones inhibit the specific binding of [^{3}H]GABA to synaptic plasma membranes from rat brains in a concentration-dependent manner. Since GABA is a major inhibitory neurotransmitter in the mammalian central nervous system, these results indicate that quinolones, as well as penicillins and cephalosporins [3–5], may induce seizures through the inhibition of postsynaptic GABA function. Recently, it was reported that several patients had severe clonic convulsions during therapy with enoxacin (a quinolone) and fenbufen, one of the non-steroidal anti-inflammatory propionic acid derivatives, and it was proposed that the concomitant use of these two drugs should be avoided [6]. Since no cases of neurotoxic side-effects of fenbufen itself have been reported and, moreover, the pharmacokinetic behavior of a new quinolone was not altered by coadministration of fenbufen in rats (unpublished observation), it is reasonable to assume that simultaneously administered fenbufen enhanced the neurotoxic potency of enoxacin via some pharmacodynamic interaction in the brain. It is important to clarify the mechanism of this interaction, because similar drug interactions between fenbufen (or its analogues) and other quinolones may arise in clinical therapeutics. In this work, therefore, we examined whether or not fenbufen could enhance the inhibitory effects of several quinolones (Fig. 1) on specific GABA binding to rat brain membranes *in vitro*, in order to obtain an insight into the mechanism of the adverse *in vivo* interaction of enoxacin and fenbufen.

Materials and methods

4-Amino-*n*-[2,3-^{3}H]butyric acid (GABA) was purchased from Amersham International Ltd. (Buckinghamshire, U.K.). Ciprofloxacin, enoxacin, norfloxacin and pipemidic acid were synthesized at the Central Research Laboratory, Hokuriku Seiyaku Co., Ltd. (Fukui, Japan). Nalidixic acid and ofloxacin were supplied by Daiichi Seiyaku Co., Ltd. (Tokyo, Japan) and cinoxacin by Shionogi & Co., Ltd. (Osaka, Japan). The purities of these quinolones were determined by high-performance liquid chromatography to be more then 99.5%. All other reagents were commercial products of analytical grade and were not purified further.

Synaptic plasma membranes were prepared by the method of Zukin *et al.* [7] from the brains of Sprague–Dawley rats (Nihon Clea, Tokyo, Japan) weighing 230–270 g. The specific bindings of [^{3}H]GABA to the synaptic plasma membranes were determined after incubation in the absence of Na^{+} at 4°, because the sodium- and temperature-dependent binding of GABA is known to represent binding to the uptake sites [8] which transport GABA into nerve terminals and glia [9]. Binding experiments were performed as follows. First, aliquots (1 ml) of the synaptic plasma membranes (1 mg protein/ml) were incubated at 4° for 20 min in 50 mM Tris-citrate buffer (pH 7.1) (designated as buffer A) containing [^{3}H]GABA (15 nM) and various concentrations of enoxacin in the absence and presence of 100 μM fenbufen. Second, to examine the effects of fenbufen on the inhibition of specific [^{3}H]GABA binding by other quinolones, the synaptic plasma membranes were incubated at 4° for 20 min in buffer A containing [^{3}H]GABA (15 nM) and a quinolone (10 μM), i.e. cinoxacin, ciprofloxacin, nalidixic acid, norfloxacin, ofloxacin or pipemidic acid, in the absence and presence of 100 μM fenbufen. Third, to examine the effect of fenbufen itself on [^{3}H]GABA binding, the membrane suspensions were incubated with [^{3}H]GABA (15 nM) and fenbufen (100 μM or 1 mM) at 4° for 20 min. As a control experiment, the membrane suspensions were incubated under the same conditions as described above with [^{3}H]GABA alone (without a quinolone or fenbufen). After incubation, the reaction was terminated by filtering 0.8 ml of the assay mixture under low vacuum through Whatman GF/C filters moistened with ice-cold buffer A. The filters were then washed four times with 5-ml portions of ice-cold buffer A. The radioactivity on the filters was determined in 10 ml of scintillation fluid (670 ml of toluene, 330 ml of Triton X-100, 5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene) by means of a liquid scintillation counter (LSC-1000; Aloka Co., Ltd., Tokyo, Japan) with a counting efficiency of approximately 54%. Nonspecific binding was determined in the presence of a large excess (1 mM) of unlabeled GABA. Bindings of [^{3}H]GABA were assayed at least in quintuplicate and evaluated as specific bindings by subtracting the nonspecific binding which was approximately 22% of the total binding. The results obtained were then expressed as specific binding percent (designated as percent of control) of the radiolabeled ligand, which is the ratio of the specific binding in the presence of a quinolone to that in the control experiment (without a quinolone or fenbufen).

