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Dopamides, Vanillylamides, Ethanolamides, and Arachidonic Acid Amides of Anti-inflammatory and Analgesic Drug Substances as TRPV1 Ligands

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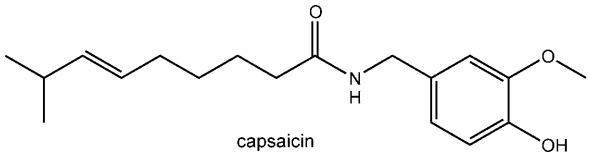
Dedicated to the memory of Professor Santosh Nigam.

Drug substances can be acylated metabolically to give derivatives with specific and strong molecular effects. We generated potentially naturally occurring acid amides of several anti-inflammatory and analgesic drugs. In the amides, the drug moieties served either as amine or acid components. All compounds were evaluated for activity toward transient receptor potential vanilloid subfamily member 1 (TRPV1) in a cell-based Ca^{2+} influx assay; TRPV1 is a key receptor in the pain pathway and a promising target for analgesic drugs. We found that dopamine amides of fenamic acids have TRPV1 agonist activity in the nanomolar range, and that the arachidonoyl amide of a dipyrone metabolite has TRPV1 antagonist activity. Flufenamic acid dopamide, the most potent TRPV1 agonist reported herein, retains the cyclooxygenase (COX) inhibition properties of the parent compound flufenamic acid. Thus it acts on two different major players in the pain processing machinery. The compounds could be further keys to understanding the mechanism of action of fenamates and dipyrone at the molecular level. The fenamic acid dopamine amides qualify as new lead structures for drug development.

Introduction

In 1997, the field of somatic sensory biology and pain research witnessed the breakthrough work of David Julius and colleagues that led to the cloning of the first vanilloid receptor, transient receptor potential vanilloid subfamily, member 1 (TRPV1).[1] TRPV1 is a nonselective cation channel displaying preference for Ca^{2+} and is activated or sensitized by a multitude of stimuli. Among them are capsaicin (the irritant principle of hot peppers), temperatures exceeding 43 °C, protons, various peptides, lipoxygenase products, and arachidonic acid derivatives.[2]



capsaicin

Capsaicin-sensitive neurons are bipolar neurons with mainly unmyelinated axons (C fibers) and somata in sensory (dorsal root and trigeminal) ganglia. Due to the expression in pain-mediating pathways, TRPV1 was selected as an attractive target for pain relief. Further, in vitro and in vivo studies show TRPV1 to be one of the most important integrators of polymodal noxious stimuli.

Some endogenous arachidonic acid derivatives are activators of TRPV1; the most prominent endovanilloids are anandamide and arachidonoyldopamine (Figure 1). They were originally designated as endocannabinoids because of their affinity for cannabinoid receptors. Both fatty acid derivatives also stimulate TRPV1, anandamide has EC_{50} values from 0.2 to 5 μM in HEK-293 cells and arachidonoyldopamine has an EC_{50} value of 30 nM in HEK-293 cells overexpressing TRPV1 receptors, which is similar to that of capsaicin.[3–6] Arvanil is a synthetic archidonate that contains the vanillylamine moiety and potently (EC_{50} = 0.5 nM) stimulates TRPV1.[7]

Another endogenous compound with vanilloid-like activity is *N*-(4-hydroxyphenyl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide, which is also known as AM404. AM404 exhibited TRPV1 agonism with an EC_{50} value of 26 nM.[7] The endogenous pres-

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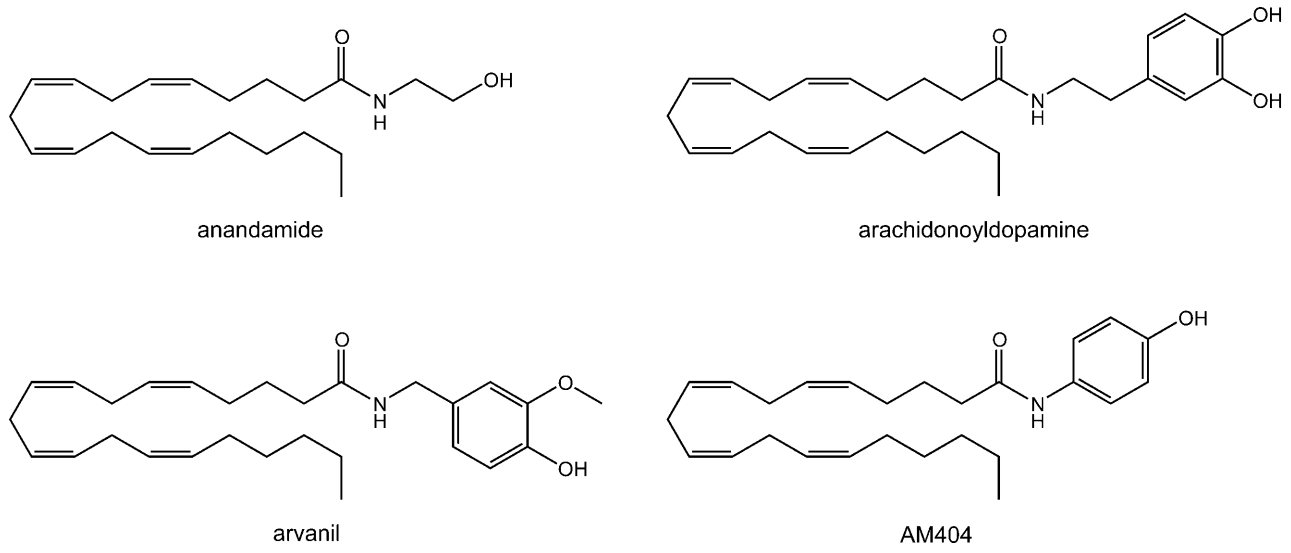


Figure 1. The naturally occurring arachidonic acid derivatives anandamide, arachidonoyldopamine, AM404, and the synthetic arachidonic acid derivative arvanil. All of them are potent agonists at TRPV1.

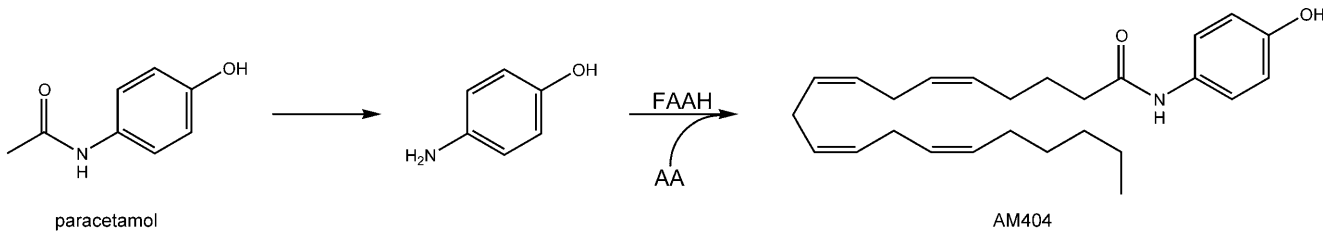
ence of AM404 is very intriguing because it is formed after administration of the widely used analgesic paracetamol (acetaminophen) to rats. This new pathway of biotransformation of paracetamol, which is depicted in Scheme 1 was described by Högestätt et al., who found a primary deacetylation step that takes place in liver, brain and spinal cord of the rats, and afterward a re-N-acylation of 4-aminophenol with arachidonic acid, which is catalyzed by fatty acid amide hydrolase, a key enzyme of the endocannabinoid system.[8]

