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Multiple actions of fenamates and other nonsteroidal anti-inflammatory drugs on GABA<sub>A</sub> receptors

Salla Mansikkamäki<sup>a</sup>, Saku T. Sinkkonen<sup>a,b</sup>, Esa R. Korpi<sup>a,b</sup>,<sup>*</sup> Hartmut Lüddens<sup>c</sup>

<sup>a</sup> Department of Pharmacology, Faculty of Medicine, University of Helsinki, Helsinki, Finland
<sup>b</sup> Department of Otorhinolaryngology – Head and Neck Surgery, Head and Neck Center, Helsinki University Hospital and University of Helsinki, Helsinki, Finland
<sup>c</sup> Department of Psychiatry and Psychotherapy, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

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ABSTRACT

The nonsteroidal anti-inflammatory drug (NSAID) niflumic acid, a fenamate in structure, has many molecular targets, one of them being specific subtypes of the main inhibitory ligand-gated anion channel, the GABA<sub>A</sub> receptor. Here, we report on the effects of other fenamates and other classes of NSAIDs on brain picrotoxininsensitive GABA<sub>A</sub> receptors, using an autoradiographic assay with [35S]TBPS as a ligand on mouse brain sections. We found that the other fenamates studied (flufenamic acid, meclofenamic acid, mefenamic acid and tolfenamic acid) affected the autoradiographic signal at low micromolar concentrations in a facilitatory-like allosteric fashion, i.e., without having affinity to the [35S]TBPS binding site. Unlike niflumic acid that shows clear preference for inhibiting cerebellar granule cell layer GABA<sub>A</sub> receptors, the other fenamates showed little brain regional selectivity, indicating that their actions are not receptor-subtype selective. Of the non-fenamate NSAIDs studied at 100 μM concentration, diclofenac induced the greatest inhibition of the binding, which is not surprising as it has close structural similarity with the potent fenamate meclofenamic acid. Using two-electrode voltage-clamp assays on Xenopus oocytes, the effect of niflumic acid was found to be dependent on the β subunit variant and the presence of γ2 subunit in rat recombinant α1β and α1βγ2 GABA<sub>A</sub> receptors, with the γ3 subunit allowing the niflumic acid inhibition and β3 the stimulation of the receptor-mediated currents. In summary, the fenamate NSAIDs constitute an interesting class of compounds that could be used for development of potent GABA<sub>A</sub> receptor allosteric agonists with other targets to moderate inflammation, pain and associated anxiety/depression.

1. Introduction

Painful conditions, such as arthritis and fibromyalgia, are comorbid with mental diseases, such as anxiety syndromes and depression (Grilli, 2017; Velly and Mohit, 2018). This interrelationship creates challenges to diagnosis and treatment options. Acute pain and inflammation are treated with nonsteroidal anti-inflammatory drugs (NSAIDs), but chronic neuropathic conditions might require drugs that have stronger actions in the central nervous system. However, some NSAIDs have been suggested to act directly e.g., on γ-aminobutyric acid type A receptors (GABA<sub>A</sub>), which constitute the main fast-acting inhibitory system in the brain. GABA<sub>A</sub> receptors are targeted by acutely efficacious anxiolytic and sedative drugs, such as benzodiazepines, hypnotics and various general anaesthetics (Korpi et al., 2002; Olsen and Sieghart, 2009). While activation of selected GABA<sub>A</sub> receptor subtypes have produced analgesia in some rodent models (Knabl et al., 2008; Munro et al., 2008; Ralvenius et al., 2015), especially in chronic pain the activation of the altered “excitatory” GABA<sub>A</sub> system might worsen the condition (Coull et al., 2003, 2005; Kahle et al., 2013). This makes the activation of this neurotransmitter system an unattractive option to treat pain conditions with anxiety. A further problem with GABA<sub>A</sub> receptor-activating drugs is caused by a rapid occurrence of tolerance and a risk of developing dependence and addiction (Korpi et al., 2015).