Results and discussion

In a previous paper [2], it was indicated that several epileptogenic quinolones competitively inhibit the sodium-independent specific binding of [^{3}H]GABA to synaptic plasma membranes from rat brains. These quinolones may be involved in the induction of epileptogenic side-effects, because a GABA "antagonistic" property of norfloxacin and pipemidic acid has been suggested by Squires and Saederup [1] on the basis of the reversal by these drugs of the inhibitory action of GABA on [^{35}S]TBPS binding to rat brain membranes. Recent case reports on the occurrence of severe clonic convulsions during therapy with enoxacin and fenbufen prompted us to examine the *in vitro* effects of fenbufen on the quinolone-induced inhibition of GABA binding to its receptor sites in the brain. The data shown in Fig. 2 demonstrate that fenbufen markedly enhanced the inhibitory effect of enoxacin on the sodium-independent [^{3}H]GABA binding to synaptic plasma membranes. Figure

* Abbreviations: quinolones, quinolonecarboxylic acid derivatives; GABA, γ-aminobutyric acid; TBPS, butylbicyclophosphorothionate.

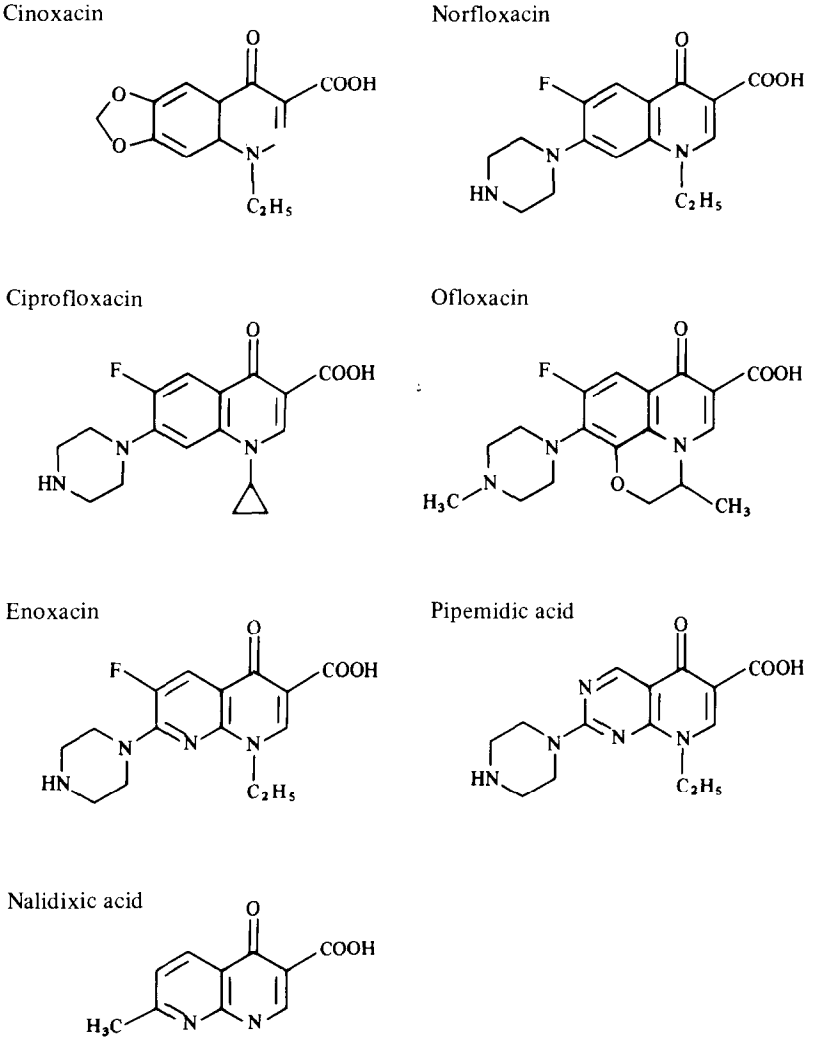

Fig. 1. Chemical structures of quinolonecarboxylic acid derivatives.