Synthetic Strategy

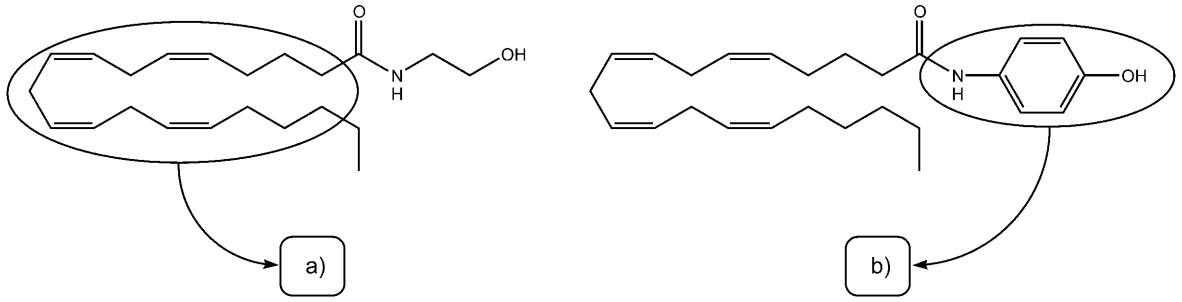
As stated, certain fatty acid derivatives have strong biological effects. Thus we reasoned that some drug substances might exert their activity at least partly through metabolic acylation with arachidonic acid as exemplified by paracetamol. Alternatively, drugs or drug metabolites might mimic fatty acid moieties and derivatives, and thus either by themselves or after metabolic conversion into amides modulate TRPV1. We chose to probe this bioequivalence concept by starting with anti-inflammatory and analgesic drug substances. Accordingly, we created new potential vanilloids that were patterned on the model molecules anandamide, arachidonoyldopamine, arvanil and AM404. We substituted either: a) the arachidonic acid moiety in anandamide, arachidonoyldopamine, or arvanil by cyclooxygenase inhibitors (in other words, arachidonic acid mimetics), or b) the 4-aminophenol moiety in AM404 by drug substances and metabolites of drug substances (Scheme 2). Mefenamic and flufenamic acid served as arachidonic acid mimetics according to Scheme 2a, and the compounds listed in Table 1 were synthesized as described in the following section.

Pharmacology

All compounds were evaluated for agonistic activity at concentrations of 1 and 0.1 μM in an intracellular Ca^{2+} assay. HEK-293 cells stably overexpressing the human recombinant TRPV1 receptor were used to assess the functional activity at TRPV1 as previously described.[9] The activity of the compounds was normalized to the maximum intracellular Ca^{2+} elevation that was generated by the application of 4 μM ionomycin. Compounds that did not show any agonistic activity were subsequently tested at concentrations of 10 and 1 μM for antagonistic activity against the effect of 0.1 μM capsaicin on intracellular Ca^{2+}. Because of its very potent activity at TRPV1 receptors, the dopamide **4**, which was derived from the COX inhibitor flufenamic acid was also tested for inhibition of cyclooxygenases in whole-blood assays. It was of special interest to evaluate if the



Scheme 1. Biotransformation of paracetamol according to Högestätt et al. First, paracetamol undergoes deacetylation in the liver, brain, and spinal cord. The resulting 4-aminophenol is then acylated with arachidonic acid (AA) by fatty acid amide hydrolase (FAAH) to obtain the arachidonic acid derivative AM404.



Scheme 2. The bioequivalence principles that were used in this work. Pathway a): Substitution of the arachidonic acid moiety by arachidonic acid mimetics was applied to anandamide, arachidonoyldopamine, and arvanil (here shown for anandamide). Pathway b): Substitution of the 4-aminophenol moiety in AM404 by drug substances.

dopamide **4** would be able to act simultaneously on two key players of the pain pathway: on TRPV1 and on cyclooxygenases.[10]

Results

TRPV1 receptor modulation

Data for the activation of TRPV1 receptors are listed in Table 2. The ethanolamides **3** and **6** and the arachidonates **7** and **9** are not included because they did not display any effect (neither agonism nor antagonism) at TRPV1 at the two concentrations that were tested. In contrast, the dopamine amide and vanillylamide of flufenamic acid (compounds **4** and **5**) and the dopamine amide of mefenamic acid **1** exhibited potent agonist activity. The vanillylamide **2** was a weak agonist. The arachidonates of mesalazine (**8**) and of methylaminoantipyrine (**10**) behaved as antagonists.

The pEC_{50} values were determined for two compounds that had shown marked activity, namely the agonists **1** and **4** (Figure 2); these proved to be potent agonists with pEC_{50} values of 7.44 ± 0.30 (36 nM) and 7.79 ± 0.02 (16 nM), respectively.

The antagonistic behavior of **8** and **10** was further characterized by determining the residual activity of a standard TRPV1 agonist, capsaicin. In this test, the mesalazine derivative **8** decreased the activity of 0.1 μM capsaicin to approximately 50% with no significant difference at the two concentrations tested (10 and 1 μM), proving it to be a weak antagonist. In contrast, the dipyrone derivative **10** showed a dose-dependent suppression of capsaicin activity that was almost complete at a concentration of 10 μM. The dipyrone derivative **10** demonstrated moderate antagonistic behavior, which is depicted in Figure 3, with a pIC_{50} value of 5.56 ± 0.12 (3 μM).

Cyclooxygenase isoenzyme inhibition

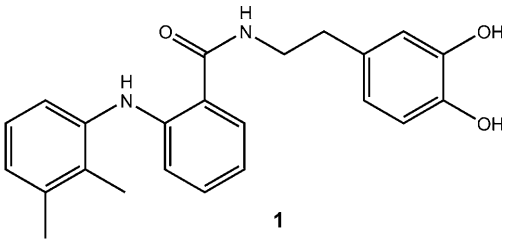
Of the compounds that were tested at the TRPV1 receptor, the flufenamic acid dopamine amide **4** displayed the most potent agonism and was therefore selected for the investigation of possible inhibitory activity against the cyclooxygenase isoenzymes. We chose a whole-blood assay because it is closer to an in vivo situation. Activities were assayed as followed: for COX-1 by monitoring, the concentration of thromboxane B_2 (TXB_2), for COX-2, of prostaglandin E_2 (PGE_2). The inhibition curves are shown in Figure 4 and Figure 5. We found inhibition of both cyclooxygenase 1 and 2 by **4** with pIC_{50} values of 4.17 ± 0.16 and 4.41 ± 0.09 for COX-1 and COX-2, which respectively corresponds to IC_{50} values of approximately 68 and 39 μM.

Discussion

By investigating the possible involvement of TRPV1 modulation by amides of some anti-inflammatory and analgesic drugs, we elucidated several possibilities. The mesalazine derivative **8** strongly resembles AM404, the recently discovered metabolite of paracetamol, but differs because it contains an added carboxylic acid function. AM404 is formed in vivo from arachidonic acid and 4-aminophenol, which are conjugated under the catalysis of fatty acid amide hydrolase. The fact that the salicylic acid derivative mesalazine is administered in doses of 1–4 g per day and that it is released in a way that is target-oriented and spatially restricted at the bowel mucosa might result in local compound concentrations that are sufficient for fatty acid amide hydrolase to conjugate arachidonic acid and mesalazine. The molecular mechanism of action of mesalazine still remains elusive, and a multifactorial basis of therapeutic action has been suggested.[11] Antagonists at TRPV1 demonstrated therapeutic utility in animal models of inflammatory bowel diseases.[12,13] Because we could show an albeit weak TRPV1 antagonism for **8**, this activity might be partly responsible for mesalazine's therapeutic activity. Animal and human studies are needed to corroborate this.