It is well known that certain NSAIDs are unselective and not restricted to inhibition of cyclooxygenases (Cryer and Feldman, 1998; Vane and Botting, 2003; Vane, 2000). For instance, they also act on GABA<sub>A</sub> receptors. Especially the fenamates, mefenamic acid and niflumic acid have been reported to either stimulate or inhibit the functions of GABA<sub>A</sub> receptors in rodent model systems as well as in recombinant receptors. Previously, we reported on a strong antagonism by niflumic acid of selected GABA<sub>A</sub> receptor subtypes (Sinkkonen et al., 2003). Mefenamic acid, flufenamic acid and niflumic acid affect GABA<sub>A</sub>...
receptors expressed from poly(A)+ RNA isolated from rat cerebral cortex and expressed in *Xenopus laevis* oocytes (Woodward et al., 1994), with low concentrations inducing potentiation and high concentrations inhibition. The potentiation was not blocked by flumazenil, indicating an effect not mediated by the high-affinity benzodiazepine sites. Flumic acid had the lowest efficacy in potentiation, but the highest in inhibition, whereas mefenamic acid was strongly potentiating and weakly inhibiting. The effects of mefenamic acid on GABA<sub>α</sub> receptor function is dependent on the β subunits in a complex fashion (Halliwell et al., 1999). It facilitates the GABA<sub>α</sub> currents in recombinant α1β2 and

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**Fig. 1.** Structures of the anti-inflammatory compounds and paracetamol studied for their effects on GABA<sub>α</sub> receptors with the 2-(phenylamino)benzoic acid moiety being the IUPAC name of fenamic acid in the first five compounds and diclofenac being a closely related structure.
Fig. 2. Effects of fenamates on basal and GABA-inhibited [35S]TBPS binding to GABA_A receptors in various mouse brain regions. (A) Representative images, demonstrating clear enhancement of the GABA effect on binding in all brain regions by 10 μM concentrations of the fenamates other than niflumic acid and in some areas flufenamic acid. Ctx, cerebral cortex; CbGCL, cerebellar granule cell layer; Hi, hippocampus; IC, inferior colliculus; Str, striatum; Th, thalamus. (B) Concentration-response curves for the effects of fenamates on basal binding. (C) Concentration-response curves for the effects of fenamates on 3-μM GABA-inhibited binding. The data points are means ± S.E.M. of the corresponding GABA values (n = 4–6).
ligand t-butylibicyclophosphoro [35S]thionate ([35S]TBPS) with special emphasis on the GABA-insensitive atypical GABA<sub>A</sub> receptor populations as these are the only defined ones which can be easily visualized with [35S]TBPS binding (Halonen et al., 2009). Furthermore, we describe in more detail the subunit-dependencies of niflumic acid actions on a number of recombinant GABA<sub>A</sub> receptor subtypes heterologously expressed in Xenopus laevis oocytes.

2. Materials and methods

2.1. Animals and tissues

The autoradiographic data were collected using six male 2-month-old C57BL/6N Hsd mice (Harlan Netherland, Horst, The Netherlands). The mice were briefly anesthetized using CO<sub>2</sub> and decapitated, the brains dissected out and frozen on solid CO<sub>2</sub> and stored at ~80 °C until sectioned. The electrophysiological data were from stage V-VI oocytes obtained from female Xenopus laevis frogs (Horst Kähler, Hamburg, Germany), anesthetized with 0.2% tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA). Animal procedures were approved by the Southern Finland provincial government (ESAVI/686/04.10.03/2012; Eläinkoelautakunta, ELLA).