2 indicates that the IC_{50} value of enoxacin was decreased in the presence of fenbufen, although the [^{3}H]GABA binding in the presence of 100 μM fenbufen alone (without enoxacin) was determined to be 85.4 ± 8.8% (mean ± SEM, N = 4) and was not significantly different from the control binding (100%). The observation that a relatively high concentration of fenbufen (100 μM) did not cause a significant inhibition of [^{3}H]GABA binding is in agreement with the fact than no clinical cases of epileptogenic side-effects of fenbufen after single administration have been reported. The [^{3}H]GABA binding in the presence of fenbufen (without a quinolone) at an extremely high concentration of 1 mM was determined to be 56.0 ± 5.5% (mean ± SEM, N = 5), indicating that the inhibition of GABA binding by fenbufen was concentration-dependent. The marked inhibition of [^{3}H]GABA binding by quinolones in the presence of fenbufen could be attributed for the most part to the influence of fenbufen on specific [^{3}H]GABA binding, because the nonspecific [^{3}H]GABA binding constituted approximately 22% of the total [^{3}H]GABA binding under the experimental conditions used. The present study could not establish how fenbufen acts specifically on the $GABA_A$ receptor. However, an apparent increase in the affinity of quinolones for the GABA receptor caused by fenbufen may be due to: (1) an allosteric action of fenbufen via interaction with some region of the GABA/benzodiazepine receptor–chloride channel complex other than the $GABA_A$ receptor, or (2) a molecular interaction of fenbufen and quinolones that may result in potentiating the binding of quinolones to the $GABA_A$ receptor.

Conformationally restricted analogs of GABA such as muscimol [10], 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) [10] and isoguavacin [11] are potent GABA agonists at the $GABA_A$ receptor site which is bicuculline sensitive [12]. All known GABA receptor agonists contain within their structure a sequence that can be superimposed on the GABA molecule [13], whereas the structural requirements that discriminate agonists from antagonists at the $GABA_A$ receptor are still unclear [14]. On the other hand, competitive antagonists at the $GABA_A$ receptor include 3-(piperazinyl-1)-9*H*-dibenz(*c,f*)triazolo(4,5-*a*)azepin or pitrazepin [15], and arylaminopyridazine derivatives such as 2-(carboxy-3'-propyl)-3-amino-4-methyl-6-phenylpyridazinium chloride or SR 95103 [16]. Squires and Saederup [17] examined the properties of thirteen GABA antagonists, including pitrazepin and SR 95103, and discussed some structural features (appropriately situated aromatic ring and nitrogen and ether groups) that seemed to increase GABA antagonistic potency. It has been proposed that the reversal of the inhibitory effect of GABA on [^{35}S]TBPS binding to rat brain membranes demonstrates GABA antagonistic

Table 1. Effect of fenbufen on the quinolone-induced inhibition of [^{3}H]GABA binding to rat synaptic plasma membranes

Drug	Percent of control binding		% Decrease in [^{3}H]GABA binding
	Without fenbufen	With fenbufen	
Cinoxacin	100.6 ± 5.0 (7)	72.8 ± 6.5* (5)	27.6
Ciprofloxacin	95.3 ± 4.5 (4)	50.8 ± 2.0† (4)	46.7
Enoxacin	91.2 ± 7.4 (11)	40.1 ± 3.5† (5)	56.0
Nalidixic acid	86.0 ± 5.6 (8)	54.9 ± 3.9‡ (5)	36.1
Norfloxacin	61.4 ± 3.3 (9)	31.2 ± 2.9† (5)	49.1
Ofloxacin	94.8 ± 2.8 (7)	64.0 ± 3.3† (4)	32.5
Pipemidic acid	98.4 ± 2.6 (10)	69.1 ± 3.6† (5)	29.8

[^{3}H]GABA (15 nM) and each quinolone (10 μM) were incubated with rat synaptic plasma membranes in 50 mM Tris–citrate buffer (pH 7.1) at 4° for 20 min. Values are means ± SEM. Each number in parentheses is the number of assays. The percent decrease in [^{3}H]GABA binding is an index of the augmentative effects of fenbufen on the quinolone-induced inhibition of GABA binding.