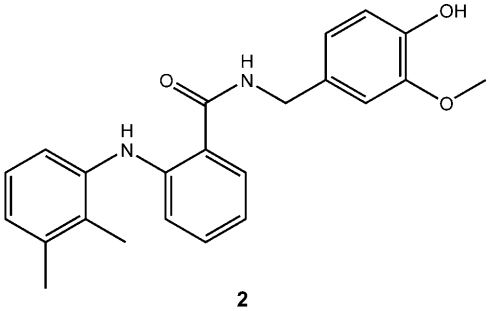
Turning to agonistic derivatives of anti-inflammatory drugs that were investigated in the present study, we found very strong agonism for **1**, which displayed an EC_{50} value of approximately 36 nM, and **4**, which stimulated TRPV1 with an EC_{50} value of approximately 16 nM. The higher TRPV1 activity of the fenamic acid dopamine amides **1** and **4**, compared with the respective vanillylamine amides **2** and **5**, was unexpected because the vanillylamine moiety hitherto was found to be a crucial unit for vanilloid binding.[14] Compounds **1** and **4** combine two properties that make them attractive as potential lead structures. First, both compounds are composed of pharmacodynamically and pharmacokinetically validated moieties. Sec-

Table 1. Synthesized amides of drug substances.



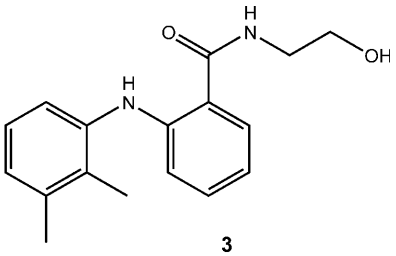
1

dopamine amide of mefenamic acid



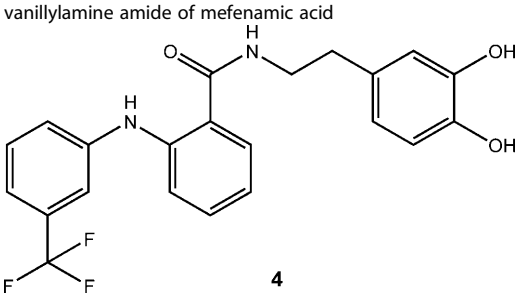
2

vanillylamine amide of mefenamic acid



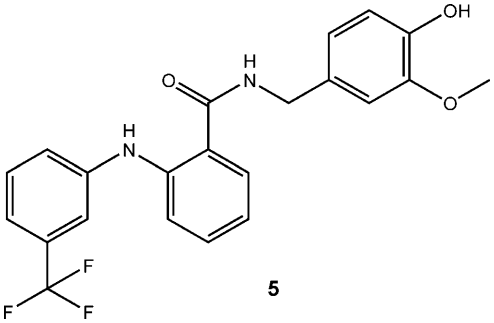
3

ethanolamine amide of mefenamic acid



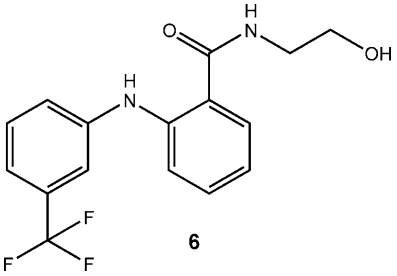
4

dopamine amide of flufenamic acid



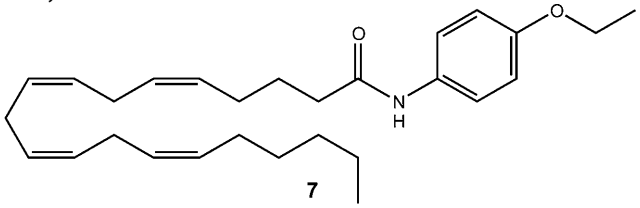
5

vanillylamine amide of flufenamic acid



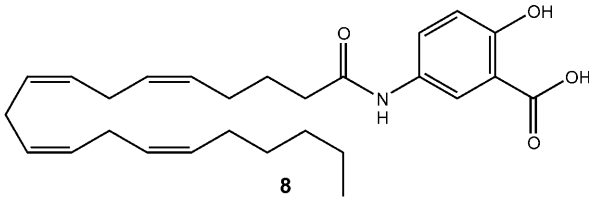
6

ethanolamine amide of flufenamic acid



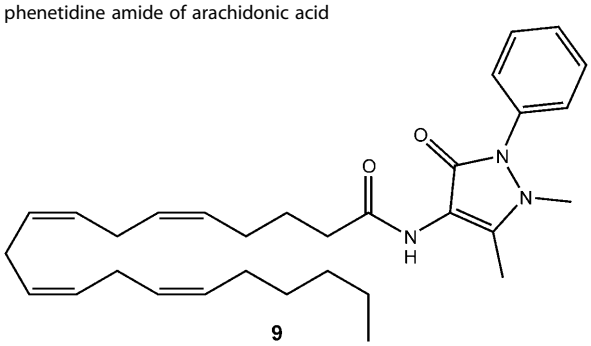
7

phenetidine amide of arachidonic acid



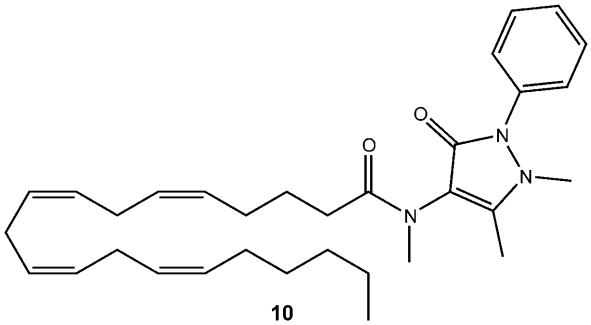
8

mesalazine amide of arachidonic acid



9

aminoantipyrine amide of arachidonic acid



10

methylaminoantipyrine amide of arachidonic acid

ondly, no pungency, usually one of the main drawbacks of TRPV1 agonists, was observed in the case of **1** and **4**.[15] Olvanil and glyceryl nonamide represent two other nonpungent TRPV1 agonists due to their slower activation kinetics at TRPV1.[16] Furthermore, *cis*-capsaicin, which is less pungent than capsaicin is being actively pursued in phase III trials for

Table 2. Activation of TRPV1 [%][a]				
Compound: c [μM]	**1**	**2**	**4**	**5**
1	72	14	81	70
0.1	55	0	71	36

[a] Ionomycin at 4 μM established activation at 100%; data are reported as the mean of $n=3$ experiments.

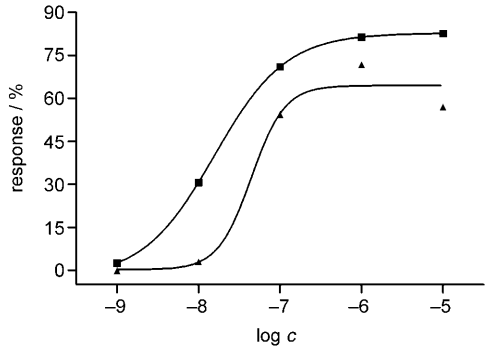


Figure 2. Dose–response curves of the agonists **1** (▲) and **4** (■) at hTRPV1-transfected HEK-293 cells. Ca^{2+} influx was determined by using Fluo-4AM. Ca^{2+} influx after application of 4 μM ionomycin was set as 100%. Data are mean values of three experiments; SEM were never >10% of the means.