2.2. [35S]TBPS autoradiography

We used 14-μm-thick horizontal cryostat sections thaw-mounted onto gelatin-coated object glasses from naive adult mice, cut with a Leica CM 3050S cryostat (Leica Microsystems, Benheim, Germany) (Korpi et al., 1992; Makela et al., 1997; Sinkkonen et al., 2001). The sections were preincubated in ice-cold buffer containing 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl for 15 min. We used two different assays. In the first one, we aimed at revealing allosteric drug sensitivities of the main GABA-sensitive GABA<sub>A</sub> receptor populations, with GABAergic agonists reducing the binding and antagonists increasing it (Korpi and Luddens, 1997). In the second one, we assessed the drug sensitivities of minor thalamic- and cerebellum-enriched atypical (GABA-insensitive, GIS) GABA<sub>A</sub> receptor populations (Halonen et al., 2009; Sinkkonen et al., 2001). The ligand t-butylibicyclophosphoro [35S]thionate ([35S]TBPS) that we used is highly selective to GABA<sub>A</sub> receptors as compared to strychnine-sensitive glycine receptors (Rienitz et al., 1987). The sections were incubated with 6 nM [35S]TBPS (Perkin-Elmer, Boston, MA, USA) in the incubation buffer (50 mM Tris–HCl, 120 mM NaCl, pH 7.4) at room temperature for 90 min, using either full or one sixth dilution radioactivity, for the GIS and main receptor populations, respectively. This was carried out in the presence and absence of GABA and drugs. Nonspecific binding was determined with 100 μM picrotoxinin (Sigma-Aldrich). For the study of the main receptor population, the sections were washed 3 × 15 s in ice-cold 10 mM Tris–HCl and quickly desalted in ice-cold distilled H<sub>2</sub>O. The GIS sections were washed 3 × 30 min in ice-cold 10 mM Tris–HCl and quickly desalted in ice-cold distilled H<sub>2</sub>O. After the washing and air-drying, the sections were exposed to Biomax MR film (Eastman Kodak, Rochester, NY, USA) for 1–6 weeks with plastic 14C-radioactivity.

Table 1

The differential effects of niflumic acid vs. Other fenamates on GABA-insensitive [35S]TBPS binding to GABA<sub>A</sub> receptors in the mouse thalamus and cerebellar granule cell layer.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>GABA 1 mM % of basal</th>
<th>Niflumic acid</th>
<th>Flufenamic acid</th>
<th>Meclomenamic acid</th>
<th>Mefenamic acid</th>
<th>Tolfenamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalamus</td>
<td>38 ± 4 5 ± 1</td>
<td>100 μM</td>
<td>3 μM</td>
<td>30 μM</td>
<td>1 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>CBGCL</td>
<td>35 ± 2 11 ± 1</td>
<td>97 ± 4</td>
<td>76 ± 11</td>
<td>11 ± 6*</td>
<td>56 ± 4*</td>
<td>0*</td>
</tr>
</tbody>
</table>

Values for the niflumic acid are mean percentages ± S.E.M. of the corresponding values in the presence of a saturating concentration of GABA alone (n = 6 mice). CBGCL, cerebellar granule cell layer. See Fig. 3 for representative images.

* P < 0.001 for the significance of the difference from the 1-mM GABA-inhibited binding (ANOVA, followed by Dunnett’s test).
standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Binding densities in selected brain regions were quantitated with MCID IAS-imaging software (Imaging Research Inc., St. Catharine’s, Ontario, Canada) and standardized with radioactivity values on the basis of the simultaneously exposed standards. Nonspecific binding was subtracted from all values.

2.3. Recombinant GABA<sub>A</sub> receptor subunits and preparation of cRNAs

Capped cRNAs coding for rat GABA<sub>A</sub> receptor subunits α<sub>1</sub>, β<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub>S (Luddens et al., 1990; Shivers et al., 1989; Ymer et al., 1989) were transcribed in vitro from pRK5 plasmids with Sp6 as promoter using mMessage mMachine kit (Ambion, Austin, TX, USA) according to manufacturer’s instructions.

2.4. Oocyte electrophysiology of recombinant GABA<sub>A</sub> receptors

Experiments using recombinant GABA<sub>A</sub> receptors were carried out as described in (Sinkkonen et al., 2003). In brief, isolated oocytes were stored in normal frog Ringer: 115 mM NaCl, 2.5 mM KCl, 18 mM CaCl<sub>2</sub>, and 10 mM HEPES, pH 7.5. Oocytes were defolliculated and injected via a glass micropipette with 46 nl of a solution containing mixtures of subunit cRNAs (0.1–2.5 μg/μl) or pure H<sub>2</sub>O with Drummond Nanoject injector (Drummond Scientific Co., Broomall, PA, USA). The oocytes were incubated at 19°C in incubation solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.91 mM CaCl<sub>2</sub>, 0.5 mM theophylline, 2 mM sodium pyruvate, 10 U/ml penicillin and 10 μg/ml streptomycin, pH 7.5]. After injection (2 h–1 day) oocytes were digested for 30 min in Ca<sup>2+</sup>-free medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, and 5 mM HEPES, pH 7.5) containing 0.3 U/ml collagenase type IA (Sigma-Aldrich). Thereafter, the oocytes were incubated in incubation solution until recordings. For each experiment, oocytes from at least two different frogs were used. Electrophysiological recordings were made 1–3 days after cRNA injection. Oocytes were perfused with normal frog Ringer + drugs at a flow rate of 1 ml/min at room temperature (22°C) using an Ismatec pump (Ismatec, Glattbrugg-Zürich, Switzerland) and 17 channel perfusion system with pinch valves.

Fig. 4. The effects of nonsteroidal anti-inflammatory drugs on the basal and GABA-inhibited [35S]TBPS binding to GABA<sub>A</sub> receptors in various mouse brain regions. (A) Representative images, demonstrating enhancement of the 3-μM GABA effect on binding by the positive control, fenamate meclofenamic acid (Mecl, at 1 μM, see Fig. 2BCE), and by 100 μM concentrations of diclofenac (Dicl), rofecoxib (Rof) and MF-tricyclic (MF), but not by paracetamol (Par, acetaminophen), which slightly increased the binding in some brain regions in the presence of GABA. Ctx, cerebral cortex; (Cb)GCL, cerebellar granule cell layer; Hi, hippocampus; IC, inferior colliculus; (Cb)MCL, cerebellar molecular layer; Str, striatum; Th, thalamus. (B) Regional effects of the studied NSAIDs on [35S]TBPS binding in selected brain regions. Bars are means ± S.E.M. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 for the significance of the difference from the basal or GABA-inhibited binding in the absence of drugs within the brain region (ANOVA, followed by Dunnett’s test).
Drug combinations were mixed before experiments. As negative control we employed furosemide which acts highly specifically on α6β2 (γ2) and α6β3 (γ2) receptors but not on α1 or β1 subunit-containing receptors (Korpi et al., 1995; Korpi and Luddens, 1997). Niflumic acid at 1000 μM concentration served as positive control. The GABA concentrations used in the experiments were chosen according to the expected EC\textsubscript{50} values, which was in good agreement with the obtained GABA concentration-response curves for the four receptor subunit combinations, respectively. F100, furosemide 100 μM. (B) Niflumic acid effects represented as mean percentage ± S.E.M., the GABA 30 μM and GABA 20 μM control response being set to 100%. F, furosemide 100 μM + GABA 30 μM or GABA 20 μM; N, 1000 μM niflumic acid alone. *P < 0.05, ***P < 0.001 for the significance of the difference from the corresponding control value (one-way ANOVA, followed by Dunnett’s test for niflumic acid in the presence of GABA, Student’s t-test for furosemide or niflumic acid alone). The dashed line represents the GABA control value. The EC\textsubscript{50} values were 6.1 ± 1.3 μM and 10.6 ± 1.1 μM for α1β1 and α1β3, respectively.

2.5. Drugs

All fenamates and other NSAIDs were obtained from Sigma-Aldrich. They were dissolved at 1 mM stock concentration into 0.1 M NaOH, and adjusted for pH 7.5 after final dilutions, if different from that.

2.6. Data analyses and statistics

Data analyses were performed using EggWorks Reader version 3.0.2 (NPI Electronic GmbH) and GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA) programs. For autoradiography, the specific \(^{35}\text{S}\)TBPS binding values were determined by subtracting the nonspecific binding values from the corresponding total binding values under each incubation condition. To assess the statistical significance of the drug effects on \(^{35}\text{S}\)TBPS binding, one-way or two-way analyses of variance (ANOVA) and Dunnett’s post hoc test were used. Student’s t-test was used for fenamate effects on GABA-insensitive binding.

For electrophysiological recordings, the amplitudes of peak currents induced by GABA and drug applications were determined from recorded traces, normalized to the corresponding GABA-induced peak currents estimated linearly between the GABA peak currents closest before and after the applications of GABA with the drugs, and presented as a percentage of the control GABA current. The peak currents induced by various GABA concentrations for each oocyte were normalized by setting the maximal GABA current without niflumic acid to 100%, and

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**Fig. 5.** (A) Representative current traces of niflumic acid and furosemide modulation on 10 μM and 20 μM GABA-induced currents in recombinant α1β1 and α1β3 GABA\textsubscript{A} receptors expressed in Xenopus laevis oocytes. n = 7 and 5 for α1β1 and α1β3 combinations, respectively. F100, furosemide 100 μM. (B) Niflumic acid effects represented as mean percentage ± S.E.M., the GABA 30 μM and GABA 20 μM control responses being set to 100%. F, furosemide 100 μM + GABA 30 μM or GABA 20 μM; N, 1000 μM niflumic acid alone. *P < 0.05, ***P < 0.001 for the significance of the difference from the corresponding control value (one-way ANOVA, followed by Dunnett’s test for niflumic acid in the presence of GABA, Student’s t-test for furosemide or niflumic acid alone). The dashed line represents the GABA control value. The EC\textsubscript{50} values were 6.1 ± 1.3 μM and 10.6 ± 1.1 μM for α1β1 and α1β3, respectively.

**Fig. 6.** (A) Representative current traces of niflumic acid and furosemide modulation on 30 μM and 20 μM GABA-induced currents in recombinant α1β1/2 and α1β3γ2 GABA\textsubscript{A} receptors, respectively, expressed in Xenopus laevis oocytes. n = 7 and 5 for α1β1γ2 and α1β3γ2 combinations, respectively. F, furosemide 100 μM. (B) Niflumic acid effects represented as mean percentage ± S.E.M., the GABA 30 μM and GABA 20 μM control response being set to 100%. F, furosemide 100 μM + GABA 30 μM or GABA 20 μM; N, 1000 μM niflumic acid alone. *P < 0.05, ***P < 0.001 for the significance of the difference from the corresponding control value (one-way ANOVA, followed by Dunnett’s test for niflumic acid in the presence of GABA, Student’s t-test for furosemide or niflumic acid alone). The dashed line represents the GABA control value. The EC\textsubscript{50} values were 14.3 ± 3.8 μM and 33.0 ± 7.6 μM for α1β1γ2 and α1β3γ2, respectively.
the GABA concentration-response curves were generated using non-linear regression fit to a sigmoidal concentration-response curve. The statistical significance of the niflumic acid modulation of the GABA response was assessed with one-way ANOVA and Dunnett’s post hoc test. Furosemide and niflumic acid effects at 1000 μM without additional GABA were assessed using Student’s t-test.

3. Results

3.1. Effects of niflumic acid and other fenamates on GABA_A receptor binding

Previously, we have reported on a strong antagonism by niflumic acid of selected GABA_A receptor subtypes (Sinkkonen et al., 2003). Now we wanted to determine whether the structurally close analogues flu- fenamic acid, meclofenamic acid, mefenamic acid and tolfenamic acid (Fig. 1) have similar effects. Concentration-response curves for these other fenamates clearly differed from those of niflumic acid in all brain regions. Niflumic acid had no effect on forebrain [35S]TBPS binding without added 3 μM GABA (Fig. 2), and only a minor inhibitory effect at the highest concentration in the presence of GABA. The other fenamates, except for flufenamic acid, robustly inhibited the binding at 10 μM concentration in both incubation conditions [one-way ANOVAs for each brain region F (6,35) > 20, P < 0.0001]. Flufenamic acid plainly differed from niflumic acid in the presence of GABA. The brain regional efficacy by the fenamates indicates a broad GABA_A receptor subtype selectivity, unlike that detected previously for niflumic acid (Sinkkonen et al., 2003) and confirmed here, as niflumic acid (10 μM) did not inhibit the [35S]TBPS binding without or with added 3 μM GABA, but rather increased it [F (6,35) = 67.5, P < 0.001; F (6,35) = 41.9, P < 0.001, respectively].

A high saturating GABA concentration (1 mM) abolished most of the picrotoxinin-sensitive [35S]TBPS binding in all brain regions, except for the thalamus and cerebellar granule cell layer, which retained 5–10% of the basal binding [Fig. 3, Table 1 (Sinkkonen et al., 2001)]. We also tested the effects of fenamates on this atypical GABA-insensitive binding component and found that the fenamates other than niflumic acid abolished this binding almost completely at micromolar concentrations [Fig. 3, Table 1; ANOVA F (8, 45) = 38.76, P < 0.0001 and F (9, 50) = 46.01, P < 0.0001 for the thalamus and cerebellar granule cell layer, respectively], whereas niflumic acid at 100 μM failed to affect the thalamic binding and robustly enhanced the cerebellar granule cell layer binding, in agreement with (Sinkkonen et al., 2001). The most potent fenamate appeared to be meclofenamic acid that reduced the binding to almost half in both brain regions at 1 μM concentration (Table 1).

3.2. Effects of non-fenamate nonsteroidal anti-inflammatory drugs on GABA_A receptor binding

We then tested non-fenamate NSAIDs (Fig. 1) for their effects on [35S]TBPS binding with or without added 3 μM GABA in various brain regions. All brain regions showed sensitivity to some of the NSAIDs [Fig. 4; 2-way ANOVAs for brain region F (6,231) = 684.7, P < 0.0001, drug F (10,231) = 18.54, P < 0.0001, and brain region × drug interaction F (60, 231) = 1.3, P > 0.05, and for brain region F (6,231) = 2115, P < 0.0001, drug F (10,231) = 113.6, P < 0.0001, and brain region × drug interaction F (60, 231) = 10.7, P < 0.0001, for the total binding and for the binding in the presence of 3 μM GABA, respectively]. Most drugs at 100 μM concentration failed to affect basal and GABA-inhibited binding (Fig. 4), but diclofenac and MF-tricyclic clearly inhibited the binding in the presence of GABA, but had hardly any effects without it. Also rofecoxib had some inhibitory effects on the binding, but less consistently than diclofenac and MF-tricyclic.

3.3. Molecular determinants of niflumic acid action on recombinant GABA_A receptors

Since niflumic acid effects are markedly different from those of other fenamates, we wanted to gain insight on which features of the GABA_A receptor subunits its actions depend. To that aim we investigated niflumic acid’s action on four defined GABA_A receptor subtypes by measuring its effect on GABA-induced Cl⁻-infux into Xenopus laevis oocytes injected with rat α1 subunit RNA together with either the rat β1 or rat β3 subunit RNA. Additionally, we looked at the action of this fenamate on GABA-induced currents of rat α1β1γ2 and α1β3γ2 receptors.

For both receptors lacking the γ2 subunit niflumic acid dose-de- pendently decreased the GABA-induced current [Fig. 5A; niflumic acid effect F (4,50) = 73.2, P < 0.0001; receptor effect F (1,50) = 87.0, P < 0.0001], though niflumic acid was more potent on β1-than on β3-containing receptors by a factor > 10 (Fig. 5B). When α1/β1 and α1/ β3 RNA was co-injected with γ2 RNA, α1β1γ2 receptor responses to GABA were still inhibited by increasing concentrations of niflumic acid [Fig. 6A, B; niflumic acid effect F (4,50) = 751.2, P < 0.0001; receptor effect F (1,50) = 6.9, P < 0.001]. However, niflumic acid stimulated the GABA response more than two-fold in α1β3γ2 receptors.

4. Discussion

Global, strong effects of the fenamates and some other NSAIDs on brain GABA_A receptors indicate either effects on the main receptor subtypes (e.g., α1β2/2 receptors) or very strong effects on minor subtypes, such as α1β3γ2 receptors. In the absence of saturating 1-mM GABA concentration, the active fenamate drugs behaved like agonists (Korpi et al., 1996; Makela et al., 1997), reducing the binding of the channel ligand [35S]TBPS practically in all brain regions, except for niflumic acid in the cerebellar cortex. We have reported on the antagonist-like action of niflumic acid on GABA_A receptors in the cerebellar granule cell layer (see also Figs. 2–3, Table 1), which effect is dependent on α6 subunit-containing GABA_A receptors (Sinkkonen et al., 2003). Here, we found that the other studied fenamates did not recapitulate this effect, but had agonist-like effect also in the cerebellum. Furthermore, the fenamates reduced the GABA-insensitive [35S]TBPS binding component, which is dependent on α1 subunit-containing receptors in the forebrain and on α6 containing receptors in the cerebellum (Halonen et al., 2009). Thus, our results suggest that the fenamates have the potential to enhance the function of a wide range of GABA_A receptor subtypes in the brain. However, we could not establish any clear structure-activity relationship for their activity.

Of the non-fenamate NSAIDs that had agonist-like activity, the clinically widely used diclofenac was the most interesting (Fig. 4). Its structure resembles those of the fenamates (Fig. 1) and it did cause a significant reduction in the binding of [35S]TBPS in most brain regions (Fig. 4B). Also the experimental MF-tricyclic and the newer cyclooxygenase-2-selective rofecoxib had some agonist-like effect, but the other drugs tested were rather inactive in this assay. Some NSAIDs have been shown to potentiate the antagonistic effects of quinoline-antibiotics on GABA_A receptors. Thus, the antagonism by enoxacin, a guinolone antibiotic, on GABA_A responses in frog dorsal root ganglion sensory neurons was potentiated by indomethacin and ibuprofen, but not by diclofenac, piroxicam and paracetamol (Yakushiji et al., 1992). It should be kept in mind that fenamates (flufenamic acid as an example (Guinamard et al., 2013)) and diclofenac have multiple ion channel targets other than GABA_A receptors that can contribute to clinical ef- ficacy and adverse effects, albeit often at higher concentrations than needed to inhibit cyclooxygenases (Gwanany et al., 2012).

The effects of niflumic acid on recombinant GABA_A receptor function was dependent on the β and γ subunits in a complex fashion. Niflumic acid had inhibitory effects on GABA-induced currents in α1γ1, α1γ3 and α1β1γ2 recombinant receptors, whereas the effect was
positive in α1β3γ2 receptors (Figs. 5 and 6). This is partially in line with the finding that mefenamic acid facilitates the GABA receptors at recombinant α1β2 and α1β2γ3 receptors, which is inactive at α1β1γ2 and inhibitory at α1β1 receptors (Halliwell et al., 1999). The activation profile was traced to a single amino acid residue ‘N290’ in the β2/3 subunits, which appears to mediate the effects of a number of drugs on GABA-α ion channels [for pharmacological listing of these compounds, see (Sieghart and Savic, 2018)]. Other fenamates have a similar profile as mefenamic acid (Smith et al., 2004). Since the β1 subunit-containing receptors have been suggested to mediate sedative drug actions more readily than β3 containing receptors, the fenamates should not be very sedative. However, the latter receptors are thought to be more involved in anaesthesia than β1 receptors (Jurd et al., 2003; Yanovsky et al., 2012).

Fenamates are also functionally active on native GABA receptors, e.g. in cultured primary hippocampal neurons from rats (Coyne et al., 2007). Oral administration of mefenamic acid to mice results in such high brain concentrations that should be relevant for potentiating GABA receptors (Gee et al., 2010). Indeed, mefenamic acid has been shown to be neuroprotective in adult male Wistar rats in a transient ischemic stroke model (Kanshari and Halliwell, 2009). As well, in a rat pilocarpine-model of seizures, intraperitoneal mefenamic acid has been protective, while ibuprofen and indomethacin not (Ikonomidou-Turski et al., 1988). In that study, high doses (30–40 mg/kg) of mefenamic acid induced sedation and reduced muscle tone. Mefenamic acid ip 5 mg/kg daily for 3 weeks reduced learning deficits in a rat model of Alzheimer’s disease (Joo et al., 2006). Tolfenamic acid has been reported to have therapeutic effect on alcohol hangover and migraine attacks (Hakkariainen et al., 1979; Parantainen, 1983). All these results suggest that fenamates are taken up into the CNS at efficient concentrations. Though the carboxy groups provide some polarity to all fenamates studied, the calculated partition coefficients (logP values ranged from 4.3 to 5.1) between water and oil ([Tietko et al., 2005] http://vclab.org) do not contradict their passage of the blood brain barrier (Pajouhesh and Lenz, 2005). We are not aware of any studies on possible GABA receptor-mediated anxiolytic effects of NSAIDs and especially those of fenamates. On the contrary, intracerebroventricular infusion in rats of niflumic acid and flufenamic acid reduced high-dose ethanol-induced loss of righting reflex as a measure of alcohol intoxication, but not low dose-induced sedation (Carter et al., 2016), which was correlated with their antagonism of calcium-activated anion channels. Thus, any agonistic effect on GABA receptor should have potentiated the alcohol sedation.

Very recently there have been efforts to develop positron emission tomography ligands for cyclooxygenase 1 and 2. The most valuable comments of Heikki Vapaatalo on the manuscript are gratefully acknowledged. We thank Aira Säisä for technical assistance in autoradiography. The study was partially supported by the Finnish Cultural Foundation, Elli Turunen Fund (S.M.), the Academy of Finland and the Sigrid Jusélius Foundation.

References


