

Ibuprofen Inhibits Rat Brain Deamidation of Anandamide at Pharmacologically Relevant Concentrations. Mode of Inhibition and Structure-Activity Relationship¹

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ABSTRACT

The ability of rat brain (minus cerebellum) homogenates to deamidate arachidonyl ethanolamide (anandamide) was determined with a custom-synthesized substrate, arachidonyl ethanolamide-[1-³H] ([³H]anandamide). Conditions whereby initial velocities were measured were established. The homogenates deamidated anandamide with a K_m value of 0.8 μ M and a V_{max} value of 1.73 nmol \cdot (mg protein)⁻¹ \cdot min⁻¹. The deamidation of 2 μ M [³H]anandamide was inhibited by phenylmethylsulfonyl fluoride and arachidonyl trifluoromethyl ketone with IC_{50} values of 3.7 and 0.23 μ M, respectively. Ibuprofen inhibited anandamide deamidation in a mixed fashion, with K_i and K'_i values of 82

and 1420 μ M. At an anandamide concentration of 2 μ M, the IC_{50} values (in μ M) of a series of compounds related in structure to ibuprofen were as follows: suprofen, 170; ibuprofen, 270; fenoprofen, 480; naproxen, 550; ketoprofen, 650; diclofenac, ~1000. Sulindac produced 27% inhibition at a concentration of 1000 μ M, whereas isobutyric acid, hydrocinnamic acid, acetylsalicylic acid and acetaminophen were essentially inactive at concentrations \leq 1 mM. We conclude that ibuprofen inhibits anandamide deamidation at pharmacologically relevant concentrations and that there is some specificity to the inhibition produced by ibuprofen and suprofen.

Since its discovery as an endogenous cannabimimetic agent (Devane *et al.*, 1992), there has been considerable interest devoted to the pharmacological properties of anandamide. In addition to producing many of the standard pharmacological and biochemical effects of cannabinoids (Mackie *et al.*, 1993; Vogel *et al.*, 1993; Smith *et al.*, 1994), anandamide has been proposed to exert neurotrophic actions in the hippocampus and to play a role in synaptic plasticity (Derkinderen *et al.*, 1996) as well as in the modulation of long-term potentiation (Terranova *et al.*, 1995) and glutamatergic transmission (Shen *et al.*, 1996) as a result of cannabinoid receptor activation.

Anandamide is metabolized by a membrane-bound amidohydrolase activity to produce arachidonic acid (Deutsch and Chin, 1993). In view of the anti-inflammatory, antinociceptive and immunosuppressive properties of cannabinoids (Dewey, 1986), inhibitors of anandamide deamidation may be of therapeutic value. Most of our knowledge concerning such inhibitors has been gleaned from the use of structural analogs of anandamide (Koutek *et al.*, 1994). Re-

cently, however, we found that the nonsteroidal anti-inflammatory drug ibuprofen inhibited the metabolism of anandamide (Fowler *et al.*, 1997). The assay, which used the PMSF-sensitive reduction in the potency of anandamide to inhibit radioligand binding to cannabinoid receptors (Childers *et al.*, 1994), was indirect in nature and did not permit quantitative data to be obtained. Preliminary data using a direct assay of anandamide deamidation (Omeir *et al.*, 1995) and a substrate concentration of 27.7 μ M (the concentration used by Omeir *et al.*, 1995) confirmed the enzyme inhibition by ibuprofen and gave an IC_{50} value of ~400 μ M (Fowler *et al.*, 1997). Because this value is similar to the value for inhibition by ibuprofen of COX-2 in cell-free systems (Mitchell *et al.*, 1994), it is important to investigate in more detail the inhibition of anandamide deamidation by ibuprofen. Consequently, we used the assay of Omeir *et al.* (1995) to investigate the nature of the inhibition of anandamide deamidation by ibuprofen and to explore the relationship between the structure of this compound and its inhibitory activity.