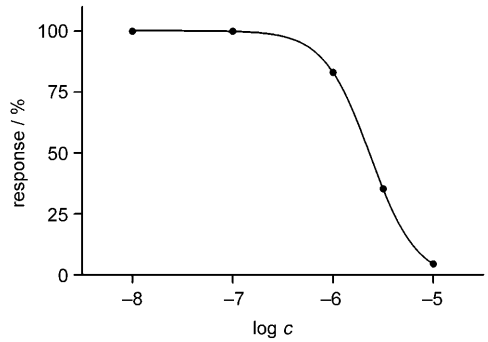


Figure 3. Dose–response curves of the antagonist **10** in the presence of 0.1 μM capsaicin in hTRPV1-transfected HEK-293 cells. Response of 100% was set at 0.1 μM capsaicin alone. Ca^{2+} influx was determined by using Fluo-4AM. Data are mean values of three experiments; SEM were never >10% of the means.

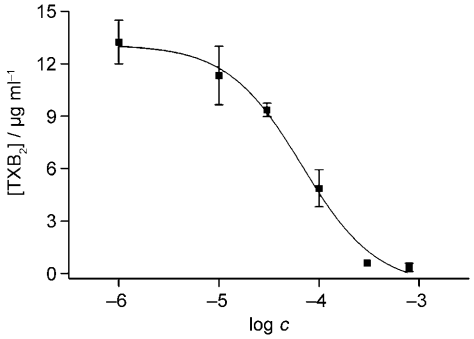


Figure 4. Inhibition of COX-1 by **4** in whole blood. Blood was incubated with **4**, and the concentration of TXB_2 was determined by GC–MS/MS. Data are means of four experiments ±SD.

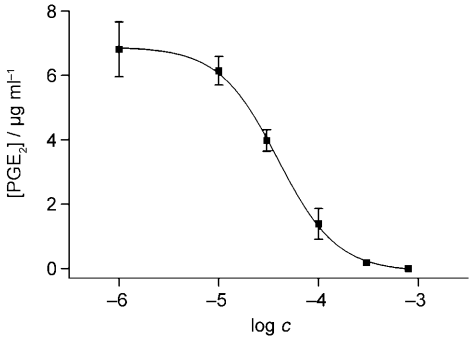


Figure 5. Inhibition of COX-2 by **4** in whole blood. Heparinized blood was incubated with **4**, and the concentration of PGE_2 was determined by GC–MS/MS. Data are means of four experiments ±SD

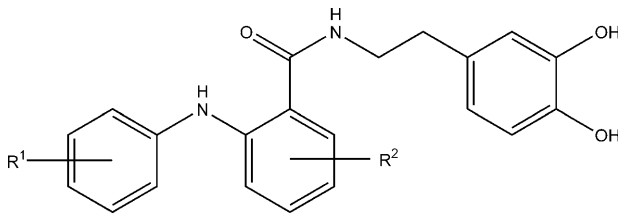


Figure 6. 2-Phenylaminobenzodopamide, a new lead structure for TRPV1 agonists on the basis of the activities of **1** and **4**.

cluster headache and osteoarthritis pain.[2,17] In view of this, we found the 2-phenylaminobenzodopamide skeleton to be a new lead for TRPV1 ligands, especially agonists (Figure 6).

To characterize **4** further, we examined its inhibitory activity toward the cyclooxygenase isoenzymes COX-1 and COX-2 in whole-blood assays. The parent compound of **4**, flufenamic acid, is known to robustly inhibit cyclooxygenases, and has IC_{50} values of 3 and 9 μM against COX-1 and COX-2, respectively.[18] For **4**, we observed inhibition of both cyclooxygenases with pIC_{50} values of 4.17±0.16 and 4.41±0.09 for COX-1 and COX-2, which corresponded to IC_{50} values of approximately 68 and 39 μM, respectively. Presently, it cannot be decided if the inhibition of the cyclooxygenases is due to the flufenamate, the catechol moiety or both. Other catechols like rooperol, hypoxoside, nordihydroguaiaretic acid and quercetin are known to inhibit cyclooxygenases.[19,20] Because catechols are easily oxidizable, they probably block the peroxidase activity of COX by reduction of the Fe^{3+} protoporphyrin complex. Again, further studies will have to reveal if **4** is formed in vivo as a pharmacologically active metabolite of flufenamic acid that is able to act on TRPV1 and cyclooxygenases.

Dipyrone (metamizole) is a well-known strong analgesic and antipyretic compound with rather weak anti-inflammatory effects relative to mesalazine and fenamates. It shares the latter characteristic with paracetamol. Dipyrone is presently experiencing a renaissance in therapy.[21] The molecular mechanism of action of dipyrone has been under debate for a long time. Both central and peripheral inhibition of prostaglandin biosyn-

thesis was shown to be at the basis of dipyrone action.[22–25] Recent studies take the biotransformation of dipyrone into consideration to explain the molecular mechanism of action. Dipyrone itself is detectable in serum for only about 15 min following intravenous administration. It is not detectable after oral intake.[26] The pharmacokinetics of dipyrone are characterized by a rapid hydrolytic retro-Mannich reaction to 4-methylaminoantipyrine, which has 85% bioavailability after oral administration, and takes a short time to achieve maximal systemic concentrations (t_{max}: 1.2–2.0 h). 4-Methylaminoantipyrine is further metabolized to 4-aminoantipyrine and both were shown to inhibit COX-1 (IC_{50}: 2.6 and 20.8 μM, respectively) and COX-2 (IC_{50}: 4.7 and 41.8 μM, respectively).[27] In a very recent paper, the possibility of forming **10** was ruled out because 4-methylaminoantipyrine was presumed to be a tertiary amine.[28] In fact, it is a secondary amine, and we were able to acylate it smoothly with arachidonic acid. Whereas the 4-aminoantipyrine derivative **9** did not demonstrate any effect at TRPV1, **10** was found to exhibit antagonist activity with an IC_{50} value of about 3 μM. TRPV1 antagonists are well known for their marked analgesic properties. Thus **10**, if formed in humans, could contribute to the molecular mechanisms of action of dipyrone. We have already proved the arachidonates **9** and **10** to be formed in small rodents after oral application of dipyrone.[29]

Conclusions

In conclusion, we have shown that some synthetic amides of widely used analgesic and anti-inflammatory drugs are capable of interacting with TRPV1 receptors by either activating it or antagonizing the activity of capsaicin. If it is proven that these compounds are formed in vivo as drug metabolites, their activities—antagonism at TRPV1 as well as agonism and consecutively desensitization of TRPV1—might contribute to their analgesic activity, and future studies will have to address this possibility. This study indicates that the synthesis, testing and finally search for metabolites of drugs that are formed by acylation is a fruitful field for the understanding of molecular mechanisms of action.

Experimental Section

Chemistry

All chemical reagents were purchased commercially unless otherwise stated. Chemical yields were not optimized. TLC was performed by using Merck Kieselgel 60 F_{254} silica plates and components were visualized by using UV light or a iodine chamber. Melting points were determined by using a Boëtius hot-stage microscope and are uncorrected. Routine NMR spectra were recorded by using a Varian Gemini 2000 or a Varian Inova Unity 500. Mass spectra were recorded by using an AMD Intectra DP 10 mass spectrometer. Combustion analyzes were performed with a Leco CHNS-932 Analyzer and were within 0.4% of theoretical values unless otherwise indicated. For combustion analysis data exceeding this range, the compound's purity was confirmed by high resolution mass spectrometry with a Waters Micromass Q-TOF-2 in the positive mode.