*–‡ The significance of differences between the binding experiments in the absence and presence of 100 μM fenbufen was assessed by means of Student's *t*-test: * $P < 0.01$, and † $P < 0.001$, and ‡ $P < 0.05$.

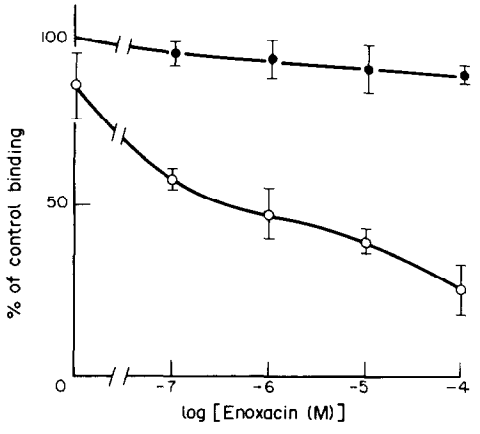

Fig. 2. Effect of fenbufen on the inhibition of [^{3}H]GABA binding to rat synaptic plasma membranes by enoxacin. [^{3}H]GABA (15 nM) and various concentrations of enoxacin were incubated with synaptic plasma membranes for 20 min at 4° in the absence (●) and presence (○) of 100 μM fenbufen. Each point and vertical bar represent the mean ± SEM (N = 5–11).

activity [18]. Recently Dalkara *et al.* [19] described novel $GABA_A$ blockers containing a mono *N*-aryl piperazine structure in their molecule, such as *N*-(*o*-chlorophenyl)-piperazine and *N*-piperazine(*p*-acetophenone); activity was demonstrated by their abilities to reverse the inhibitory action of GABA on [^{35}S]TBPS binding to rat brain membranes and by an iontophoretic (population spike) test in the rat hippocampus. Strikingly, a conspicuous structural similarity exists between these $GABA_A$ blockers and the quinolones except for cinoxacin and nalidixic acid, supporting the possibility that the quinolones possess $GABA_A$ antagonistic activity. Furthermore, it is noteworthy that oxolinic acid, a structurally related quinolone, produces insomnia in humans as well as locomotor stimulation and stereotypic behavior in rats and mice, and that diazepam antagonizes the stimulant properties of oxolinic acid [20]. These findings are certainly consistent with our present findings. However, in contrast to the inhibitory effect of quinolones on GABA binding, it is still not known how fenbufen interacts with the GABA receptors.

The effects of fenbufen on the inhibition of [^{3}H]GABA binding by several quinolones were expressed as percent decrease in [^{3}H]GABA binding, and are summarized in Table 1. Since [^{3}H]GABA binding was not inhibited markedly by a 10 μM concentration of the quinolones except for norfloxacin, this concentration of quinolones was chosen for evaluation of the effect of fenbufen on the quinolone-induced inhibition of GABA binding. Comparison of the extent of [^{3}H]GABA binding (as percent of control) in the presence of a quinolone (10 μM) between the experiments performed without and with 100 μM fenbufen revealed that the augmentative effect of fenbufen on the quinolone-induced inhibition of GABA binding, i.e. the decrease (ranging from 30–60% of the control binding) in GABA binding caused by fenbufen, was prevalent among all the quinolones examined and greatest in the case of enoxacin. This result indicates the possible occurrence of similar adverse interactions between other quinolones and fenbufen in clinical therapeutics. However, we cannot exclude the possibility that biphenylacetic acid, the major active metabolite of fenbufen, is responsible for the enhancement of the enoxacin-induced epileptogenic action *in vivo*, instead of fenbufen itself.