Materials and Methods

Compounds. Arachidonyl ethanolamide [1-³H] ([³H]anandamide, specific activity 30 Ci/mmol) was custom-synthesized by American Radiolabeled Chemicals Inc., St. Louis, MO. The chemical purity of

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ABBREVIATIONS: Anandamide, arachidonyl ethanolamide; PMSF, phenylmethylsulfonyl fluoride; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase.

the compound was found to be 97.3% using HPLC on a Zorbax SB C-18 column with acetonitrile/water/acetic acid (85:15:0.05) as the mobile phase. Arachidonyl ethanolamide-[1,2- ^{14}C] (^{14}C)anandamide, specific activity 120 mCi/mmol) was a kind gift from Dr. D. G. Ahern, E.I. DuPont de Nemours Co., Biomedical Product Division, Boston, MA. Anandamide (in ethanol) and arachidonyl trifluoromethyl ketone were purchased from Research Biochemicals International, Natick, MA. Ibuprofen (sodium salt), naproxen, suprofen, ketoprofen, fenoprofen, diclofenac, sulindac, isobutyric acid, hydrocinnamic acid, acetylsalicylic acid, acetaminophen and PMSF were obtained from the Sigma Chemical Co., St. Louis, MO. The NSAID compounds were dissolved in ethanol before dilution with buffer and were then further diluted with the ethanol/buffer mix to keep the ethanol concentration constant through the experiments. PMSF was dissolved in either ethanol or butanol (for assay blanks) and diluted with buffer to give a stock solution of 12 mM. Subsequent experiments demonstrated that the assay butanol concentration itself produced a 30% to 50% inhibition of [^3H]anandamide amidation. However, a series of experiments using a [^3H]anandamide concentration range of 1 to 27.7 μM demonstrated that the assay blank value was the same when PMSF was dissolved in ethanol (which did not itself affect the anandamide amidation rate) as when PMSF was dissolved in butanol.

Assay of [^3H]anandamide deamidation. The assay used was that of Omeir *et al.* (1995). Briefly, rat brains minus cerebella (for [^3H]anandamide) or cerebella (for [^{14}C]anandamide) were homogenized in 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, and aliquots were stored frozen at -70°C until used for assay. Assay mixtures contained homogenate, the appropriate concentration of test compound, or carrier [ethanol diluted with buffer] (25 μl), and radiolabeled anandamide (25 μl) containing 10 mg/ml fatty acid-free bovine serum albumin (Pinal assay volume 200 μl). Blanks contained PMSF (final concentration 1.5 mM) in place of the NSAIDs. The mixtures were incubated at 37°C , after which reactions were stopped by placing the tubes in ice and adding 400 μl of chloroform/methanol (1:1, v/v). The tubes were vortex-mixed, after which the phases were separated by centrifugation in a bench centrifuge. Aliquots (200 μl) of the methanol/buffer phase were removed for analysis of radioactivity by liquid scintillation spectroscopy with quench correction.

Determination of K_m and V_{\max} values. K_m and V_{\max} values of the mean data, compiled from three separate experimental series, were analyzed using the direct linear plot of Eisenthal and Cornish-Bowden (1974). Analyses were conducted using the Enzyme Kinetics computer program (Trinity Software, Campton, NH). K_i and K'_i values were calculated from replots of $K_m^{\text{app}}/V_{\max}^{\text{app}}$ vs. [ibuprofen] and $1/V_{\max}^{\text{app}}$ vs. [ibuprofen], respectively (see Cornish-Bowden, 1976).

Results

Assay conditions. Because the substrate used in the present study is the result of a custom synthesis, initial experiments were undertaken to establish the validity of the assay. In the absence of enzyme, there was no time-dependent accumulation of radioactivity in the aqueous/methanol phase, which suggests that the substrate is stable under the conditions used. At a concentration of 1 μM , product formation was dependent on incubation time and protein concentration (fig. 1), a result consistent with other studies of anandamide deamidation (*e.g.*, Desarnaud *et al.*, 1995; Maurelli *et al.*, 1995; Ueda *et al.*, 1995; Omeir *et al.*, 1995; Lang *et al.*, 1996). Complete metabolism of [^3H]anandamide, blocked by the presence of PMSF, was seen after incubation for 20 min at a protein concentration of 20 $\mu\text{g}/\text{assay}$ (fig. 1). It is noteworthy that linearity was preserved until surprisingly high rates of substrate utilization were reached. A similar pattern

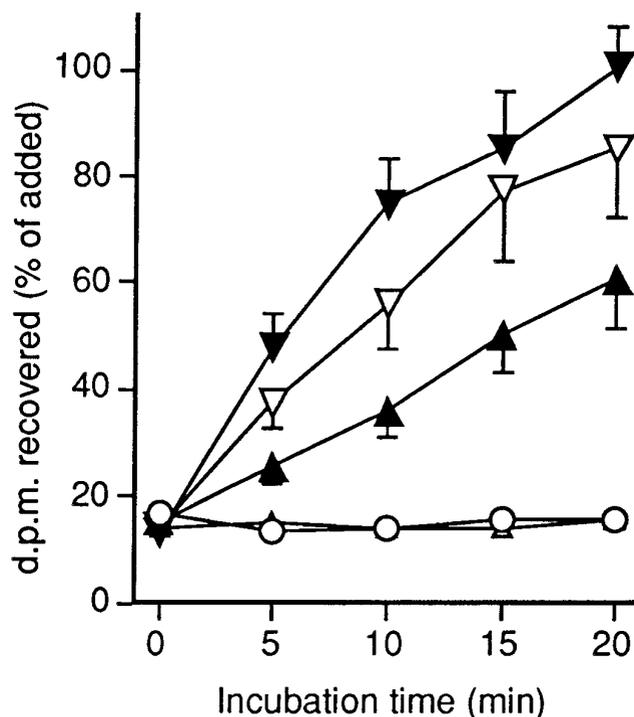


Fig. 1. Time courses of the deamidation of 1 μM [^3H]anandamide by rat brain minus cerebellum homogenates. Protein concentrations of 0 (\circ), 6.7 (\blacktriangle), 13.3 (∇), 20 (\blacktriangledown) $\mu\text{g}/\text{assay}$, and 20 $\mu\text{g}/\text{assay}$ in the presence of 1.5 mM PMSF (\triangle) were used. Shown are means \pm S.E. (unless enclosed by the symbol); $n = 3$ to 4.

was found when either [^3H]anandamide (whole brain minus cerebellum) or [^{14}C]anandamide (cerebellum) were used at a concentration of 27.7 μM (data not shown). In the remaining experiments, incubation times of 5 to 10 min were used, and protein concentrations were chosen to ensure that initial velocities were measured.

The effects of PMSF and arachidonyl trifluoromethyl ketone on the deamidation of 2 μM [^3H]anandamide are shown

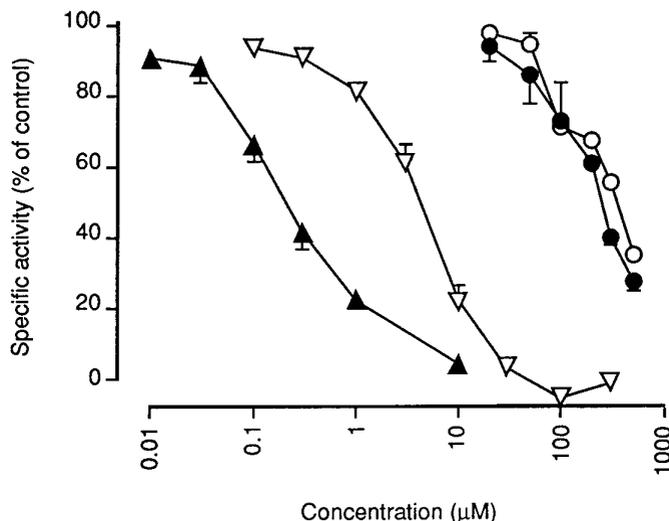


Fig. 2. Inhibition of the deamidation of 2 μM [^3H]anandamide by arachidonyl trifluoromethyl ketone (\blacktriangle), PMSF (∇) and ibuprofen (\bullet), no preincubation phase; \circ , 90-min preincubation phase at 37°C). Shown are means \pm S.E. (unless enclosed by the symbol); $n = 3$. The ethanol concentrations used to dissolve the compounds was kept constant throughout each experimental series.

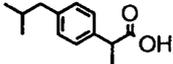
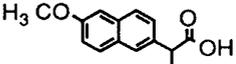
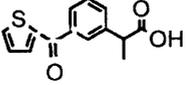
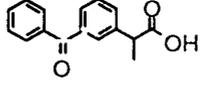
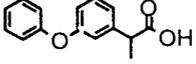
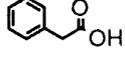
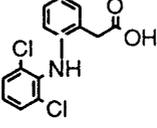
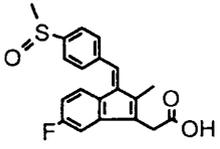
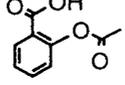
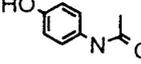
in figure 2. Both compounds inhibited the deamidation of the substrate, with IC_{50} values (calculated as described in the legend to table 1) of 3.7 and 0.23 μM , respectively.

Inhibition of anandamide deamidation by ibuprofen. Ibuprofen produced a concentration-dependent inhibition of the deamidation of 2 μM [^3H]anandamide (fig. 2). The inhibition was not changed upon preincubation of ibuprofen with homogenate for up to 90 min at 37°C before the addition of [^3H]anandamide. The data for a 90-min preincubation period are presented in figure 2; similar concentration-re-

sponse curves were found after preincubations of 30 and 60 min. This lack of time-dependence was also seen for cerebellar homogenates with 27.7 μM [^{14}C]anandamide (data not shown), although ibuprofen was a less potent inhibitor at this high substrate concentration.

Data from three experimental series, all using the same four homogenate preparations, are shown in figure 3A. Direct linear plot analyses of the combined mean data gave a K_m value of 0.8 μM and a V_{max} value of 1.73 $\text{nmol} \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$. Ibuprofen was found to produce inhibition of

TABLE 1
Effect of a series of compounds related to ibuprofen on rat brain (minus cerebellum) anandamide deamidation

Compound	Structure	% of Control Anandamide Deamidation ^a at Concentration (μM) of:							IC_{50} (μM) ^b
		30 (20*)	50	100	200	300	500	1000	
Ibuprofen		94 ± 4*	86 ± 8	73 ± 3	61 ± 2	40 ± 2	27 ± 3 39 ± 1	25 ± 0.6	270
Naproxen		99 ± 4	92 ± 6	82 ± 6	70 ± 2	66 ± 2	53 ± 5 51 ± 3	36 ± 2	550
Suprofen		97 ± 3	80 ± 4	62 ± 0.1	48 ± 3	30 ± 3	26 ± 6 23 ± 3	12 ± 3	170
Ketoprofen		98 ± 3	92 ± 2	96 ± 3	84 ± 4	78 ± 4	72 ± 3 51 ± 3	33 ± 5	650
Fenoprofen		103 ± 9	88 ± 2	85 ± 9	75 ± 1	66 ± 3	41 ± 5		480
Isobutyric acid		98 ± 1	96 ± 4	100 ± 0.7	110 ± 11	111 ± 12	101 ± 2 94 ± 5	87 ± 4	—
Hydrocinnamic acid		103 ± 1	101 ± 4	99 ± 5	102 ± 5	104 ± 4	98 ± 5 90 ± 4	96 ± 3	—
Diclofenac		103 ± 2	98 ± 5	99 ± 3	87 ± 4	74 ± 5	67 ± 6 67 ± 3	54 ± 3	~1000
Sulindac				81 ± 2	85 ± 3		79 ± 4	73 ± 3	>1000
Acetylsalicylic acid				101 ± 1	103 ± 2		101 ± 4	96 ± 2	—
Acetaminophen				99 ± 2	103 ± 4		98 ± 1	101 ± 5	—

^a Shown are means ± S.E., $n = 3$ using a [^3H]anandamide concentration of 2 μM . The ethanol carrier concentration was kept constant for each experimental series. For compounds where two values are given at the concentration of 500 μM , two sets of experiments were undertaken: one over a range of 30 to 500 μM and one with 500 and 1000 μM , the second series using a higher ethanol concentration to permit solubilization of the compounds.

^b IC_{50} values were calculated by Hill analysis of the mean data at each concentration. Only concentrations giving an inhibition in the range of 10% to 90% were included to prevent data bias at the ends of the scales. In all cases, the regression lines gave r^2 values >0.91.

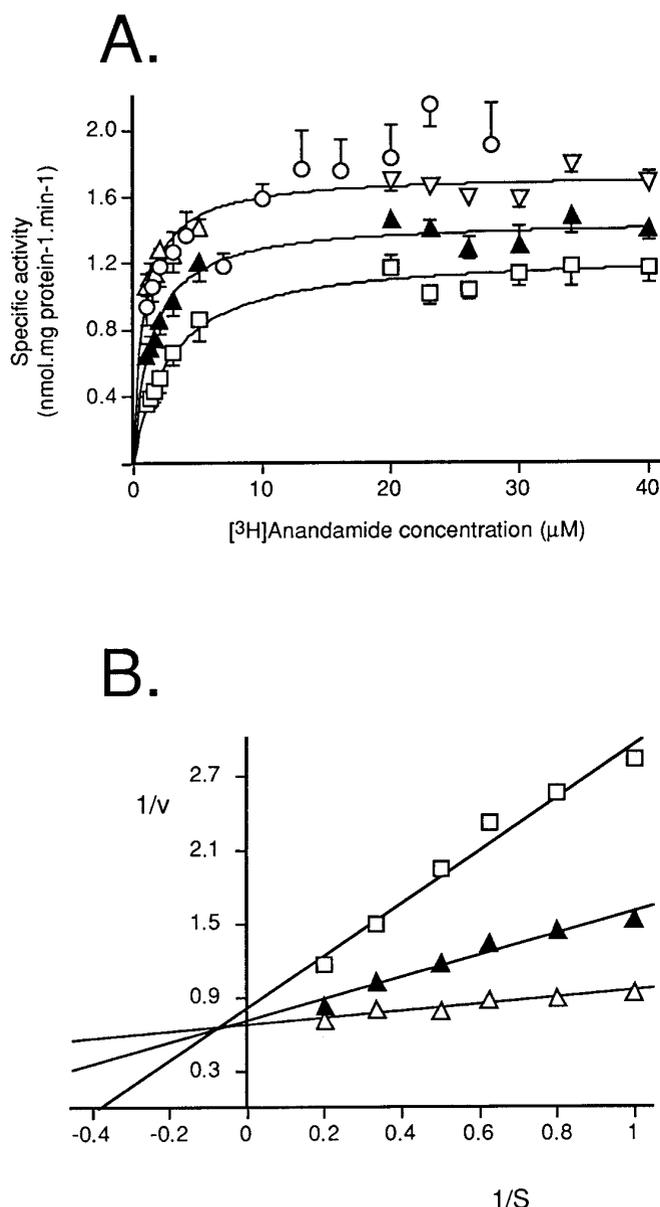


Fig. 3. Mode of inhibition of anandamide deamidation by ibuprofen in homogenates of rat brain minus cerebellum. A) Deamidation of [³H]anandamide in the absence (Δ , \circ , ∇) or presence of 200 (\blacktriangle) or 500 (\square) μM ibuprofen. Three separate experimental series (substrate concentration ranges of 1–5 μM [controls shown as Δ], 1–27.7 μM [controls shown as \circ , no ibuprofen tested] and 20–40 μM [controls shown as ∇]) were run using samples from the same homogenate preparations. Shown are means \pm S.E. (unless enclosed by the symbol), $n = 4$. Incubation times were in all cases 10 min, and protein concentrations of 10, 10 and 40 $\mu\text{g}/\text{assay}$ were used for the three series, respectively. B) 1/S vs. 1/v replots for the mean data from the 1 to 5 μM [³H]anandamide series, with 0 (Δ), 200 (\blacktriangle) and 500 (\square) μM ibuprofen.

enzyme activity at all concentrations tested, the inhibition appearing to be mixed in nature (fig. 3B). When the low (1–5 μM) and high (20–40 μM) experiments were combined (data shown in fig. 3A), the K_m and V_{\max} values calculated by direct linear plot from the mean data were as follows: [μM and $\text{nmol} \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$, respectively] control, 0.7 and 1.70; 200 μM ibuprofen, 1.3 and 1.46; 500 μM ibuprofen, 2.7 and 1.25. One-way ANOVA for repeated measures, calculated using the individual values, showed a significant

effect of ibuprofen on V_{\max} ($F_{3,6} = 50$, $P > .0005$), and a nearly significant effect on K_m ($F_{3,6} = 4.3$, $P = .069$). From the mean K_m and V_{\max} data, K_i and K'_i values of 82 and 1420 μM can be calculated.

Structure-activity relationships. The potencies of a series of compounds related in structure to ibuprofen are shown in table 1. Suprofen was the most potent, with an IC_{50} value of 170 μM . This was followed by ibuprofen (270 μM), fenoprofen (480 μM), naproxen (550 μM), ketoprofen (650 μM) and diclofenac (~ 1000 μM). Sulindac produced 27% inhibition at a concentration of 1000 μM , whereas isobutyric acid, hydrocinnamic acid, acetylsalicylic acid and acetaminophen were essentially inactive at concentrations ≤ 1 mM.

Discussion

In the present study, anandamide deamidation in homogenates of whole brain minus cerebellum has been measured using a custom-synthesized substrate, arachidonyl ethanolamide-[1-³H]. Metabolism of this substrate gives arachidonic acid and [³H]ethanolamine, which can easily be separated from [³H]anandamide. This method is based on the assay of Omeir *et al.* (1995), who used arachidonyl ethanolamide-[1,2-¹⁴C] as substrate. The validity of the assay was confirmed with respect to 1) the sensitivity to PMSF and arachidonyl trifluoromethyl ketone, 2) the linearity of product formation with time up to relatively high rates of substrate utilization and 3) the saturability of the activity at high substrate concentrations. The sensitivities to PMSF and arachidonyl trifluoromethyl ketone found in the present study are consistent with the literature. Thus IC_{50} values for PMSF on the order of 12 and 25 μM have been reported for rat brain homogenates and microsomes, respectively (Hillard *et al.*, 1995; Desarnaud *et al.*, 1995). With respect to arachidonyl trifluoromethyl ketone, a concentration of 7.5 μM was found to inhibit the deamidation of 27.7 μM anandamide by $\sim 90\%$ in rat brain homogenates (Koutek *et al.*, 1994).

The K_m and V_{\max} values found in the present study (0.8 μM and $1.73 \text{ nmol} \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$, respectively) are in reasonable agreement with the values of 3.4 μM and $2.2 \text{ nmol} \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$ found at 30°C *via* an assay where [¹⁴C]arachidonic acid was separated from [¹⁴C]anandamide by thin-layer chromatography (Hillard *et al.*, 1995). On the other hand, higher values ($K_m = 12.7 \mu\text{M}$ and $V_{\max} = 5.6 \text{ nmol} \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$) were found when rat brain microsomes were used (Desarnaud *et al.*, 1995). This raises the possibility of subcellular heterogeneity, especially given that anandamide amidohydrolase activity is found in all subcellular fractions of rat brain except the cytosol (Hillard *et al.*, 1995).

In their study, Hillard *et al.* (1995) found that arachidonic acid inhibited 3 μM anandamide deamidation, with an inhibition of $\sim 30\%$ over the concentration range 1 to 10 μM arachidonic acid and increasing to $\sim 40\%$ and $\sim 95\%$ at 30 and 100 μM arachidonic acid, respectively, being found. The linearity with time found in the present study, however, would suggest that there is little functional product inhibition by arachidonic acid under the conditions used. Whether this is due to insufficient build-up of arachidonic acid (in the case of 1 μM anandamide) or to a mode of product inhibition that

decreases with increasing substrate concentration (in the case of 27.7 μM anandamide) awaits investigation.

It can thus be concluded, in agreement with the study of Omeir *et al.* (1995), that the use of anandamide labeled in the ethanolamide position provides a simple and robust assay of anandamide deamidation. The assay has been used to establish the nature of inhibition of anandamide deamidation by the NSAID ibuprofen and to explore the relationship between the structure of this compound and its inhibitory activity. Ibuprofen was found to be a mixed-type inhibitor of anandamide deamidation, with K_i and K'_i values of 82 and 1420 μM being found. Whether the mixed-type nature of the inhibition reflects the absolute mode of inhibition or a mixture of actions on a heterogeneous enzyme population awaits elucidation. Be that as it may, the K_i value (and the IC_{50} value of 270 μM found at 2 μM [^3H]anandamide) is lower than the IC_{50} value of ~ 800 μM for inhibition by ibuprofen of COX-2 activity in broken cells (Mitchell *et al.*, 1994). With respect to clinical data, peak plasma ibuprofen concentrations of 110 and 150 μM have been reported after 2×200 -mg single doses of two over-the-counter ibuprofen preparations (Karttunen *et al.*, 1990). Thus the data presented here suggest that ibuprofen is a sufficiently potent inhibitor of anandamide deamidation to affect anandamide metabolism *in vivo*, particularly in the case of individuals taking higher doses for rheumatoid arthritis.

In order further to explore the effect of ibuprofen on anandamide deamidation, we evaluated the potencies of a series of related compounds. All of the compounds tested with an isobutyric acid side-chain coupled to an aromatic moiety inhibited anandamide deamidation with IC_{50} values ranging from 170 to 650 μM . The aromatic moiety was necessary for inhibition, because isobutyric acid itself was inactive. For the compounds with an isobutyric acid side-chain, some structure-activity elements can be seen. Thus replacement of the isobutylphenyl group of ibuprofen with the more bulky 6-methoxynaphthyl group of naproxen reduced the inhibitory potency by a factor of 2. On the other hand, replacement of the isobutyl group of ibuprofen with a thienecarbonyl group (suprofen) produced a slight increase in potency and resulted in the most potent compound tested, despite the more bulky nature of this compound. The potency of suprofen is presumably a consequence of the polarity of the thiophene, because its replacement with a phenyl group (ketoprofen) reduced the potency about 4-fold. On the other hand, the ketone linkage of ketoprofen can be replaced with an ether linkage (fenoprofen) with only marginal effect on the inhibitory potency.

Diclofenac and sulindac were considerably less potent than ibuprofen and suprofen, although the molecules contained some inhibitory activity. This was not the case for hydrocinnamic acid, which produced no inhibition at all, even at the highest concentration tested. Thus the inhibition of anandamide deamidation requires more than simply a carboxyl moiety coupled to an aromatic ring. These data suggest that there is some specificity to the inhibition produced by ibuprofen and suprofen, a conclusion reinforced by the finding that neither acetylsalicylic acid nor acetaminophen showed inhibitory effects even at the highest concentration tested.

In conclusion, the present findings demonstrate that ibuprofen is able to inhibit anandamide deamidation in a mixed manner at pharmacologically relevant concentrations. The clinical relevance of this finding is unclear: although ibupro-

fen (and suprofen) have been shown to be superior to aspirin with respect to analgesic efficacy in studies of oral surgical pain after removal of impacted third molars (Cooper *et al.*, 1986; Forbes *et al.*, 1992), there is no obvious clinical correlate for the differences in inhibitory potencies with respect to anandamide deamidation of the various propionic acid NSAIDs (Insel, 1996). Nevertheless, the finding that pravadoline, which has agonist efficacy at cannabinoid receptors in addition to its COX-inhibitory properties, has greater antinociceptive efficacy than other NSAIDs (see D'Ambra *et al.*, 1992) raises the possibility that a combined anandamide amidohydrolase/COX-2 inhibitor may be a therapeutically interesting prospect. This possibility is underlined by the recent finding that intrathecal administration of the cannabinoid receptor antagonist SR 141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide) produces hyperalgesia in mice as assessed by the hot-plate test (Richardson *et al.*, 1997), which suggests a tonic cannabinoid receptor activity and, by extension, a role of endogenous cannabimimetics in some aspects of nociception.

Acknowledgments

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