***N*-[2-(3,4-Dihydroxyphenyl)ethyl]-2-[(2,3-dimethylphenyl)amino]-benzamide (1)**: Initially, mefenamic acid pentafluorophenyl ester was prepared and was treated with dopamine hydrochloride afterward. The preparation of the pentafluorophenyl ester was chosen due to its selectivity for aminolysis compared with alcoholysis. Mefenamic acid (1.21 g, 5 mmol) and pentafluorophenol (1.10 g, 6 mmol) were dissolved in dry $CHCl_3$ (50 mL) and the mixture was ice cooled for 10 min. Dicyclohexylcarbodiimide (DCC; 1.03 g, 5 mmol) was added, and the reaction was stirred for 1 h with ice cooling and then overnight at room temperature. The precipitated dicyclohexylurea (DCU) was filtered off, and the organic solvent was evaporated. The crude product was purified by column chromatography on silica gel (hexane). Crystallization from MeOH afforded yellowish crystals. Yield: 599.9 mg (29%); $R_f = 0.48$ (hexane); mp: 123–125 °C (MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.87$ (s, 1H, N**H**), 8.17–8.13 (m, 1H, Ar), 7.37–7.31 (m, 1H, Ar), 7.16–7.04 (m, 3H, 3×Ar), 6.76–6.71 (m, 2H, 2×Ar), 2.32 (s, 3H, C**H$_3$**), 2.15 ppm (s, 3H, C**H$_3$**); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 164.3$, 151.0, 138.4, 137.7, 136.1, 132.9, 132.1, 127.6, 126.1, 123.8, 116.4, 113.9, 107.0, 20.6, 14.0 ppm; EIMS (70 eV), *m/z* (%): 224 (100), 407 (56.4, $[M]^+$), 209 (39.3); Anal. ($C_{21}H_{14}F_5NO_2$) C, H, N.

Mefenamic acid pentafluorophenyl ester (305.5 mg, 0.75 mmol) was dissolved in acetonitrile/*t*-butyl methyl ether (TBME; 2:1, 15 mL). Dopamine hydrochloride (213.3 mg, 1.12 mmol) and Et_3N (151.7 mg, 1.5 mmol) were added, and the mixture was stirred at room temperature for 3 h. The organic phase was extracted consecutively with 0.1 M HCl (10 mL), sat. $NaHCO_3$ (10 mL), and H_2O (10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/hexane, 2:3). Crystallization from EtOAc/hexane afforded yellowish crystals. Yield: 105.7 mg (39%); $R_f = 0.25$ (EtOAc/hexane, 2:3); mp: 208–210 °C (EtOAc/hexane); ^{1}H NMR (400 MHz, $CDCl_3$/[D$_6$]DMSO): $\delta = 9.41$ (s, 1H, N**H**), 8.40 (br s, 2H, 2×O**H**), 8.27 (t, $^3J = 5.5$ Hz, 1H, CON**H**), 7.51–7.48 (m, 1H, Ar), 7.13–6.93 (m, 3H, 3×Ar), 6.83–6.75 (m, 2H, 2×Ar), 6.63–6.58 (m, 3H, 3×Ar), 6.45–6.41 (m, 1H, Ar), 3.41–3.35 (m, 2H, NHC**H$_2$**CH$_2$), 2.65 (t, $^3J = 7.5$ Hz, 2H, NHCH$_2$C**H$_2$**), 2.22 (s, 3H, C**H$_3$**), 2.08 ppm (s, 3H, C**H$_3$**); ^{13}C NMR (100 MHz, CD_3OD), $\delta = 171.7$, 147.7, 146.0, 144.5, 140.7, 138.8, 132.7, 132.0, 131.5, 129.1, 126.7, 126.5, 121.8, 120.9, 119.0, 117.9, 116.7, 116.2, 115.4, 42.4, 35.8, 20.4, 13.8 ppm; EIMS (70 eV), *m/z* (%): 224 (100), 223 (65.7), 376 (64.3, $[M]^+$); Anal. ($C_{23}H_{24}N_2O_3$) C, H, N.

***N*-(4-Hydroxy-3-methoxybenzyl)-2-[(2,3-dimethylphenyl)amino]-benzamide (2)**: Mefenamic acid (241.3 mg, 1 mmol) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 520.4 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL). Vanillylamine hydrochloride (189.6 mg, 1.0 mmol) was added, and the solution was stirred for 10 min under ice cooling. Et_3N (303.6 mg, 3.3 mmol) was added, and the mixture was stirred at room temperature for 3 h. The organic solvent was evaporated and the crude residue was dissolved in Et_2O. The organic phase was extracted with sat. $NaHCO_3$ (2×10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/cyclohexane, 7:3). Crystallization from TBME afforded white crystals. Yield: 116.5 mg (32%); $R_f = 0.57$ (EtOAc/cyclohexane, 7:3); mp: 140–142 °C (TBME); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.19$ (s, 1H, N**H**), 7.39–7.35 (m, 1H, Ar), 7.21–6.82 (m, 8H, 8×Ar), 6.68–6.63 (m, 1H, Ar), 6.31 (br s, 1H, CON**H**), 5.57 (s, 1H, O**H**), 4.53 (d, $^3J = 5.4$ Hz, 2H, NHC**H$_2$**), 3.86 (s, 3H, OC**H$_3$**), 2.31 (s, 3H, C**H$_3$**), 2.19 ppm (s, 3H, C**H$_3$**); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.4$, 145.2, 139.5, 138.0, 132.3, 131.0, 127.2, 125.71, 125.69, 121.1, 120.9, 116.74, 116.71,

115.0, 114.5, 110.7, 56.0, 43.9, 20.6, 13.9 ppm; EIMS (70 eV), *m/z* (%): 223 (100), 376 (97.9, $[M]^+$), 240 (50.7); Anal. ($C_{23}H_{24}N_2O_3$) C, H, N.

***N*-(2-Hydroxyethyl)-2-[(2,3-dimethylphenyl)amino]benzamide (3)**: Mefenamic acid (241.3 mg, 1 mmol) was dissolved in dry acetonitrile (25 mL). Et_3N (303.6 mg, 3 mmol) and PyBOP (520.4 mg, 1 mmol) were added, and the solution was stirred at room temperature for 5 min. 2-Aminoethanol (61.1 mg, 1 mmol) was added, and the mixture was stirred for 3 h at room temperature. The organic solvent was evaporated, and the crude residue was dissolved in brine/TBME (1:2, 30 mL). The organic phase was extracted consecutively with 0.1 M HCl (10 mL) and sat. $NaHCO_3$ (10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/cyclohexane, 6:4). Crystallization from MeOH/hexane afforded yellow crystals. Yield: 111.9 mg (40%); R_f = 0.25 (EtOAc/cyclohexane, 6:4); mp: 89–90 °C (MeOH/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.13 (s, 1H, N**H**), 7.43–7.40 (m, 1H, Ar), 7.22–6.88 (m, 5H, 5×Ar), 6.95–6.87 (m, 1H, Ar), 6.58 (br s, 1H, CON**H**), 3.85–3.80 (m, 2H, C$\mathbf{H_2}$OH), 3.63–3.58 (m, 2H, NHC$\mathbf{H_2}$), 2.38 (br s, 1H, O**H**), 2.30 (s, 3H, C$\mathbf{H_3}$), 2.18 ppm (s, 3H, C$\mathbf{H_3}$); ^{13}C NMR (100 MHz, CD_3OD): δ: 171.9, 147.8, 140.5, 138.7, 132.7, 131.4, 129.1, 126.6, 126.4, 121.8, 118.5, 117.8, 115.3, 61.5, 43.0, 20.5, 13.8 ppm; EIMS (70 eV), *m/z* (%): 223 (100), 284 (98.6, $[M]^+$), 208 (88.6); Anal. ($C_{17}H_{20}N_2O_2$) C, H, N.

***N*-[2-(3,4-Dihydroxyphenyl)ethyl]-2-{[3-(trifluoromethyl)phenyl]amino}benzamide (4)**: Initially, flufenamic acid pentafluorophenyl ester was prepared that was treated with dopamine hydrochloride afterward. The preparation of the pentafluorophenyl ester was chosen due to its selectivity for aminolysis compared with alcoholysis. Flufenamic acid (4.22 g, 15 mmol) and pentafluorophenol (3.31 g, 18 mmol) were dissolved in dry CH_2Cl_2 (15 mL), and the mixture was ice cooled for 10 min. DCC (3.10 g, 15 mmol) was added, and the solution was stirred for 1 h with ice cooling, and then overnight at room temperature. The precipitated DCU was filtered off, and the organic solvent was evaporated. The crude product was purified by column chromatography on silica gel (EtOAc/hexane, 1:9). Crystallization from MeOH afforded yellowish crystals. Yield: 4.69 g (70%); R_f = 0.52 (EtOAc/hexane, 1:9); mp: 53–55 °C (MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.15 (s, 1H, N**H**), 8.21–8.18 (m, 1H, Ar), 7.51–7.23 (m, 6H, 6×Ar), 6.91–6.86 ppm (m, 1H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.2, 148.5, 142.8, 140.6, 136.7, 135.8, 132.6, 132.4, 132.2, 131.9, 130.6, 129.6, 126.3, 125.2, 125.1, 122.4, 120.2, 119.8, 119.2, 118.8, 113.6, 109.0 ppm; EIMS (70 eV), *m/z* (%): 264 (100), 167 (18.6), 244 (15.7), 447 (10.7, $[M]^+$); Anal. ($C_{20}H_9F_8NO_2$) C, H, N.

Flufenamic acid pentafluorophenyl ester (894.6 mg, 2 mmol) was dissolved in dry acetonitrile (10 mL). Dopamine hydrochloride (569 mg, 3 mmol) and Et_3N (404.6 mg, 4 mmol) were added and stirred at room temperature for 2.5 h. The organic phase was diluted with TBME (10 mL) and then extracted with H_2O (10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/hexane, 2:3). Crystallization from Et_2O/pentane afforded beige crystals. Yield: 135.4 mg, (16%); R_f = 0.20 (EtOAc/hexane, 2:3); mp: 50–53 °C (Et_2O/pentane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.35, (s, 1H, N**H**), 7.41–7.17 (m, 7H, 7×Ar), 6.82–6.73 (m, 3H, 3×Ar), 6.65–6.63 (m, 1H, Ar), 6.12 (br s, 1H, CON**H**), 5.27 (s, 1H, O**H**), 5.06 (s, 1H, O**H**), 3.65–3.60 (m, 2H, NHC$\mathbf{H_2}$CH$_2$), 2.80 ppm (t, ^3J = 6.6 Hz, 2H, NHCH$_2$C$\mathbf{H_2}$); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.6, 144.0, 143.9, 142.6, 142.4, 132.4, 131.9, 131.6, 131.2, 129.8, 127.6, 122.8, 121.0, 119.9, 119.5, 118.5, 118.4, 116.3, 116.13, 116.09, 115.63, 115.57, 41.2, 34.8 ppm; EIMS (70 eV), *m/z* (%): 264 (100), 263 (40), 416 (19.3, $[M]^+$); HRMS: *m/z*: calcd for $C_{22}H_{19}F_3N_2O_3$: 416.1348 $[M]^+$; found: 416.1360; combustion analysis calcd (%) for $C_{22}H_{19}F_3N_2O_3$: C 63.46, H 4.60, N 6.73; found: C 63.53, H 5.20, N 6.39.

***N*-(4-Hydroxy-3-methoxybenzyl)-2-{[3-(trifluoromethyl)phenyl]amino}benzamide (5)**: Flufenamic acid (281.2 mg, 1.0 mmol) and PyBOP (520.4 mg, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL). Vanillylamine hydrochloride (189.6 mg, 1.0 mmol) was added, and the solution was stirred for 10 min under ice cooling. Et_3N (303.6 mg, 3.3 mmol) was added, and the solution was stirred at room temperature for 3 h. The organic solvent was evaporated, and the crude residue was dissolved in Et_2O. The organic phase was extracted with sat. $NaHCO_3$ (2×10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/cyclohexane, 7:3). Crystallization from TBME afforded white crystals. Yield: 110.5 mg (31%); R_f = 0.55 (EtOAc/cyclohexane, 7:3); mp: 123–125 °C (TBME); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.52 (s, 1H, N**H**), 7.43–7.18 (m, 7H, 7×Ar), 6.89–6.80 (m, 4H, 4×Ar), 6.33 (br s, 1H, CON**H**), 5.58 (s, 1H, O**H**), 4.51 (d, ^3J = 5.4 Hz, 2H, NHC$\mathbf{H_2}$), 3.84 ppm (s, 3H, OC$\mathbf{H_3}$); ^{13}C NMR (100 MHz, $CDCl_3$), δ = 170.0, 144.2, 142.2, 132.4, 131.8, 131.5, 131.2, 129.7, 127.7, 125.3, 122.9, 122.6, 119.1, 119.0, 118.5, 118.43, 118.39, 118.36, 116.3, 116.2, 115.9, 62.1, 42.6 ppm; EIMS (70 eV), *m/z* (%): 263 (100), 152 (86.4), 137 (82.9), 416 (71.4, $[M]^+$). Anal. ($C_{22}H_{19}F_3N_2O_3$) C, H, N.

***N*-(2-Hydroxyethyl)-2-{[3-(trifluoromethyl)phenyl]amino}benzamide (6)**: Flufenamic acid (281.2 mg, 1 mmol) was dissolved in dry acetonitrile (20 mL). Et_3N (303.6 mg, 3 mmol) and PyBOP (520.4 mg, 1 mmol) were added and stirred at room temperature for 5 min. 2-Aminoethanol (61.1 mg, 1 mmol) was added, and the solution was stirred for 3 h at room temperature. The organic solvent was evaporated, and the crude residue was dissolved in brine/TBME (1:2, 30 mL). The organic phase was extracted consecutively with 0.1 M HCl (10 mL) and sat. $NaHCO_3$ (10 mL). The organic phase was separated, dried with $MgSO_4$, filtered and concentrated to dryness. The crude product was purified by column chromatography on silica gel (EtOAc/cyclohexane, 4:1). Crystallization from MeOH/hexane afforded yellow crystals. Yield: 163 mg (50%); R_f = 0.35 (EtOAc/cyclohexane, 4:1); mp: 92–93 °C (MeOH/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.45 (s, 1H, N**H**), 7.48–7.18 (m, 7H, 7×Ar), 6.86–6.82 (m, 1H, Ar), 6.59 (br s, 1H, CON**H**), 3.83 (dt, ^3J = ^3J = 5.0 Hz, 2H, C$\mathbf{H_2}$OH), 3.62–3.58 (m, 2H, NHC$\mathbf{H_2}$), 2.18 ppm (t, ^3J = 5.0 Hz, 1H, O**H**); ^{13}C NMR (100 MHz, $CDCl_3$), δ = 168.9, 146.7, 145.3, 144.3, 142.3, 132.3, 131.8, 131.5, 131.2, 129.7, 129.6, 127.4, 125.3, 122.8, 122.6, 120.9, 119.3, 119.1, 118.4, 118.3, 116.2, 116.1, 116.0, 114.5, 110.6, 56.0, 44.0 ppm; EIMS (70 eV), *m/z* (%): 263 (100), 324 (67.1, $[M]^+$), 235 (13.6); Anal. ($C_{16}H_{15}F_3N_2O_2$) C, H, N.

***N*-(4-Ethoxyphenyl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (7)**: The reaction was carried out under an argon atmosphere. Arachidonic acid (201 mg, 0.66 mmol) and DMF (48.2 mg, 0.66 mmol) were dissolved in dry TBME (10 mL) and stirred under ice cooling for 15 min. Oxalyl chloride (167.5 mg, 1.32 mmol) was added, and the mixture was stirred under ice cooling for 1 h. 4-Ethoxyaniline (452.7 mg, 3.3 mmol) and 4-dimethylaminopyridine (24.2 mg, 0.2 mmol) were added, and the reagents were allowed to react for 20 min at room temperature. The solution was diluted with TBME (10 mL) and filtered. The filtrate was extracted consecutively with 0.1 M HCl (10 mL), sat. $NaHCO_3$ (10 mL) and H_2O (10 mL). The organic layer was separated, dried with $MgSO_4$ and filtered, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (EtOAc/hexane, 3:7) to afford a brownish oil. Yield: 205.7 mg

(74%); R_f = 0.42 (EtOAc/hexane, 3:7); ^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.26 (dd, ^3J = 7.1 Hz, ^4J = 2.1 Hz, 2H, 2×Ar), 6.98 (br s, 1H, CONH), 6.71 (dd, ^3J = 7.1 Hz, ^4J = 2.1 Hz, 2H, 2×Ar), 5.34–5.17 (m, 8H, 4×CH=CH), 3.88 (q, ^3J = 7.1 Hz, 2H, OCH_2CH_3), 2.75–2.64 (m, 6H, 3×CH=CHCH$_2$CH=CH), 2.20 (t, ^3J = 7.5 Hz, 2H, CH_2CH_2CONH), 2.08–1.89 (m, 4H, 2×CH=CHCH$_2$), 1.68 (tt, ^3J = ^3J = 7.5 Hz, 2H, CH_2CH_2CONH), 1.32–1.10 (m, 9H, OCH_2CH_3 + $CH_2CH_2CH_2CH_3$), 0.76 ppm (t, ^3J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.8, 155.7, 130.9, 130.5, 129.0, 128.9, 128.6, 128.2, 128.1, 127.8, 127.5, 121.7, 114.8, 63.7, 36.9, 31.5, 29.3, 27.2, 26.7, 25.7, 25.4, 22.6, 14.8, 14.1 ppm; EIMS (70 eV), *m/z* (%): 137 (100), 179 (84.3), 423 (35.0, $[M]^+$); Anal. ($C_{28}H_{41}NO_2$) C, H, N.

2-Hydroxy-5-[((5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enoyl)amino]-benzoic acid (8): The reaction was carried out under an argon atmosphere. Arachidonic acid (201 mg, 0.66 mmol) and DMF (48.2 mg, 0.66 mmol) were dissolved in dry THF (10 mL) and stirred under ice cooling for 15 min. Oxalyl chloride (83.8 mg, 0.66 mmol) was added, and the mixture was stirred under ice cooling for 1 h. 5-Amino-2-hydroxybenzoic acid (505.3 mg, 3.3 mmol) was added, and the mixture was stirred for 20 min at room temperature. The organic phase was diluted with TBME (10 mL) and then extracted with 0.1 M HCl (2×10 mL). The organic layer was separated, dried with $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on reversed-phase RP-18 silica gel (MeOH/H_2O, 7:3). Precipitation from hexane afforded a brownish solid. Yield: 144.4 mg (49%); R_f = 0.51 (RP-18; MeOH/H_2O, 7:3); mp: 143–146 °C (hexane); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 9.78 (s, 1H, CONH), 8.07 (d, ^4J = 2.9 Hz, 1H, Ar), 7.64 (dd, ^3J = 9.1 Hz, ^4J = 2.9 Hz, 1H, Ar), 6.86 (d, ^3J = 9.1 Hz, 1H, Ar), 5.40–5.24 (m, 8H, 4×CH=CH), 2.81–2.72 (m, 6H, 3×CH=CHCH$_2$CH=CH), 2.26 (t, ^3J = 7.5 Hz, 2H, CH_2CONH), 2.11–1.96 (m, 4H, 2×CH=CHCH$_2$), 1.63 (tt, ^3J = ^3J = 7.5 Hz, 2H, CH_2CH_2CONH), 1.32–1.10 (m, 6H, $CH_2CH_2CH_2CH_3$), 0.83 ppm (t, ^3J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.1, 173.1, 159.7, 131.4, 131.1, 130.1, 129.8, 129.6, 129.4, 129.11, 129.08, 128.8, 128.7, 123.3, 118.2, 113.5, 37.1, 32.6, 30.4, 28.2, 27.7, 26.7, 26.61, 26.57, 23.6, 14.4 ppm; EIMS (70 eV), *m/z* (%): 195 (100), 153 (91.4), 135 (57.1), 439 (48.6, $[M]^+$); Anal. ($C_{27}H_{37}NO_4$) C, H, N.

***N*-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (9)**: The reaction was carried out under an argon atmosphere. Arachidonic acid (201 mg, 0.66 mmol) and DMF (48.2 mg, 0.66 mmol) were dissolved in dry THF (10 mL) and stirred under ice cooling for 15 min. Oxalyl chloride (167.5 mg, 1.32 mmol) was added, and the mixture was stirred under ice cooling for 1 h. A solution of 4-aminoantipyrine (670.7 mg, 3.3 mmol) in CH_2Cl_2 (5 mL) was added, and the reagents were allowed to react for 30 min at room temperature. The organic phase was extracted consecutively with 0.1 M HCl (10 mL), sat. $NaHCO_3$ (10 mL) and H_2O (10 mL). The organic layer was separated, dried with $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (EtOAc/MeOH, 98:2) to afford a white wax. Yield: 179.7 mg (54%); R_f = 0.24 (EtOAc/MeOH, 98:2); ^{1}H NMR (400 MHz, $CDCl_3$), δ = 8.26 (br s, 1H, CONH), 7.45–7.25 (m, 5H, 5×Ar), 5.42–5.27 (m, 8H, 4×CH=CH), 3.04 (s, 3H, NCH_3), 2.84–2.76 (m, 6H, 3×CH=CHCH$_2$CH=CH), 2.29 (t, ^3J = 7.5 Hz, 2H, CH_2CH_2CONH), 2.20 (s, 3H, C=CCH_3), 2.11–2.00 (m, 4H, 2×CH=CHCH$_2$), 1.70 (tt, ^3J = ^3J = 7.5 Hz, 2H, CH_2CH_2CONH), 1.38–1.22 (m, 6H, $CH_2CH_2CH_2CH_3$), 0.87 ppm (t, ^3J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$), δ = 172.1, 161.8, 149.6, 134.6, 130.4, 129.3, 129.2, 128.51, 128.48, 128.3, 128.1, 127.9, 127.5, 126.8, 124.2, 109.0, 36.2, 35.6, 31.5, 29.3, 27.2, 26.8, 25.7, 25.6, 22.6, 14.1, 12.5 ppm; EIMS (70 eV), *m/z* (%): 203 (100), 204 (82.9), 56 (70), 489 (35.7, $[M]^+$); Anal. ($C_{31}H_{43}N_3O_2$) C, H, N.

***N*-Methyl-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-(5*Z*,8*Z*,11*Z*,14*Z*)-icosatetra-5,8,11,14-enamide (10)**: Initially, 4-methylaminoantipyrine had to be synthesized. Thus dipyrone (6.3 g, 19 mmol) was dissolved in NaOH (0.1 M, 250 mL) and heated at 100 °C for 30 min. Hydrolysis was finished after this period, and the aqueous phase was extracted with $CHCl_3$ (500 mL). The organic phase was separated, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (EtOAc/MeOH, 95:5). Crystallization from hexane afforded yellow crystals. Yield: 2.45 g (60%); R_f = 0.14 (EtOAc/MeOH, 95:5); mp: 50–53 °C (hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45–7.36 (m, 4H, 4×Ar); 7.21–7.17 (m, 1H, Ar), 2.82 (s, 3H, NCH_3), 2.79 (s, 3H, $NHCH_3$), 2.21 ppm (s, 3H, CCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 162.3, 139.2, 135.4, 128.9, 125.7, 123.3, 122.7, 37.9, 34.9, 10.9 ppm; EIMS (70 eV), *m/z* (%): 217 (100, $[M]^+$), 56 (72.1), 83 (30.0); Anal. ($C_{12}H_{15}N_3O$) C, H, N.

The reaction was carried out under an argon atmosphere. Arachidonic acid (201 mg, 0.66 mmol) and DMF (48.2 mg, 0.66 mmol) were dissolved in dry CH_2Cl_2 (10 mL), and the solution was stirred under ice cooling for 15 min. Oxalyl chloride (167.5 mg, 1.32 mmol) was added, and the mixture was stirred under ice cooling for 1 h. 4-Methylaminoantipyrine (717.0 mg, 3.3 mmol) was then added, the reagents were allowed to react 30 min at room temperature. The organic phase was extracted consecutively with 0.1 M HCl (10 mL), sat. $NaHCO_3$ (10 mL), and H_2O (10 mL). The organic layer was separated, dried with $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (EtOAc/MeOH, 95:5) to afford a yellow oil. Yield: 156.4 mg (45%). R_f = 0.29 (EtOAc/MeOH, 95:5); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.48–7.28 (m, 5H, 5×Ar), 5.41–5.27 (m, 8H, 4×CH=CH), 3.29 (s, NCH_3, peak of rotational isomer of the product), 3.13 (s, 3H, NCH_3), 3.11 (s, 3H, $CONCH_3$), 3.07 (s, $CONCH_3$, peak of rotational isomer of the product), 2.82–2.73 (m, 6H, 3×CH=CHCH$_2$CH=CH), 2.27 (dt, ^2J = 15.0 Hz, ^3J = 7.5 Hz, 1H, $CH_2CH_2CONCH_3$), 2.18 (s, 3H, C=CCH_3), 2.12 (dt, ^2J = 15.0 Hz, ^3J = 7.5 Hz, 1H, $CH_2CH_2CONCH_3$), 2.10 (s, C=CCH_3, peak of rotational isomer of the product), 2.07–2.00 (m, 4H, 2×CH=CHCH$_2$), 1.67 (tt, ^3J = ^3J = 7.5 Hz, 2H, $CH_2CH_2CONCH_3$), 1.36–1.23 (m, 6H, $CH_2CH_2CH_2CH_3$), 0.87 ppm (t, ^3J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$), δ = 174.3, 161.7, 151.7, 134.6, 130.5, 129.6, 129.3, 129.1, 128.6, 128.3, 128.1, 127.9, 127.6, 127.1, 124.1, 115.4, 36.0, 35.7, 32.8, 31.6, 29.4, 27.3, 26.8, 25.7, 25.0, 22.6, 14.1, 10.6 ppm; EIMS (70 eV), *m/z* (%): 57 (100), 218 (82.9), 503 (21.4, $[M]^+$); HRMS: *m/z*: calcd for $C_{32}H_{45}N_3O_2$: 503.3512; found: 503.3494; combustion analysis calcd (%) for $C_{32}H_{45}N_3O_2$: C 76.30, H 9.00, N 8.34; found: C 75.85, H 9.03, N 8.18.

Pharmacology

Chemicals that were used in the biological assays, nonessential amino acids, glutamine, Tyrode, dimethyl sulfoxide (DMSO) and lipopolysaccharides (LPS), were purchased from Sigma–Aldrich (St. Louis, USA). Essential medium and fetal calf serum were from BioWhittaker Europe (Verviers, Belgium). Fluo-4AM and ionomycin were obtained from Invitrogen (Carlsbad, USA). Acetylsalicylic acid (aspirin) was purchased from Merck (Darmstadt, Germany) and capsaicin was purchased from Tocris (Bristol, UK). HEK-293 cells were from LGC Promochem (Teddington, UK).

Assays for activity at hTRPV1: HEK-293 cells stably overexpressing recombinant human TRPV1 cDNA were grown as monolayers in

minimum essential medium that was supplemented with nonessential amino acids, fetal calf serum (10%) and glutamine (2 mM), and were maintained under air/CO_2 (95:5) at 37 °C. The effect of the test substances on Ca^{2+} influx was determined by using Fluo-4AM, a selective intracellular fluorescent probe for Ca^{2+}. On the day of the experiment, $1–2\times10^6$ cells were loaded for 1 h at 25 °C with Fluo-4AM (4 μM in DMSO that contained 0.02% Pluronic®), in minimum essential medium without fetal bovine serum. After the loading, cells were washed twice with Tyrode (pH 7.4), and resuspended in Tyrode (10 mL). This HEK-293 cell suspension (1 mL) and saline (1 mL) was transferred to the quartz cuvette of the fluorescence detector (Luminescence Spectrometer LS50B; PerkinElmer). Experiments were carried out by measuring cell fluorescence at 25 °C ($\lambda_{ex}=488$ nm, $\lambda_{em}=516$ nm) under continuous stirring before and after the addition of the test compounds (2 μL dissolved in DMSO or MeOH) at various concentrations.

Agonist activity was determined in comparison with the maximum Ca^{2+} influx due to the application of ionomycin (4 μM). Antagonist behavior was evaluated against the agonist (EC_{50}: 26 nM)[7] capsaicin (0.1 μM). All determinations were performed in triplicate. Non-transfected HEK-293 cells served as negative controls in cases of the potent TRPV1 agonists **1** and **4**. Both compounds were not able to evoke a Ca^{2+} influx exceeding 30% of the influx that was observed in hTRPV1-transfected HEK-293 cells.

Whole-blood assay of COX-1 inhibition: Blood was drawn from a 50-year-old healthy volunteer who had not taken any NSAID for two weeks prior to blood sampling. Aliquots (1 mL) of whole blood without anticoagulant were immediately transferred to plastic tubes that contained **4** (2 μL) in DMSO. Blood was allowed to clot for 1 h at 37 °C. Serum was separated by centrifugation, and serum TXB_2 levels were determined by GC–MS/MS.[30] Every data point was determined in quadruplicate.

Whole-blood assay of COX-2 inhibition: Aliquots (1 mL) of heparinized whole blood from a 50-year-old healthy volunteer who had not taken any NSAID for two weeks prior to blood sampling were incubated with LPS (10 μg mL^{-1}; from *Escherichia coli* 026:B26), acetylsalicylic acid (10 μg mL^{-1}) plus **4** (2 μL) dissolved in DMSO for 24 h at 37 °C. LPS was used as a stimulant for COX-2 and the contribution of platelet COX-1 activity was inhibited by acetylsalicylic acid. Plasma was separated by centrifugation, and PGE_2 levels were determined per GC–MS/MS.[30] Every data point was determined in quadruplicate.

Data analysis: All dose–response curves were fitted by a sigmoidal regression with variable slope, and 50% inhibitory or effective concentration (IC_{50} or EC_{50}) values were derived by use of Prism® Version 3.0 (GraphPad, San Diego, CA, USA).

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