In conclusion, our findings suggest that the augmentative effect of fenbufen on the quinolone-induced inhibition of GABA-receptor binding *in vitro* may be related to the adverse interaction between enoxacin and fenbufen which has resulted in seizures in some patients, although the mechanism of this *in vitro* effect of fenbufen is the object of much speculation at present.

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Unique sensitivity of nitrogen mustard-resistant human Burkitt lymphoma cells to novobiocin

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Overcoming resistance to chemotherapeutic agents is a major objective of clinical oncology. If the mechanism of resistance to a particular drug is understood, it may be possible to specifically treat the resistant fraction when it arises, or even prevent its development. Recently Frei *et al.* [1] developed Raji-HN2, a human Burkitt lymphoma cell line, that is approximately 10-fold resistant to HN2*. These cells have been characterized in our laboratory as containing approximately 3-fold higher specific activity of extractable topoisomerase II than that of topoisomerase II extracted from the parental Raji cells [2]. This difference in topoisomerase II activity was correlated with the difference between Raji and Raji-HN2 cells in survival and in the ability to repair HN2-induced damage leading to DNA interstrand crosslink formation [2, 3]. In the course of further characterizing Raji-HN2 cells, we found that they were hypersensitive to novobiocin, an inhibitor of eukaryotic topoisomerase II [4]. In this communication, we present our further characterization of the unique hypersensitivity to novobiocin demonstrated by Raji-HN2 cells, evidence suggesting the probable biochemical basis for this phenomenon, and discuss the clinical implications of this observation.

Methods

Cells. Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Raji-HN2 cells were treated weekly with 10 μM HN2 to maintain the resistant phenotype [1, 2]. Treatment of cells with drugs was in serum-free medium. All cells used were in the exponential growth phase [2].

Clonogenic assay. Cell survival was determined by colony formation in soft agarose. After drug treatment, cells were diluted 500-fold into 0.15% low-melting-point agarose (Sea Plaque, FMC Corp., Rockland, ME) prepared in RPMI medium containing 20% fetal bovine serum. After 7–9 days of incubation at 37°, cell colonies were stained with tetrazolium salts and counted in a Biotran counter [2]. Each treatment was assayed in triplicate, and the individual values did not vary by more than 15% from the mean.

Topoisomerase II activity. Enzyme activity contained in the 0.35 M NaCl extracts of isolated nuclei was measured by the P4 DNA unknotting assay as described previously [2]. Specific activity is expressed as units of enzyme per microgram of protein of the nuclear extract. One unit of enzyme activity is that which unknots 200 ng of knotted P4 DNA.

Results and discussion

Raji-HN2 cells are approximately 10-fold more resistant to the cytotoxicity of HN2 and contain 3-fold more extractable topoisomerase II activity than Raji cells [1, 2]. The enzyme is of vital importance to Raji-HN2 cells because of the cytotoxicity achieved in this cell line by a number of topoisomerase II inhibitors, including novobiocin [2, 3]. In a preliminary experiment, Raji-HN2 cells were treated for various lengths of time with 400 μg/ml novobiocin to determine the kinetics of novobiocin-induced cell killing. Novobiocin exerted its cytotoxic effects rapidly; a 30-min treatment resulted in approximately 80% reduction of cell survival (unpublished data). Novobiocin at 800 μg/ml had no effect on the survival of Raji (Burkitt lymphoma), HeLa (cervical carcinoma), K562 (erythroleukemia), and KG1a (bone marrow stem cells) but reduced the survival of Raji-HN2 cells by 90% (data not shown).

Because Raji and Raji-HN2 cells demonstrated different sensitivities to novobiocin or HN2, we were interested in determining the response of these cells to treatment with a combination of novobiocin and HN2. Two types of experiments were performed: (i) pretreatment with a single dose of novobiocin followed by treatment with increasing concentrations of HN2, and (ii) pretreatment with a single dose of HN2 followed by treatment with increasing concentrations of novobiocin. When used in combination with

* Abbreviation: HN2, nitrogen mustard [2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine].