## Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain

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Abstract | Chronic pain remains unsatisfactorily treated, and few novel painkillers have reached the market in the past century. Increasing the levels of the main endogenous opioid peptides — enkephalins — by inhibiting their two inactivating ectopeptidases, neprilysin and aminopeptidase N, has analgesic effects in various models of inflammatory and neuropathic pain. Stemming from the same pharmacological concept, fatty acid amide hydrolase (FAAH) inhibitors have also been found to have analgesic effects in pain models by preventing the breakdown of endogenous cannabinoids. Dual enkephalinase inhibitors and FAAH inhibitors are now in early-stage clinical trials. In this Review, we compare the effects of these two potential classes of novel analgesics and describe the progress in their rational design. We also consider the challenges in their clinical development and opportunities for combination therapies.

#### Fibromyalgia

A disorder of unknown aetiology that is characterized by widespread pain, abnormal pain processing, sleep disturbance, fatigue and often psychological distress.

Neuropathic pain Pain caused by a lesion or a disease of the somatosensory nervous system.

<sup>1</sup>Pharmaleads SAS, 11 Rue Watt, 75013 Paris, France. <sup>2</sup>Université Paris-Descartes, 4 Avenue de l'Observatoire, 75006 Paris, France. Correspondence to B.P.R. e-mail: <u>bernard.roques@</u> <u>pharmaleads.com</u> doi:10.1038/nrd3673 Pain is a unique, conscious experience with sensorydiscriminative, cognitive-evaluative and affectiveemotional components<sup>1</sup>. Transient and acute pain can be effectively alleviated by activating the endogenous opioid system, which has a key role in discriminating between innocuous and noxious sensations<sup>2,3</sup>. However, chronic pain can occur after several pathophysiological processes, as well as without any identifiable cause (such as in fibromyalgia)<sup>4</sup>. One example is neuropathic pain — a frequent complication of shingles, diabetes, antiviral or antitumour chemotherapy, as well as surgery or lowerback disorders — which is unsatisfactorily treated with morphine<sup>5,6</sup>.

At present, tricyclic antidepressants, the anticonvulsants gabapentin and pregabalin, and the antidepressant duloxetine are the only available treatments for neuropathic pain. However, their efficacy and tolerability are often mediocre and it is therefore not surprising that more than 100 new chemical entities have been under investigation for the treatment of neuropathic pain in recent years. Among these, neurokinin 1 receptor (also known as TACR1) antagonists<sup>7</sup>, sodium channel blockers and NMDA (*N*-methyl-D-aspartate) receptor antagonists<sup>8,9</sup> have failed in clinical trials despite showing signs of efficacy in preclinical studies. Other compounds targeting acid-sensitive channels or vanilloid receptors<sup>10</sup> are being investigated, but none of them has yet made it to the market. There is an urgent need for novel treatments for all types of pain, particularly neuropathic pain, that show greater efficacy, better tolerability and wider safety margins<sup>11</sup>.

One innovative approach<sup>12</sup> for the development of analgesics is based on the fact that the painkillers found in *Papaver somniferum* (morphine) or *Cannabis sativa* ( $\Delta$ 9-tetrahydrocannabinol;  $\Delta$ 9-THC) mimic endogenous opioids and endogenous cannabinoids, respectively. Indeed, exogenous agonists of opioid and cannabinoid receptors elicit marked analgesic effects but they may excessively stimulate these ubiquitously distributed receptors, thereby inducing serious side effects such as respiratory depression, sedation, constipation, nausea, tolerance and dependence in the case of opiates<sup>13</sup>, or dysphoria, changes in motor coordination and memory disorders in the case of cannabinoids<sup>14</sup>. So, alternative strategies to harness the endogenous opioid and cannabinoid systems are desirable.

*Pain reduction by endogenous enkephalins.* The main endogenous opioids endowed with antinociceptive properties are Met-enkephalin and Leu-enkephalin. They are expressed as pre-propeptides (preproenkephalin (PENK)), which are processed within specific neurons and released by a Ca<sup>2+</sup>-dependent mechanism<sup>15</sup> to interact specifically with two G protein-coupled

receptors (GPCRs): the  $\mu$ -opioid receptors (MORs) and the  $\delta$ -opioid receptors (DORs)<sup>16</sup>. The affinity of enkephalins for MORs is similar to that of morphine, whereas their affinity for DORs is about tenfold higher<sup>16</sup>.

The crucial role of enkephalins in physiological pain control is supported by the increase in sensitivity to noxious stimuli elicited by *PENK* gene ablation<sup>17,18</sup>. Furthermore, when Met-enkephalin is injected into the rodent brain, it produces a morphine-like transient antinociceptive effect<sup>19</sup>. The limited duration of this effect is due to the rapid interruption of endogenous opioidergic signalling by the concomitant action of two zinc metallopeptidases — the neutral endopeptidase neprilysin (NEP; also known as CD10) and aminopeptidase N (APN; also known as CD13) — which break down enkephalins to produce the inactive metabolites Tyr-Gly-Gly and Tyr, respectively<sup>20,21</sup> (FIG. 1a).

Blocking the enzymatic inactivation of enkephalins increases their basal extracellular levels near the release site, so the effect of blocking NEP and APN is limited to local opioid receptors<sup>21</sup>. The intensity of the response therefore depends on: first, the levels of enkephalins released by a given stimulus; second, the levels of opioid receptors; and third, the activity of inactivating enzymes. All three of these factors vary according to the neuronal pathways involved and the type of stimulation<sup>21-24</sup>. When administered systemically, selective NEP inhibitors have no significant analgesic effects in rodents<sup>25</sup> or in humans<sup>26</sup> because the level of protected enkephalins is too low<sup>23</sup> and the stimulation of opioid receptors is therefore insufficient. This finding led to the proposal that dual inhibitors targeting both NEP and APN<sup>27</sup> might be more effective, and promising results were obtained with some of these compounds in various animal models of pain<sup>21,25,27-32</sup>. Accordingly, in a noncontrolled open-label study, intrathecal administration of the combination of a selective NEP inhibitor<sup>12</sup> and a nonspecific APN inhibitor elicited marked and lasting pain relief in terminally ill patients with cancer who were unresponsive to morphine<sup>33</sup>.

Pain reduction by endogenous cannabinoids. The major endogenous substances recognizing the same receptors as  $\Delta$ 9-THC are the endogenous cannabinoids N-arachidonoyl ethanolamide (also known as anandamide (AEA)) and 2-arachidonoylglycerol. Like Δ9-THC, AEA interacts with two GPCRs, namely cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R)<sup>34</sup>. In brain homogenates, the affinity of AEA for cannabinoid receptors is about 100 times weaker than that of  $\Delta$ 9-THC (2-6 nM)<sup>35</sup>. The signal conveyed by AEA is rapidly interrupted mainly by the intracellular fatty acid amide hydrolase (FAAH)<sup>36,37</sup>, which generates two inactive metabolites: arachidonic acid and ethanolamine (FIG. 1b), both of which are devoid of affinity for cannabinoid receptors. However, FAAH is not specific for AEA and is able to cleave many other substrates, including oleoylethanolamide, which leads to a decrease in food intake, and palmitoylethanolamide (PEA), which exerts anti-inflammatory actions through its interaction with the nuclear peroxisome proliferator-activated receptor-a (PPARa).

The synaptic concentrations of AEA are crucially dependent on a recently characterized reuptake system known as FAAH-like anandamide transporter (FLAT)38. The basal release of AEA is very low in the brain and requires a stimulus before neuronal secretion<sup>39</sup>. When it is delivered into the brain by intravenous (i.v.) injection, AEA alone does not reduce acute pain but it elicits a significant antinociceptive response when it is coadministered with a compound that inhibits its catabolism<sup>40</sup>. Nevertheless, even after stressful stimulation<sup>41</sup>, endogenous cannabinoid-mediated analgesia<sup>19,42</sup> is never as efficacious as the morphine-like analgesia induced by enkephalins or dual enkephalinase (DENK) inhibitors<sup>21,30</sup> in acute pain models. By contrast, in a chronic pain model in which cannabinoid receptors are permanently stimulated by protected endogenous cannabinoids, significant analgesic effects were observed<sup>43-49</sup>.

*Harnessing the endogenous opioid and cannabinoid systems.* These results have encouraged the development of dual NEP–APN inhibitors (now usually described as DENK inhibitors) and reversible or irreversible FAAH inhibitors<sup>25,27,30,34,37,42,50</sup>. To this end, the structure of the metabolizing enzymes in complex with an inhibitor<sup>51–54</sup>, their central<sup>21,55</sup> and peripheral distribution<sup>56,57</sup> as well as their molecular mechanisms of hydrolysis<sup>21,37,52,58</sup> have been taken into account.

Interestingly, both endogenous opioids and endogenous cannabinoids are present in primary sensory neurons<sup>57,59-65</sup>, offering the possibility to relieve, or at least reduce, the noxious inputs at their initial stage<sup>34,49,58-61,66-68</sup>. Indeed, more than 50% of the effects of morphine are attributable to the stimulation of peripheral neurons<sup>32,69,70</sup>. Therefore, the development of DENK inhibitors and FAAH inhibitors has focused on the treatment of neuropathic pain and inflammatory pain with compounds that are unable to enter the brain (and are thus devoid of possible behavioural adverse effects)<sup>30,49,58,63,66,67,71</sup>.

Results with one of the first synthetic orally active DENK inhibitors — PL37 (REF. 72) — in various animal models of pain have revealed interesting analgesic effects<sup>32,73,74</sup>; PL37 is the first DENK inhibitor to reach clinical trials. The first orally active FAAH inhibitor, URB597, was developed following a structure–activity study<sup>42</sup> and remains the most studied FAAH inhibitor for both its antinociceptive and anxiolytic properties. The orally active FAAH inhibitor PF-04457845 was in Phase II development for the treatment of osteoarthritic pain when it was found to be inactive<sup>75</sup>.

Here, we briefly describe the molecular similarities and differences between signalling by endogenous opioids and endogenous cannabinoids [FIG. 1c,d], before discussing the strategies used to rationally design potent inhibitors of their metabolizing enzyme (or enzymes) and their ability to relieve neuropathic pain and inflammatory pain. We also examine the possibility of reinforcing the analgesic potential of DENK inhibitors or FAAH inhibitors by combining them with different substances targeting biochemical systems involved in pain control, such as gabapentin, purinergic or cholecystokinin (CCK) antagonists, NMDA receptor antagonists, PPARα agonists and



Figure 1 | Endogenous opioid and endogenous cannabinoid signalling: differences in synthesis, secretion mechanisms and metabolism. a | Both of the endogenous enkephalins, Met-enkephalin and Leu-enkephalin, bind to  $\mu$ -opioid receptors (MORs) and  $\delta$ -opioid receptors (DORs). Enkephalins have a higher affinity for DORs (~tenfold higher) than for MORs, whereas morphine has a higher affinity for MORs than for DORs. The arrows denote the sites of enkephalin cleavage by aminopeptidase N (APN) and neprilysin (NEP). **b** | *N*-arachidonoyl ethanolamide (AEA), like  $\Delta$ 9-tetrahydrocannabinol ( $\Delta$ 9-THC), binds to cannabinoid receptor 1 (CB1R) and CB2R with about 100 times lower affinity than  $\Delta$ 9-THC. Fatty acid amide hydrolase (FAAH) cleaves AEA (as illustrated by the arrow) into the metabolites ethanolamine and arachidonic acid, which are both devoid of affinity for AEA targets. c | Enkephalins are synthesized intracellularly from enzymatic processing of the gene-derived precursor preproenkephalin (PENK). Stored in large synaptic vesicles, they are released (under basal or phasic conditions) by a  $Ca^{2+}$ -dependent exocytosis mechanism. Outside the cells, enkephalins interact with opioid receptors only, and their signal is interrupted by the concomitant action of two zinc metallopeptidases — NEP and APN — that generate inactive metabolites. The circulating concentrations of enkephalins, which modulate the physiological analgesic response, are enhanced by dual enkephalinase (DENK) inhibitors. d | AEA is synthesized from membrane phosphoglycerides through a multi-enzymatic process involving N-arachidonoyl-phosphatidyl-ethanolamine transferase (NAT) and a selective phospholipase D (PLD)<sup>88,130</sup>. AEA is released from the cells both by passive membrane diffusion and using the catalytically silent intracellular transporter FAAH-like anandamide transporter (FLAT)<sup>38</sup>. The same dual mechanisms are also used for the reuptake of synaptic AEA and delivering it to FAAH. FLAT may act as a shuttle delivering AEA to the cell membrane for secretion or, conversely, desorbing it from the membrane to transport it to the FAAH site. Outside the cells, AEA binds to various receptors such as cannabinoid receptors, transient receptor potential subfamily V member 1 receptor (TRPV1) and peroxisome proliferator-activated receptor-a (PPARa). The AEA signal is interrupted inside the cells by FAAH-induced degradation.

opiates. Finally, the advantages and limitations of these approaches, possible pitfalls and foreseeable difficulties in the clinical development of DENK and FAAH inhibitors are also discussed.

#### Endogenous opioid and cannabinoid signalling

*Similarities in receptor structure and signal transduction.* The main elements of the endogenous opioid system and the endogenous cannabinoid system, endogenous agonists and their degrading enzymes are present at all three (peripheral, spinal and brain) levels of pain control (FIG. 2a). MOR, DOR, CB1R and CB2R are part of the same GPCR family<sup>76,77</sup>; they are negatively coupled via  $G_i$  and  $G_o$  proteins to similar intracellular signalling pathways that inhibit adenylyl cyclase activity and ion channel phosphorylation, and elicit changes in gene expression mediated by cAMP-responsive element binding protein and mitogen-activated protein kinase<sup>2,15,76,77</sup>. The stimulation of opioid receptors and cannabinoid receptors blocks the conversion of noxious stimuli into electrochemical signals by inhibiting voltage-gated Ca<sup>2+</sup> channels, stimulating K<sup>+</sup> inward channels and subsequently inhibiting the Ca<sup>2+</sup>-dependent release of pro-nociceptive effectors such as substance P, calcitonin gene-related peptide and bradykinin<sup>2,15,61,76–78</sup>. Knocking out the *Oprm1* gene (the gene encoding MOR) in mice abolishes the antinociceptive effects of morphine and enkephalins, highlighting the role of MORs in the control of acute pain<sup>79,80</sup>. Both MORs and DORs are associated with the regulation of chronic pain<sup>81–86</sup>.

Variations in the endogenous levels of opioids and cannabinoids in specific nociceptive pathways (FIG. 2a,b) are likely to account for the variability in the efficacy of DENK inhibitors and FAAH inhibitors<sup>21,24,85,86</sup>. Opioid receptors bind to a limited number of endogenous opioids (for example, enkephalins,  $\beta$ -endorphin and, to a much lesser extent, small fragments of  $\beta$ -endorphin or dynorphin ending with the enkephalin sequence). All of these neuropeptides have no pharmacologically relevant affinity for binding sites other than opioid receptors, which makes the effector–receptor signalling of the endogenous opioid system highly specific.

Cannabinoid receptors, however, are recognized by several polyunsaturated fatty acid amides and triacylglycerol esters with different affinities<sup>87-89</sup>, and the binding of endogenous cannabinoids is not limited to cannabinoid receptors<sup>50,90,91</sup>. Depending on its concentration, AEA can interact with the transient receptor potential subfamily V member 1 receptor to induce pro-nociceptive responses<sup>50,92</sup> and/or with PPARa<sup>93</sup> to reduce inflammatory pain<sup>88,94</sup>. At high concentrations, AEA also behaves as a substrate of cyclooxygenase 2 (REF. 95), resulting in the generation of biologically active oxygenated derivatives of AEA<sup>96</sup>.

Differences in the synthesis, release and catabolism of endogenous opioids and cannabinoids. The greatest difference between the endogenous opioid system and the endogenous cannabinoid system lies at the levels of effector synthesis, secretion process and metabolism. Enkephalins are derived from the PENK precursor by processing enzymes, and then stored in large vesicles from which the active enkephalins are released, in a Ca<sup>2+</sup>dependent manner, by exocytosis<sup>15,86,97</sup>. Similarly to other neuropeptides, enkephalins diffuse into the extended synaptic area to interact with opioid receptors located on axon terminals, dendrites and even neuronal perikarya<sup>58,97</sup>. Their affinity for their targets is in the nanomolar range<sup>77</sup>, about 1,000 times stronger than that of classical neurotransmitters, which is in the micromolar range<sup>97</sup>.

Endogenous cannabinoids such as AEA are formed from glycerophospholipid precursors of unknown origin by an incompletely characterized enzymatic process. AEA is therefore not embedded in vesicles; rather, in contrast to enkephalins, it diffuses from the cytosol to the external cell membrane and from there to the synapse, as convincingly demonstrated using [<sup>3</sup>H]AEA<sup>42,88</sup> and the transporter FLAT<sup>38</sup>. FLAT is structurally related to FAAH — it has a similar affinity for AEA, but lacks the enzymatic activity. Outside the cell, AEA interacts with local cannabinoid receptors<sup>97</sup>. Interruption of AEA signalling is ensured by a two-step mechanism that involves AEA reuptake by FLAT<sup>38</sup> and cleavage by cytosolic FAAH, which is probably located in membranes near the AEA synthesis site<sup>37</sup> (FIG. 1d). Inhibition of intracellular FAAH<sup>42</sup> or selective blockade of FLAT<sup>38</sup> enhances the synaptic concentration of AEA, some of which is immediately taken up into the cell. Extracellular concentrations of AEA are probably dependent on both diffusion and FLAT-mediated reuptake and secretion<sup>38,42</sup>. The fact that numerous external factors can modify the synthesis, release and metabolism of endogenous cannabinoids might explain the differences in AEA concentrations reported in different issues<sup>87,98</sup>.

By contrast, interrupting endogenous opioid signalling is simple: extracellular circulating enkephalins are catabolized into inactive fragments by the externally accessible catalytic site of membrane-bound NEP or APN<sup>21,52,53</sup> (FIG. 1c). Opioid receptors and inactivating enzymes (NEP and APN) can be located close to or far from the enkephalin release site, which enables enkephalins to modulate physiological responses over larger regions than neurotransmitters or endogenous cannabinoids<sup>58,97</sup>, and allows them to potentially exert longlasting effects because of their high affinity for opioid receptors and subsequent slow dissociation.

However, enkephalins are not the only substrates of NEP and APN, and AEA is not the only substrate of FAAH. *In vitro*, NEP and APN cleave several natural peptides such as substance P, neurotensin, CCK and bradykinin<sup>86</sup>, which are cleaved *in vivo* by their own peptidases<sup>99,100</sup>. The other endogenous opioid peptides dynorphin and  $\beta$ -endorphin — which are often regarded as NEP and/or APN substrates — also have their own metabolizing enzymes<sup>101,102</sup>. *In vivo*, NEP modulates the activity of atrial natriuretic peptide and endothelins<sup>103,104</sup>, whereas APN contributes to angiotensin metabolism in the brain and fluid homeostasis in the kidney<sup>104</sup>. Importantly, DENK inhibitors enhanced the analgesic effect of enkephalins without increasing neurokinin 1 receptor activation by endogenous substance P<sup>105</sup>.

Given the broad distribution of NEP and APN in the body, their involvement in other peptidergic pathways that have not yet been characterized cannot be excluded. FAAH has a broad enzymatic activity, comprising well-characterized substrates such as AEA, oleoylethanolamide and PEA<sup>106,107</sup> as well as other substrates and derived metabolites that remain to be characterized; nevertheless, some of them may contribute to the analgesic effects of FAAH inhibitors<sup>87</sup>.

#### Enhancing 'physiological' analgesia

*Endogenous opioids and adaptation.* Physiological analgesia can be defined as a form of pain relief induced by endogenous effectors that stimulate the same targets (opioid receptors) as natural (for example, morphine) or synthetic opiates. Blocking the targets of enkephalins by naloxone lowers the pain threshold in patients, and this opioid receptor antagonist was shown to increase postoperative pain in patients who had not received



Figure 2 | Endogenous opioids and endogenous cannabinoids are present at all three levels of pain control. a | Endogenous opioids and endogenous cannabinoids are widely distributed in the central nervous system, spinal cord and peripheral organs<sup>21,55,61,223,224</sup> At the periphery, endogenous opioids and endogenous cannabinoids are present in epithelial cells of the intestine and kidney<sup>21</sup>, in the joints<sup>21,225</sup>, lung, testis and skin<sup>199,226</sup> as well as on various types of immune cells<sup>65,199</sup>, including oligodendrocytes and Schwann cells surrounding nerve fibres<sup>227</sup>. Cannabinoid receptors and fatty acid amide hydrolase (FAAH) are found at the nociceptor level<sup>63,223,228</sup> and in immune cells<sup>229</sup> where inhibitors can block the noxious inputs<sup>49,230</sup>. Noxiously stimulated cutaneous fibres converge to the dorsal horn, sometimes along with non-stimulated fibres from distant cutaneous, muscular or visceral areas<sup>3</sup>.  $\mu$ -opioid receptors (MORs) and  $\delta$ -opioid receptors (DORs) are mainly located at the presynaptic end of afferent fibres in the spinal dorsal horn, whereas neprilysin (NEP) is found in interneurons<sup>231</sup>. The distribution of NEP and aminopeptidase N (APN) in the brain overlaps with that of MORs and DORs in structures involved in the control of pain and emotions such as the periaqueductal grey, thalamus, cortex and limbic system<sup>21,55,61,65</sup>. FAAH is also highly expressed near neurons enriched in cannabinoid receptor 1 (CB1R), including structures involved in fear and emotions<sup>42</sup> but also in non-neural cells<sup>226,229</sup>. **b** | A simplified mechanism is shown whereby dual enkephalinase inhibitors and FAAH inhibitors inhibit the spinal relay of peripheral noxious inputs to the brain. Opioid receptors and cannabinoid receptors are synthesized in the dorsal root ganglion and transported to the spinal afferent terminals. Stimulation of opioid receptors by enkephalins from interneurons (shown in gold) inhibits the release of pro-nociceptive peptides such as substance P (SP) and calcitonin gene-related peptide (CGRP). Enkephalins are also released near the spinothalamic neurons, where they block the transfer of nociceptive inputs to the brain via an increase in K<sup>+</sup> conductance and subsequent hyperpolarization<sup>2,83,232,233</sup>. Regarding the endogenous cannabinoids, it is hypothesized that, like enkephalins, N-arachidonoyl ethanolamide (AEA) from presynaptic neurons (shown in light red) or from afferent terminals (autocrine mechanisms) inhibits the release of pro-nociceptive substances. AEA may also block noxious transfer from spinothalamic neurons by inhibiting their glutamate-dependent excitation. Glutamate may also be released from interneurons or from glial cells<sup>234</sup>.

exogenous opioids<sup>108</sup>. Furthermore, the enkephalinmediated beneficial effects on pain of acupuncture, electrical nerve stimulation or long and intensive body stimulation are reversed by naloxone. Anticipation and expectancy of pain relief also strongly decreased noxious sensations in humans, and reduced the requested doses of morphine. All of these situations were shown to be related to a physiological increase in enkephalin levels in pain and reward pathways<sup>24,109</sup>. Accordingly, increasing the levels of enkephalins using DENK inhibitors in these circuits enhances analgesic responses, as demonstrated in all animal models of pain studied<sup>30</sup> (TABLE 1).

Systemic administration of DENK inhibitors will lead to a homogenous distribution of the drug in the body. However, unlike morphine or exogenous opiates, which directly stimulate any available receptor, DENK inhibitors will act where there is an abundance of enkephalins and their degrading enzymes. Therefore, their analgesic effects will be crucially dependent on the phasic release of enkephalins and the subsequent stimulation of opioid receptors, and will be restricted to the structures and pathways involved in the control of pain. This has been investigated by looking at whether the main side effects associated with exogenous opiates occur with DENK inhibitors. To date, unlike morphine, even very high doses of DENK inhibitors in rodents<sup>110,111</sup> (reviewed in REFS 30,112) or PL37 in humans did not result in tolerance, sedation, respiratory depression, emesis, constipation or dependence.

*Putative limitations of nonspecific endogenous signalling modulation.* Deletion of the genes encoding NEP or APN in mice also provides indirect information on the physiological roles of the enzymes and can be used to assess the effects of DENK inhibitor-evoked blockade of NEP or APN activity. NEP-knockout mice<sup>113</sup> exhibit limited abnormalities, such as an increased sensitivity to endotoxic shock, enhanced sensitivity in a model of hypertension, an exacerbation of intestinal inflammation and increased sensitivity to pancreatitis-associated lung injury (reviewed in REF. 114). However, most of these effects appear under induced stress, suggesting that excessive compensatory genetic adaptations are involved. In APN-knockout mice, angiogenesis is impaired under pathological hypoxic conditions<sup>115</sup>.

It must be noted that the effects observed in NEPknockout mice were not observed in humans after treatment with the selective NEP inhibitor thiorphan<sup>12</sup> (with an  $IC_{50}$  (half-maximal inhibitory concentration) of 2 nM, and an  $IC_{50}$  within and below the micromolar range for other identified peptidases), which was marketed in 1992 as an antidiarrhoeal medicine for adults, children and newborns<sup>116</sup>. Similarly, the effects observed in APN-knockout mice were not observed with the APN inhibitor bestatin, which has been chronically used in patients with cancer<sup>117</sup>. Thousands of patients have been treated in clinical trials with NEP inhibitors or dual NEP–angiotensin converting enzyme (ACE) inhibitors (reviewed in REF. 118), and none of the serious effects observed in NEP-knockout mice has been reported.

Table 1   Pharmacological activity of various classes of DENK inhibitors									
Compound	Models	Dose (route)	Tests; animal	Result (% MPE)	Refs				
Kelatorphan	Neuropathic pain (CCI model)	5–15 mg per kg (i.v.)	Paw pressure test; rats (vocalization)	60% ↑ in pain threshold (10 mg per kg maximum)	145				
Kelatorphan	Inflammatory pain (CFA; i.p.)	2.5 mg per kg (i.v.)	Paw pressure test; rats (vocalization)	244% 1 in pain threshold	28				
	Inflammatory pain (without CFA; i.p.)	2.5 mg per kg (i.v.)	Paw pressure test; rats (vocalization)	144% $\uparrow$ in pain threshold					
PC12	Inflammatory pain (polyarthritic)	5–20 mg per kg (i.v.)	Paw pressure test; rats (vocalization)	70% ↑ in pain threshold (5 mg per kg maximum)	144				
RB-101	Acute pain	0.5–10 mg per kg (i.v.)	HPT; mice	85% analgesia maximum (ED <sub>50</sub> : 2*–8 <sup>‡</sup> mg per kg)	25				
RB-101	Acute pain	50–150 mg per kg (s.c.)	HPT; pregnant mice	50% analgesia (at 150 mg per kg)	236				
RB-101	Acute pain	7–30 mg per kg (i.v.)	Electromyographic C-fibre reflex; rats	↑ in pain threshold (ED <sub>si</sub> : 17 mg per kg)	237				
RB-101	Neuropathic pain (chemically induced	5–20 mg per kg (i.v.)	Paw pressure test; rats (vocalization)	100% reduction (20 mg per kg)	150				
	diabetes)		Von Frey test; rats	35% mechanical allodynia					
RB-101	Postoperative pain (paw incision)	20 mg per kg (i.v.)	Von Frey test; mice	26% $\downarrow$ in mechanical allodynia	151				
RB-101	Inflammatory pain (CFA; i.p.)	20 mg per kg (i.v.)	Paw pressure test Analgesia metre	156%↑in control	66				
RB-120	Inflammatory pain and/or neuropathic pain (formalin)	80 mg per kg (p.o.)	Paw pressure test Analgesia metre	60% analgesia	147				
RB-120	Acute pain	80 mg per kg (i.v.)	TFT; rats	40% analgesia	147				
		400 mg per kg (p.o.)	TFT; mice	50% analgesia					
RB-120	Abdominal pain	30–250 mg per kg (p.o.)	Writhing test	90% $\downarrow$ in writhing (ED $_{ m 50}$ : 53 mg per kg)	147				
PL37	Inflammatory pain and/or neuropathic pain (formalin)	50–200 mg per kg (p.o.)	Licking test; mice	Phase I: 30–80% analgesia Phase II: 30–70% analgesia	72				
PL37	Acute pain	8* mg per kg (i.v.)	HPT; mice	80% analgesia (dose-dependent at 200 mg per kg)	72				
		50–100 <sup>‡</sup> mg per kg (p.o.)	HPT; mice	80% analgesia (dose-dependent at 200 mg per kg)					
PL37	Acute pain	17* mg per kg (i.v.)	TFT; rats	40% analgesia (dose-dependent at 200 mg per kg)	72				
		50 <sup>‡</sup> mg per kg (p.o.)	TFT; rats	10% analgesia (dose-dependent at 200 mg per kg)					
PL37	Inflammatory pain (CFA; i.p.)	20–80 mg per kg (p.o.)	Paw pressure test	100% MPE (no tolerance, long duration)	72				
PL37	Neuropathic pain (diabetes)	100 mg per kg (p.o.)	Paw pressure test; rats	30% $\downarrow$ in hyperalgesia	72				
PL37	Neuropathic pain (CCI model); Seltzer model	20–40 mg per kg (p.o.)	Von Frey test; mice	90%↓in allodynia	72				
		10–20 mg per kg (p.o.)	Plantar test; mice	70% $\downarrow$ in thermal hyperalgesia					
PL37	Neuropathic pain (tibial osteosarcoma)	25 mg per kg (p.o.)	HPT; mice	100% $\downarrow$ in thermal hyperalgesia	32,73				
PL37	Neuropathic pain (vincristin)	60 mg per kg (i.p.)	Von Frey test; rats	40% $\downarrow$ in mechanical allodynia	74				
		100 mg per kg (p.o.)	Von Frey test; rats	60% $\downarrow$ in mechanical allodynia					
		100 mg per kg (p.o.)	'Paint-brush' test; rats	75% $\downarrow$ in mechanical allodynia					
PL37	Inflammatory pain (carrageenan; i.p.)	84 mg per kg (p.o.)	Von Frey test; rats	100% $\downarrow$ in mechanical allodynia	72				
PL37	Neuropathic pain (capsaicin)	6–8 mg per kg (p.o.)	Healthy human subjects	↓ in neurogenic flare area ↓ in mechanical allodynia ↓ in total pain score	ş				
PL37	Plasma NEP and/ or APN activity	3–12 mg per kg (p.o.)	Healthy human subjects	100% MPE (1 hour post-dosing) 10% MPE (6 hours post-dosing)	ş				

APN, aminopeptidase N; CCI, chronic constrictive injury; CFA, complete Freund's adjuvant; DENK, dual enkephalinase; ED<sub>s0</sub>, half-maximal effective dose; HPT, hot plate test; i.p., intraperitoneal; i.v., intravenous; MPE, maximum possible effect; NEP, neprilysin; p.o., per os (by mouth); s.c., subcutaneous; TFT, tail-flick test. \*Vehicle: EtOH/Tween80/H<sub>2</sub>O (1/1/8). <sup>+</sup>EtOH/PEG400/H<sub>2</sub>O (1/4/5). <sup>§</sup>Unpublished data, Debiopharm Group.

Enhancement of basal and phasic levels of enkephalins through dual NEP–APN inhibition. The potential usefulness of DENK inhibitors as a new class of analgesics, without the side effects associated with morphine, was based on the reasonable assumption that they would be able to increase the extracellular concentrations of enkephalins, whether released tonically or after stimulus-evoked depolarization (phasic release)<sup>22,23</sup>. Owing to the relatively low basal concentrations of released Met-enkephalin which are in the femtomolar range<sup>23,119–123</sup> — and the large, rapidly renewable intracellular pool of enkephalins<sup>23,124</sup>, repetitive stimulation of enkephalin-containing neurons is unable to exhaust the intracellular content of opioid peptides that can be mobilized<sup>119</sup>. This is a prerequisite for the use of DENK inhibitors as analgesics.

Without noxious stimuli, the intraperitoneal (i.p.) administration of the disulphide DENK inhibitor RB-101 (FIG. 3) induces a long-lasting two- to threefold increase in extracellular Met-enkephalin levels within the nucleus accumbens — a structure involved in the rewarding (that is, euphorigenic) effects of opiates<sup>120</sup>. During noxious stimulation, the aminophosphinic DENK inhibitor PL253 (REF. 125) (FIG. 3) increased the basal enkephalin concentration by 88% in the periaqueductal grey (PAG), an area of the brain that is involved in pain modulation<sup>121</sup>. These direct demonstrations of an induced increase in extracellular concentrations of endogenous opioids were recently confirmed in the human brain by neuroimaging studies in patients with neuropathic pain<sup>126</sup>. Kelatorphan (FIG. 3) almost completely prevented the spinal degradation of exogenous [3H]Met-enkephalin in the superfused spinal cord of halothane-anaesthetized rats<sup>23</sup>. The recovery of the spontaneous outflow of endogenous Metenkephalin in the spinal cord was 2.5-fold higher in the presence of kelatorphan and fivefold higher during noxious stimulation, with no apparent change in the release process itself, suggesting that the inhibitor did not have a significant effect on enkephalin secretion<sup>23</sup>.

DENK inhibitors have also been used to explore the tone of enkephalinergic pathways. The synaptic concentrations of enkephalins, even after the administration of DENK inhibitors, were found to be very low in the brain structures involved in respiratory or cardiac control<sup>22</sup>, which may explain why DENK inhibitors do not induce respiratory depression127 - a severe side effect of morphine. Likewise, there is little or no tonic endogenous opioid receptor activation in the locus coeruleus<sup>22</sup> — an area involved in physical dependence to morphine<sup>128</sup>. Unlike morphine, DENK inhibitors do not induce constipation, even at high and repeated doses<sup>30</sup>. This is probably due to a restricted release of enkephalins in intestinal plexi, where MOR stimulation influences transit and may elicit constipation<sup>129</sup>. All of these results emphasize the correlation between the amount of enkephalins released and the physiological responses induced.

# Basal and phasic release of AEA: enhancement of extracellular levels by FAAH inhibition and effects on nociception. Unlike endogenous opioids, the role of endogenous cannabinoids in the tonic regulation of pain remains unclear<sup>130</sup>. The 15-fold increase in AEA levels in

the brain of FAAH-knockout mice131 is not reflected in the effects of FAAH inhibitors on acute pain, which are generally absent<sup>40,132</sup> or weak<sup>40,133,134</sup> after a single dose. Moreover, changes in AEA concentration in mouse models of sciatic nerve chronic constrictive injury and sham-operated mice treated with the FAAH inhibitor URB597 are surprisingly similar in brain and spinal cord tissues<sup>134</sup>. This may be related to the rapid degradation of AEA, not only by FAAH but also by other mechanisms<sup>87</sup>. Moreover, owing to the intracellular synthesis and metabolism of AEA, knocking out the FAAH gene induces an accumulation of AEA. Surprisingly, the antinociceptive effects of AEA in the supraspinal hot plate test (HPT) are not reduced in Cb1r<sup>-/-</sup> mice, suggesting that the action of AEA is mediated by other receptors in the brain (very few CB2Rs are found in the brain)<sup>91</sup>.

The extracellular concentrations of endogenous cannabinoids, measured by microdialysis following painful stimuli and/or FAAH inhibitor administration, are more consistent with the AEA-related pharmacological effects than the total (essentially intracellular) amounts of AEA. Thus, electrical stimulation of the PAG or intraplantar formalin injection leads to a weak but significant 0.5- to 1.3-fold increase in AEA concentration in the PAG<sup>135,136</sup>. In a murine chronic constrictive injury model of neuropathic pain, the plasma levels of orally administered URB597, the magnitude of FAAH inhibition, the enhanced spinal levels of AEA and the analgesic effect (albeit weak) were significantly correlated<sup>133</sup>.

The URB597-induced reduction in allodynia and hyperalgesia is not reproduced by *FAAH* gene deletion, which suggests that adaptive changes during development and/or alterations in the pathways of pain transmission are involved<sup>134</sup>. In the hypothalamus of mice, the basal concentration of synaptic AEA in the absence of a painful stimulus was increased by 88% after i.p. administration of URB597.

The increase in extracellular amounts of enkephalins that are triggered by noxious stimuli is higher than that of AEA. This may be due to the simpler mechanisms of enkephalin synthesis, release and inactivation described above<sup>37,58</sup>. Furthermore, the affinity of AEA for cannabinoid receptors is in the submicromolar range, whereas the affinity of enkephalins for opioid receptors is in the nanomolar range<sup>77,135</sup>. Both of these factors may contribute to the higher receptor occupancy and subsequent stimulation by enkephalins than by AEA, which is consistent with the greater analgesic effects of DENK inhibitors reported in animal models of pain<sup>30,71,74,133,134,136</sup> (TABLES 1,2).

### Rational design of various DENK inhibitors

NEP<sup>137</sup> and APN<sup>52,138</sup> are membrane-bound zinc metallopeptidases with a catalytic site on the outer part of the cell, allowing them to cleave extracellular peptides such as enkephalins. The amino acid sequence of NEP is highly conserved and the structure is stabilized by five or six disulphide bonds<sup>53,137</sup>. Site-directed mutagenesis of rabbit NEP<sup>139</sup>, computer modelling<sup>140</sup> and crystallographic studies<sup>52,53</sup> have shown that the catalytic sites of NEP and APN are very similar but there are subtle

#### Chronic constrictive injury model

An animal model of mononeuropathic pain in rodents resulting from ligation of the sciatic nerve, which induces a painful syndrome analogous to that observed in humans. Chronic constrictive injury models may differ according to the location and the tightness of the ligation along the sciatic nerve.

#### Mononeuropathic rats

Rats that mimic the symptoms induced by nerve injury in humans. Symptoms are restricted to the area innervated by the injured nerve. differences in their S<sub>1</sub>, S<sub>1</sub>' and S<sub>2</sub>' subsites<sup>141</sup>. In pig APN, the Glu350 residue in the S<sub>1</sub> site is essential for the exoaminopeptidase activity<sup>58</sup> of the enzyme, whereas the Arg102 residue in the S<sub>1</sub> site of NEP is essential for the carboxydipeptidase activity of NEP (FIG. 1a).

R<sup>+</sup> 102

#### Selective inhibitors



Heterodisulphide DENK inhibitors



Hydroxamate DENK inhibitors



Aminophosphinic DENK inhibitors



Figure 3 | Main selective dual NEP-APN inhibitors. The knowledge of detailed mechanisms of substrate hydrolysis by zinc metallopeptidases<sup>21,235</sup> was used for rationally designing dual enkephalinase (DENK) inhibitors. Specific neprilysin (NEP) or aminopeptidase N (APN) inhibitors (thiorphan and PC18, respectively) and the three main classes of DENK inhibitors are represented with their zinc-chelating groups and their side chains interacting with the binding subsites  $S_1$ - $S_2'$  of NEP and APN, as inferred from site-directed mutagenesis<sup>21,139</sup>, docking and molecular modelling studies<sup>140</sup>, crystallographic data<sup>52,53</sup> and K<sub>i</sub> values. Hydroxamate inhibitors (kelatorphan and RB-38) and aminophosphinic DENK inhibitors (PL253, PL254 and PL265) have been designed to fit the active sites of both APN and NEP, whereas RB-101, RB-120 and PL37 are prodrugs that release potent and selective NEP and APN inhibitors after cleavage of a disulphide bond (see main text). All the DENK inhibitors described here have nanomolar affinities for both NEP and APN.

Taking into account the substantial similarities in the active sites of zinc metallopeptidases<sup>52,53,139,140</sup>, the rational design of potent selective or dual inhibitors of NEP and APN<sup>21,30,58,142</sup> has led to the selection of molecules that contain a strong metal-coordinating group (for example, a thiol, carboxyl, hydroxamate or phosphinic group) and are able to satisfy all possible energetically favourable interactions with at least one of the  $S_1 - S_2'$  subsites surrounding the catalytic site, as evidenced by inhibitor co-crystallization52,53 (reviewed in REFS 30,31,58,138,142).

The first DENK inhibitors (FIG. 3) were designed in 1984 (REF. 27) using the hydroxamate group as a zincchelating moiety, assuming that the strength of its coordination to the metal should counterbalance a 'less than perfect' fit of the inhibitor side chains to the active sites of the two metallopeptidases<sup>21</sup> that are obviously not identical<sup>52,53</sup>. Accordingly, kelatorphan strongly inhibits NEP (IC<sub>50</sub> = 1.8 nM) and less efficiently inhibits APN  $(IC_{50} = 380 \text{ nM}).$ 

Kelatorphan was the first compound that completely inhibited enkephalin catabolism<sup>23</sup>. It had antinociceptive effects in numerous acute nociceptive animal models<sup>27,143</sup> and, after intrathecal administration, it induced longerlasting analgesia in patients with cancer (M.C.F.Z. and J. Meynadier, unpublished observations) than the combination of both bestatin and thiorphan<sup>33</sup>. Kelatorphan was also active in complete Freund's adjuvant-induced arthritis in rats - a widely used model of chronic pain<sup>28,144</sup> — and it reduced nociception by 60% in mononeuropathic rats<sup>28,145</sup>. The entrance of kelatorphan into the brain is very limited and therefore the analgesic effects observed in arthritic rats are assumed to be due to a peripheral effect at the level of injured tissues<sup>66</sup>.

DENK inhibitors with improved brain penetration were developed from 1992 onwards by linking two highly potent inhibitors ( $K_1 < 10 \text{ nM}$ ) for each peptidase<sup>30,146</sup> by a disulphide bridge (co-drug) (FIG. 3), which is rapidly cleaved in vivo by an enzymatic process<sup>146</sup>. The pharmacokinetic properties of these disulphides were modulated by introducing hydrophobic ester groups<sup>30,72</sup>. One of these DENK inhibitors, RB-101 (FIG. 3), when administered intravenously, has analgesic effects that are three times larger than symmetric disulphides combining APN or NEP inhibitors<sup>25,30,146</sup>. The pain-alleviating effects of RB-101 or oral RB-120 (REF. 147) were completely reversed by the non-selective opioid antagonist naloxone, but only partially reversed in the tail-flick test (TFT) and in the motor response to electrical stimulation of the tail by the DOR-selective antagonist naltrindole. This suggests that signalling through MORs predominantly mediates these analgesic effects, probably at the spinal and/or brain level<sup>82,85</sup>.

In the HPT, i.v. administration of RB-101 elicited a maximum 85% analgesia with an ED<sub>50</sub> (half-maximal effective dose) between 1.6 mg per kg and 10 mg per kg, depending on the vehicle — a dose that is only two times higher than the equipotent dose of morphine<sup>110</sup>. This is consistent with binding experiments demonstrating that RB-101 does not completely displace [<sup>3</sup>H]-diprenorphine bound to opioid receptors in the

Table 2   Pharmacological activity of various classes of FAAH inhibitors								
Compound	Model	Dose (route)	Tests (animal)	Result (% MPE)	Refs			
OL-135	Inflammatory pain (acute)	10 mg per kg (i.p.)	<ul> <li>Mild thermal injury</li> <li>Paw pressure test (rats)</li> </ul>	50%↓in allodynia (opioid receptor-dependent)	170			
	Neuropathic pain (SNL)	20 mg per kg (i.v.)	<ul> <li>Paw pressure test (rats)</li> <li>Von Frey test</li> </ul>	80%↓in allodynia (opioid receptor-dependent)				
OL-135	Acute pain	10 mg per kg (i.p.)	• Tail immersion test (mice)	~30% 1 in latency	238			
			• HPT (mice)	~30%↑in latency				
	Viceral pain (formalin)	10 mg per kg (i.p.)	• Paw licking test (mice)	~30% $\downarrow$ in response				
OL-135	Neuropathic pain (CCI model)	10 mg per kg (i.p.)	• Paw pressure test (mice)	~60%↓in allodynia	134			
		10 mg per kg (i.p.)	Paw lifting test	~30% $\downarrow$ in allodynia (CB1R- or CB2R-dependent)				
URB597	Neuropathic pain (CCI model)	10 mg per kg (i.p.)	<ul> <li>Paw pressure test (mice)</li> <li>Von Frey test</li> </ul>	~35% ↓ in allodynia	134			
		10 mg per kg (i.p.)	<ul><li>Paw lifting test (mice)</li><li>Cold acetone test</li></ul>	~80%↓in allodynia (CB1R- or CB2R-dependent)				
URB597	Inflammatory pain (CFA; i.p.)	0.3 mg per kg (i.p.)	<ul><li>Paw pressure test (rats)</li><li>Von Frey test</li></ul>	90%↓in allodynia	132			
		0.3 mg per kg (i.p.)	<ul> <li>Plantar test (rats)</li> </ul>	50% $\downarrow$ in allodynia				
	Neuropathic pain (CCI model)	0.3 mg per kg (i.p.)	<ul> <li>Paw pressure test (rats)</li> <li>Von Frey test</li> </ul>	No effect				
URB597	Visceral pain	Pretreatment: 1–10 mg per kg (s.c.)	<ul> <li>Acetic acid-induced stretching test (mice)</li> </ul>	90% reduction in stretching (at 10 mg per kg)	192			
URB597	Inflammatory pain	10 mg per kg (s.c.)	• HPT (mice)	40% $\downarrow$ in hyperalgesia	194			
	(lipopolysaccharide; i.p.)	10 mg per kg (s.c.)	<ul> <li>Paw thickness test</li> </ul>	No reduction in oedema				
		Three times (cumulative)	<ul> <li>Paw thickness test</li> </ul>	20–40% reduction in oedema				
URB597	Neuropathic pain (CCI model)	10–50 mg per kg (p.o.) once daily for 4 days	• Paw pressure test	35% ↓ in allodynia (10 mg per kg)	133			
			• Electronic Von Frey test	70% ↓ in allodynia (50 mg per kg)				
	Neuropathic pain (CCI model)	10 mg per kg (p.o.) once daily for 4 days	<ul><li>Thermal hyperalgesia</li><li>Paw withdrawal latency</li></ul>	75% ↓ in hyperalgesia				
URB597	Inflammatory pain; iodoacetic acid-induced osteoarthritis	5 mg per kg (s.c.)	Paw pressure incapacitance test	19% MPE	239			
URB597	Inflammatory pain (carrageenan)	3 mg per kg (i.p.)	• Paw oedema measurement (mice)	80% reduction in oedema (CB2R-dependent)	168			
URB597	Bone cancer	$9\mu g$ per bone infusion	• Paw pressure test	50–60%↓in mechanical allodynia	47			
URB597	Acute pain	40 mg per kg (i.p.)	• TFT (mice)	No effect	40			
URB597 plus AEA	Acute pain	URB597: 10 mg per kg (i.p.) AEA: 40 mg per kg (i.p.)	• TFT (mice)	68% MPE				
URB937 (strictly peripheral	Neuropathic pain (SNL)	n 1 mg per kg (single dose; i.p.)	<ul><li>Paw pressure test;</li><li>Withdrawal latency</li></ul>	~50% $\downarrow$ in hyperalgesia	49			
FAAH inhibitor)			<ul><li>Thermal stimulation</li><li>Withdrawal latency</li></ul>	100% $\downarrow$ in hyperalgesia				
			<ul><li>Paw pressure tests</li><li>Von Frey test</li></ul>	1,000% $↓$ in hyperalgesia				
URB937	Neuropathic pain (SNL)	1 mg per kg (i.p.) once a day for 7 days	<ul> <li>Paw pressure and withdrawal latency</li> <li>Thermal stimulation and withdrawal latency</li> <li>Paw pressure and Von Frey tests</li> </ul>	Long-lasting similar effects (lack of tolerance)	49			

lable 2 (cont.)   Pharmacological activity of various classes of FAAH inhibitors							
Compound	Model	Dose (route)	Tests (animal)	Result (% MPE)	Refs		
JNJ-1661010	Mild thermal injury	20 mg per kg (i.v.)	<ul> <li>Paw pressure test (rats)</li> <li>Von Frey test</li> </ul>	90% ↓ in allodynia; naloxone (reversible): 3 mg per kg morphine	169		
JNJ-1661010	Neuropathic pain (SNL)	20 mg per kg (i.v.)	<ul> <li>Paw pressure test (rats)</li> <li>Von Frey test</li> </ul>	60% ↓ in allodynia; naloxone (reversible): 49 mg per kg; gabapentin: 300 mg per kg (p.o.)	169		
PF-04457845	Inflammatory pain (CFA; i.p.)	0.1–10 mg per kg (p.o.)	<ul><li>Paw pressure test</li><li>Von Frey test (rats)</li></ul>	~50% reduction in allodynia (not dose-dependent)	48		
	Osteoarthritis (MIA-induced injury in knee)	0.3 mg per kg and 3 mg per kg (p.o.)	<ul> <li>Joint compression threshold (rats)</li> </ul>	~35% reduction in mechanical hyperalgesia (not dose-dependent)			
ARN272	Inflammatory pain (formalin; i.p.)	0.01–1 mg per kg (i.p.)	• Paw withdrawal latency (mice)	Dose-dependent reduction in thermal hyperalgesia in Phase I and II trials	38		
FLAT inhibitor	Inflammatory pain (carrageenan; i.p.)	0.01–1 mg per kg (i.p.)	<ul> <li>Paw withdrawal latency (mice)</li> </ul>	Dose-dependent alleviation of hyperalgesia and oedema	38		
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AEA, N-arachidonoyl ethanolamide; CB1R, cannabinoid receptor 1; CCl, chronic constrictive injury; CFA, complete Freund's adjuvant; FAAH, fatty acid amide hydrolase; FLAT, FAAH-like anandamide transporter; HPT, hot plate test; i.p., intraperitoneal; i.v., intravenous; MIA, monoiodoacetic acid; MPE, maximum possible effect; p.o., per os (by mouth); s.c., subcutaneous; SNL, spinal nerve ligation; TFT, tail-flick test.

mouse brain<sup>122,148</sup>. As DENK inhibitors do not modify enkephalin secretion, and only modify its extracellular concentrations<sup>23</sup>, they may be of great interest for *in vivo* studies of opioid receptor occupation in various situations (for example, pain, anger, stress or emotion) using positron emission tomography (PET) scans<sup>149</sup>. In various animal models of inflammatory pain and neuropathic pain, RB-101 suppressed mechanical hyperalgesia and reduced allodynia, mainly by recruiting peripheral opioid receptors<sup>32,66,73,74,112,144,150,151</sup> (BOX 1; TABLE 1).

The oral bioavailability of DENK inhibitors was improved by introducing cascade esters, which are known to enhance intestinal absorbance<sup>152</sup>. This yielded PL37 (FIG. 3), the first orally active DENK inhibitor<sup>72</sup>. Single oral doses of PL37 — between 12 mg per kg and 50 mg per kg — induce marked antihyperalgesic and anti-allodynic effects in mice and rats, particularly in models of neuropathic and neuroinflammatory pain<sup>32,72,74,153</sup> (TABLE 1). Antinociception is observed at doses higher than those resulting in complete antihyperalgesia, suggesting spinal or central participation<sup>32</sup>. As for RB-101 (REFS 30,111,112,154), repeated administration of PL37 does not induce tolerance or any crosstolerance with morphine72. Although DOR expression and functionality is increased during chronic pain<sup>82</sup>, all antinociceptive responses<sup>32,74,153</sup> are prevented only by a selective MOR antagonist and by methylnaloxonium, which is an opioid antagonist that does not enter the central nervous system (CNS), thus underpinning the hypothesis that enkephalins are active at the nociceptor level<sup>66,67,69</sup> (BOX 1). As tolerance to morphine may contribute to the transition to chronic pain, DENK inhibitors, which do not induce tolerance<sup>30,111,112</sup>, are likely to be devoid of this risk155.

Another series of orally active DENK inhibitors was synthesized in 1998 (REF. 125) using a phosphinic group as a zinc-chelating moiety and by taking into account the active-site characteristics of both enzymes. These  $\alpha$ -aminophosphinic DENK inhibitors with  $K_i$  values in the nanomolar range<sup>125</sup> are transition-state analogues of substrates, as shown by the structures of their complexes with APN<sup>52</sup> and NEP<sup>53</sup>. They are very soluble in water and do not enter the brain. The introduction of reversible protecting groups on the amino, carboxyl and phosphinic acid groups modulates their analgesic effects and duration of action in neuropathic and inflammatory pain models<sup>65,81,121,125</sup>. These (and all DENK inhibitors described above) have no affinity (>10  $\mu$ M) for endogenous opioid receptors and other GPCRs.

Other DENK inhibitors that have been developed<sup>31</sup> or purified from different sources include opiorphin (QRFSR), which potently inhibits NEP and APN at micromolar concentrations and was isolated from human saliva. It is nevertheless surprisingly active in a naloxone-reversible manner in some nociceptive tests<sup>156</sup>. All DENK inhibitors displayed in FIG. 3 are reversible inhibitors of NEP or APN, as shown by classical enzymatic methods or using radiolabelled inhibitors<sup>57,157</sup>.

Compared with morphine, enkephalins have a lower propensity to induce tolerance and addiction<sup>111,158</sup>. This may be related to their ability to stimulate the internalization and recycling of active opioid receptors at the cell surface<sup>159</sup>, thus reducing receptor reactivation and preventing the widespread changes in neural plasticity that are associated with tolerance and addiction to opiates<sup>160</sup>. Moreover, the limited occupation of opioid receptors in the brain by enkephalins that are protected by DENK inhibitors<sup>148</sup>, at doses that completely block the *in vivo* catabolism of enkephalins<sup>23</sup>, prevents any risk of opioid receptor overstimulation<sup>148</sup>. Finally, DENK inhibitors induce a weaker dopamine release in the reward system than morphine<sup>161</sup> (BOX 2).

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#### Box 1 | Peripheral reduction of inflammatory or neuropathic pain

Pain results from an initial noxious stimulation of nociceptors on primary afferent nerve endings that are present in skin, joints, muscles and viscera<sup>2</sup>. Noxious stimuli can be blocked or largely reduced at their source<sup>2,66,67,69,196</sup> by enhancing the extracellular concentrations of enkephalins. This may result from a constant upregulation of opioid receptor expression in the dorsal root ganglion during inflammation<sup>197</sup>, and their efficient transport to peripheral nerve endings<sup>198</sup> where protected enkephalins could act. In chronic constrictive injury models of neuropathic pain<sup>199</sup>, opioid receptors are also strongly augmented on both sides of the nerve injury<sup>200</sup>, with  $\mu$ -opioid receptor recycling preserving the antinociceptive effects of continuously available enkephalins and thus counteracting peripheral opioid tolerance<sup>201</sup>.

The enhanced availability of enkephalins induced by inflammation or nerve injury is due to various concomitant mechanisms. For instance, opioid-containing immune cells migrate from surrounding blood vessels; this is facilitated by the expression of endothelial adhesion molecules and triggered by neuropeptides such as substance P, which is released from noxiously stimulated nerve terminals<sup>199,202</sup>. Chemokines, cortico-tropin-releasing factor (CRF)<sup>202,203</sup>, interleukins, leukotrienes and protons are released by membrane disruption of the insulted tissue or nerve. They interact with lymphocyte receptors (for example, CRF receptors)<sup>204</sup> or ion channels, leading to a release of enkephalins<sup>61</sup>. Along with the enkephalins issuing from inflamed keratinocytes and the stimulated nerve fibre<sup>195,205</sup>, they bind to opioid receptors and reduce or eliminate the transfer of noxious inputs to the spinal cord (peripheral desensitization)<sup>67</sup>.

Neprilysin and aminopeptidase N, which break down enkephalins, are located on fibroblasts, keratinocytes, lymphocytes and neurons<sup>65,206</sup>. By increasing the levels of enkephalins, dual enkephalinase (DENK) inhibitors such as PL37 and PL265 induce long-lasting antihyperalgesic and anti-allodynic responses in complete Freund's adjuvant (CFA)-induced paw inflammation and chronic constrictive injury models, even after a single oral administration<sup>30,32,72-74</sup> (TABLE 1). Furthermore, DENK inhibitors lead to the diffusion of protected enkephalins stimulating opioid targets located along the sensory nerves, which could be beneficial in the treatment of neuropathic pain<sup>2,58</sup>.

Cannabinoid receptor 1 and N-arachidonoyl ethanolamide (AEA) are synthesized in the dorsal root ganglion and transported to peripheral terminals. Endogenous cannabinoids are also released from inflamed skin, in particular from keratinocytes. Activation of cannabinoid receptor 2 located on these cells, mast cells and macrophages<sup>206</sup> was also shown to release enkephalins, thus enhancing the pool of these antinociceptive peptides.

Local administration of URB597 increases peripheral AEA levels, thus reducing hyperalgesia in a model of bone cancer<sup>47</sup> as well as in a rat model of osteoarthritis. However, the complexity of endogenous cannabinoid signalling and the rapid inactivation of AEA at synapses may be less favourable for the treatment of neuropathic pain and inflammatory pain than the diffusion of endogenous opioids away from their storage and secretion site<sup>86,97</sup>.

### Pain-reducing effects of FAAH inhibitors

Many reviews have been devoted to the enzymatic<sup>37,162</sup> and pharmacological properties of FAAH inhibitors<sup>34,68,88</sup>. FAAH belongs to a large group of enzymes characterized by a Ser217-Ser241-Lys252 catalytic triad (also known as an amidase signature) that is different from the classical Ser-His-Asp triad found in serine proteases<sup>37</sup>. FAAH is embedded almost exclusively in internal membranes of the cell by a transmembrane segment. At the catalytic site, the hydroxyl group of Ser241 has a crucial role in AEA amide bond hydrolysis and in the binding of irreversible or reversible inhibitors. The hydrolysis reaction involves a proton exchange between Lys142, Ser217 and Ser241, leading to the formation of a tetrahedral intermediate with the carbonyl group of AEA<sup>37</sup> (FIG. 4a).

FAAH inhibitors are classified as reversible or irreversible compounds according to their half-life inside the enzyme's catalytic site<sup>37,136</sup>. The early FAAH

inhibitors (developed in 1994-1999) were designed to mimic the arachidonic part of AEA by introducing a trifluoroketone (CF<sub>2</sub>CO) group in place of the AEA amide or other fatty acid amide groups to inhibit the catalytic process<sup>163</sup>. A first breakthrough in FAAH inhibition was the discovery in 2000 (REF. 164) that ketones substituted by heterocycles potentiate binding to Ser241. This led to the development of potent inhibitors such as OL-135 (FIG. 4b), which is a selective and reversible FAAH inhibitor<sup>165</sup>. In 2003, the substitution of the amide group with a carbamate group was assessed based on the inhibition of FAAH by serine hydrolase inhibitors<sup>42</sup>. This led to URB597 (FIG. 4b), which is a potent FAAH inhibitor as a result of its almost irreversible binding of the carbamate group to Ser241 (FIG. 4b). URB597 is considered as the standard FAAH inhibitor<sup>42</sup> but other FAAH inhibitors stemming from URB597 have been developed<sup>34,37</sup>.

A new potent family of FAAH inhibitors has recently been designed by substituting the carbamate group with a urea group; these inhibitors include JNJ-1661010, PF-750 and PF-04457845 (FIG. 4b). The increased efficacy of these FAAH inhibitors is mainly due to the rigidity of the inhibitor–enzyme complex ensured by the planar carbamate (URB597) or urea groups, which not only facilitates the irreversible binding to Ser241 but also prevents the reverse hydrolysis of the carbamylated enzyme<sup>54,166</sup>.

The antinociceptive potency of FAAH inhibitors was investigated in acute and chronic animal models of pain but results in acute central pain have been inconsistent<sup>132</sup>. Possible reasons for these discrepancies include the possible involvement of receptors that are different from CB1R and CB2R<sup>91</sup>, as well as the need for prior endogenous cannabinoid mobilization by slight stressful or noxious stimuli to trigger endogenous cannabinoid signalling before testing<sup>88</sup>.

Experiments with URB937, a derivative of URB597, have shed light on the debated involvement of cannabinoid receptors in peripheral versus central or spinal pain reduction<sup>49,133,134,167</sup>. The marked and long-lasting effects of URB937 on neuropathic pain and inflammatory pain (TABLE 2) are due to the increase in AEA and PEA levels, which activate CB1R (or CB2R) and PPARα, respectively<sup>49</sup>. As URB937 does not enter the CNS, these effects take place unambiguously at the peripheral level (BOX 1).

Except for URB597, few oral FAAH inhibitors have been investigated. It is therefore difficult to compare the efficacy of DENK inhibitors and FAAH inhibitors in the alleviation of neuropathic pain or inflammatory pain, except in the model of intraplantar injection of carrageenan and in various models of chronic constrictive injury. Unlike DENK inhibitors<sup>30,32,74,81,121,153</sup>, URB597 and other FAAH inhibitors (except for URB937) are not very efficacious at a single dose on neuropathic pain, and only show significant analgesia after repeated administration for 3 to 10 days<sup>132,133,167,168</sup>. URB597 seems to be devoid of the main unwanted behavioural and reinforcing effects of  $\Delta$ 9-THC<sup>71</sup> but was reported to facilitate alcohol consumption in animals<sup>34</sup>. Such a central effect is not to be feared with the strictly

#### Box 2 | Potential antidepressant effects of DENK inhibitors

Opium and morphine have euphoric and disinhibitory properties, which suggests that there is some enkephalin deficit during mood disorders<sup>207-209</sup>. Accordingly, enkephalins that are protected from their metabolizing enzymes by kelatorphan or RB-101 are active after a single administration in all screening tests for antidepressant drugs<sup>29,80,210-214</sup>. Opiorphin is also active in some antidepressant-like (ADL) assays<sup>156</sup>. These ADL effects, which are also observed with  $\delta$ -opioid receptor (DOR) agonists<sup>209,214,215</sup>, are reversed by the selective DOR antagonist NTI or by dopamine antagonists<sup>210,216</sup>, suggesting that the regulation of mood is mainly DOR-dependent, and involves the dopamine-dependent mesolimbic pathway. The ADL effects elicited by dual enkephalinase (DENK) inhibitors are facilitated by deafferentation of the dopaminergic mesolimbic pathway, which increases the levels of preproenkephalin (PENK) and enkephalins<sup>217</sup>, suggesting that the phasic control of the dopaminergic mesolimbic pathway by enkephalin-mediated activation of DORs might be altered in depressive syndromes<sup>208,212,216</sup>. This is supported by the increase in both motor activity and extracellular dopamine levels in the nucleus accumbens, which is elicited by the injection of kelatorphan into the ventral tegmental area, and the prevention of these effects by a selective DOR antagonist<sup>161</sup>. Indeed, the selective DOR agonist SNC-80, which displays potent anxiolytic and ADL effects, also induces seizures with simultaneous epileptiform activity<sup>213</sup>. None of these side effects was observed with RB-101, indicating that DENK inhibitors may be an interesting alternative to alleviate depressive syndromes<sup>208,213,218</sup>.

RB-101 has also shown anxiolytic effects mainly through DOR stimulation<sup>80,219,220</sup>, as DOR effects remain present in mice in which the gene encoding the  $\mu$ -opioid receptor has been knocked out<sup>30,80</sup>. Consistent with these results, PENK-knockout mice exhibit anxiogenic responses, increased aggressiveness<sup>17,221</sup>, stronger anxiety and depressive post-traumatic stress disorder<sup>222</sup>.

peripherally acting URB937. A urea FAAH inhibitor, JNJ-1661010, has been shown to be very effective in reducing allodynia and/or hyperalgesia (TABLE 2). All of these responses were antagonized by naloxone, indicating again the crucial involvement of opioid receptors in the effects of these FAAH inhibitors<sup>40,169,170</sup>.

Irreversible FAAH inhibition induces a longer duration of action than the reversible DENK inhibitors, as shown with URB937. However, the slowness of FAAH synthesis moderates this possible advantage, making it problematic to achieve accurate dosing with FAAH inhibitors and impossible to use cannabinoid receptor antagonists in case of overdosing. Moreover, most FAAH inhibitors are not totally selective and interact with liver carboxylesterases, which may inhibit the hydrolytic activation of ester prodrugs<sup>171</sup>.

#### Synergistic effects of DENK and FAAH inhibitors

The effects of enkephalins released in injured tissues can be enhanced synergistically by analgesic substances such as gabapentin, non-steroidal anti-inflammatory drugs (NSAIDs) or antagonists of pro-nociceptive compounds; for example, ATP or CCK.

*Synergistic effects of morphine or DENK inhibitors with exogenous or endogenous cannabinoids.* Functional interactions between endogenous opioids and endogenous cannabinoids have been demonstrated following genetic deletion of opioid receptors or CB1R<sup>172</sup>. CB1R and MORs have a similar distribution and are often colocalized at the different levels of pain control<sup>173</sup>. Thermal nociception induced in a rat TFT and mouse HPT is synergistically reduced by the combination of  $\Delta$ 9-THC with morphine<sup>174</sup> or DENK inhibitors<sup>122</sup>, and a similar facilitation is observed in models of inflammatory pain and neuropathic pain<sup>174-176</sup>. In the mouse HPT, a single co-administration of subanalgesic doses of RB-101 (2.5 mg per kg; i.v. administration) or PL37 (0.4 mg per kg; i.p. administration) with  $\Delta$ 9-THC (1.25-5 mg per kg; i.v. administration) produces 60-80% of analgesia, whereas 10-15-fold higher doses of each individual compound are required to achieve the same response<sup>72,122</sup>. The synergistic effects are reversed by a MOR antagonist but not by a DOR or κ-opioid receptor antagonist. The replacement of  $\Delta$ 9-THC with AEA in combination with the FAAH inhibitor URB597 elicits a similar antinociceptive potentiation, which is also reversed by MOR but not DOR antagonists<sup>40,175</sup>.

Several explanations have been proposed to account for the synergistic facilitation of endogenous opioid and endogenous cannabinoid signalling<sup>177,178</sup>: reciprocal enhancement of extracellular levels of endogenous opioids and endogenous cannabinoids (as demonstrated by microdialysis)<sup>122,179</sup> or of *PENK* gene expression<sup>176</sup>; creation - by MORs and CB1R - of membrane-bound heterodimers with increased pharmacological efficiency<sup>178</sup>; and amplification of transduction pathways downstream of opioid receptors and cannabinoid receptors when both receptors are colocalized on the same cell, resulting in greater antinociceptive responses<sup>173,174,178</sup>. This probably also occurs at the periphery and may account for the naloxone-reversed responses observed in inflammatory pain and neuropathic pain with the FAAH inhibitors OL-135 and URB597 (REF. 170).

The synergistic responses obtained by combining opiates with exo- or endocannabinoids seem to occur in those tissues and pathways in which their physiological role is the most important (for example, pain control, mood regulation, adaptive behaviours, intestinal motility and secretion), and where their release (tonic or phasic) is the highest. Therefore, the combination of FAAH inhibitors and DENK inhibitors might induce stronger pharmacological responses at lower doses, thus reducing or eliminating the risk of the unwanted effects of endogenous opioids and cannabinoids on other structures<sup>40,174,180</sup>.

#### Synergistic analgesic effects of DENK inhibitors combined

*with opioids.* The synergy of RB-101 and subactive doses of morphine (0.5 mg per kg; subcutaneously administered) or methadone on thermal, mechanical and inflammatory nociceptive stimuli was demonstrated using an isobolographic plot<sup>121,181</sup>. This may partly be due to the increase in enkephalin levels evoked by morphine as demonstrated in the PAG, where chronic morphine administration triggers a three- to fivefold increase in the basal levels of enkephalins<sup>123</sup> and increases by 43% the levels of released Met-enkephalin measured by microdialysis<sup>121</sup>. This contributes to the spinal control of pain by afferent neurons from the PAG<sup>119</sup>. Moreover, the activation of opioid receptors by the protected enkephalins enhances receptor trafficking<sup>182</sup> and increases the amounts of active opioid receptors at the cell surface<sup>159,183</sup>. By elevating enkephalin

#### Isobolographic plot

A method of determining drug synergy. The theoretical additive  $ED_{50}$  value (the half-maximal effective dose) is estimated from the dose– response curves of each drug administered individually. This theoretical  $ED_{50}$  value is compared with the experimental  $ED_{50}$  value. If a statistically significant difference is observed, synergy is present.



Figure 4 | **Main FAAH inhibitors. a** | The scheme depicts the main steps of the irreversible binding of a urea fatty acid amide hydrolase (FAAH) inhibitor to Ser241 of FAAH. **b** | Structures of six representative FAAH inhibitors are shown. OL-135 is a reversible FAAH inhibitor and was the first to be used for pharmacological experiments. URB597 was rationally designed on the basis of carbamate-containing serine protease inhibitors and was co-crystallized with humanized FAAH. It is commonly used as a standard for the evaluation of new FAAH inhibitors. Its derivative URB937 is the first FAAH inhibitor that has been shown to act selectively on peripheral FAAH. ARN272 is the first inhibitor of FAAH-like anandamide transporter (FLAT) to be described. FLAT is an *N*-arachidonoyl ethanolamide transporter that is structurally similar to FAAH but devoid of its enzymatic activity. JNJ-1661010, PF-3845 and PF-04457845 are irreversible FAAH inhibitors<sup>75</sup>. Their very long half-life inside the FAAH active site is due to thermodynamic features deduced from crystallographic data<sup>54</sup>. PF-04457845 was tested in clinical trials for the treatment of osteoarthritic pain but was found to be inactive.

levels, DENK inhibitors may facilitate MOR–DOR heterodimerization<sup>184</sup>, hence inducing a greater pharmacological response than individual stimulation of each opioid receptor<sup>81</sup>. This synergy may allow a reduction of the therapeutic doses of morphine, thereby limiting its unwanted side effects.

*Synergistic analgesic effects induced by DENK inhibitors with non-opioid modulators of pain.* ATP released by cell damage or nerve injury excites nociceptors<sup>2,3</sup>. Consistently, A-317491 — an antagonist of the purinergic P2X3 receptor that inhibits the nocifensive effect of ATP and is almost unable to enter the CNS<sup>185</sup> — enhances the PL37-induced alleviation of thermal hyperalgesia<sup>32</sup>. This effect is antagonized by pre-administration of methylnaloxonium, suggesting a peripheral contribution of the endogenous opioid system to the analgesic effect (BOX 1). An important finding is that the coadministration of an anti-enkephalin antibody with either PL37 or A-317491 completely blocks their antihyperalgesic effects, proving that the action of PL37 selectively involves endogenous enkephalins<sup>32</sup>. Similar results have been observed with another DENK inhibitor, PL253 (REF. 121) (FIG. 3).

It has been hypothesized that the synergy of gabapentin or A-317491 with enkephalins that are protected by PL37 is due to the induction of nitric oxide (NO) synthesis at the periphery73 and the subsequent reduction of noxious inputs. Consistently, NO from various biochemical donors reduces nociceptive transmission and potentiates the analgesic properties of morphine in neuropathic pain symptoms caused by cancer in humans<sup>186</sup>. The synergies observed with PL37 may also be due to a NEP-dependent inhibition of bradykinin cleavage at the periphery, as bradykinin increases NO production from endothelial microvessels in injured tissues. Moreover, P2X3 receptor activation by ATP induces hyperalgesia by pro-nociceptive-dependent sensitization of the primary afferent nociceptors<sup>187</sup>. Reduction of these processes by endogenous opioids decreases the pain threshold and thereby the doses of PL37 necessary to elicit analgesia32.

Opioid and CCK systems are counteracting<sup>30,188-190</sup>, which explains the antagonism between enkephalins and CCK8 — the amino terminal fragment of CCK. This was unambiguously established using DENK inhibitors<sup>190</sup>. Selective activation of the CCK2 receptor reduces the analgesic effects of RB-101, whereas CCK2 receptor antagonists strongly potentiate them in models of acute<sup>121,188</sup> and chronic pain<sup>150</sup>. Furthermore, an increased release of CCK8 in primary sensory neurons may contribute to neuropathic pain symptoms and explain the relative inefficacy of opiates and, by contrast, the efficacy of an RB-101-CCK2 antagonist combination in experimental neuropathic pain<sup>189</sup>. The synergy between inactive doses of the CCK2 receptor antagonist PD-134308 (3 mg per kg; i.p. administration) and RB-101 (5 mg per kg; i.p. administration) is illustrated in the rat TFT, where the combination has an eightfold higher analgesic effect than RB-101 alone. In the HPT, which is thought to be more supraspinal and in which the spinal nociceptive neurons have a lesser role, the synergy is lower (only 250% higher). Glutamate also has a key function in conveying noxious inputs at the spinal and brain levels, and NMDA receptor antagonists have been shown to strongly improve the antinociceptive effects of RB-101 in inflammatory pain<sup>191</sup>.

The combination of the FAAH inhibitor URB597 and the cyclooxygenase inhibitor diclofenac elicited synergistic analgesic responses that were supported by isobolographic analysis in a model of acetic acid-induced visceral nociception in mice. This suggests that the gastric toxicity of NSAIDs could be significantly reduced by combining them with FAAH inhibitors<sup>192</sup>.

### Conclusions

It has taken over 20 years since the conception of DENK inhibitors as analgesics, and 10 years in the case of FAAH inhibitors, for these compounds to reach clinical testing. The hurdles that DENK inhibitors have had to overcome are as follows: an assumption that their analgesic properties would be far less than those of morphine; doubts about a possible renewal of synaptic enkephalin levels; wariness about the *in vivo* specificity of NEP and APN for enkephalins; and the

risk of morphine-like adverse effects. All of these concerns have been addressed in the studies reviewed in this article.

Primary afferent nociceptors are an important target for the development of novel pain therapeutics<sup>69</sup>, for the following reasons: nociceptors contain functionally important molecules that are not found in other cells (for example, the voltage-gated sodium channel Nav1.8); only a subpopulation of nociceptors may be involved in a given pain syndrome, which might allow for preservation of protective pain sensation; analgesics working at this level in the pain pathway (that is, on the primary afferent nociceptors) act before pain signals enter the CNS to diverge over multiple pathways; and peripherally restricted analgesics avoid their many CNS-related side effects. Thus, inhibiting the breakdown of endogenous opioids and/or endogenous cannabinoids at this level seems to be a promising approach for alleviating pain.

The endogenous cannabinoid system appeared much later than the endogenous opioid system during evolution<sup>193</sup>. Nevertheless, the former is not a duplication of the latter; rather, it acts as a local regulating mechanism and as a paracrine system at the peripheral level<sup>89</sup>. Several recent studies indicate that FAAH inhibitors may preferably find their clinical indication as anti-inflammatory agents44,49 for reducing both oedema and nociception194 and/or as anxiolytic drugs42. The efficacy of FAAH inhibitor-protected endogenous cannabinoids in inflammatory pain treatment is due to the synergistic action of two substrates - AEA and PEA - the concentrations of which are enhanced by FAAH inhibitors. Clinical use of the different families of FAAH inhibitors or FLAT inhibitors requires knowledge of the physiological roles of all substrates of FAAH. Moreover, additional information is necessary on the pathophysiological conditions requiring stimulation or blockade of endogenous cannabinoids<sup>50</sup>. Indeed, unlike opiate antagonists, which are devoid of clinically significant pharmacological effects in healthy individuals, CB1R and CB2R antagonists are endowed with numerous positive and negative effects in humans<sup>50,89</sup>.

DENK inhibitors are more potent analgesics than FAAH inhibitors, in particular when a central involvement is required (for example, in acute nociceptive pain), and are effective after a single-dose administration in almost all pharmacological tests performed. All combinations tested with DENK inhibitors (cannabinoids, morphine, gabapentin, CCK antagonists and purinergic receptor antagonists) show synergistic responses, thus allowing significant dose reductions.

There are several advantages of DENK inhibitors over FAAH inhibitors. First, DENK inhibitors act on both the basal and phasic release of enkephalins, the former being far more abundant than that of endogenous cannabinoids at all three levels of pain control. Second, enkephalins, as neuropeptides, diffuse far away from their release site. Both of these characteristics make enkephalins attractive targets for alleviating chronic pain, including neuropathic pain, when they are enhanced by DENK inhibitors. PL37 is entering Phase II

trials in neuropathic pain, whereas PL265 is entering Phase I trials.

Enkephalins are more suitable than endogenous cannabinoids for treating acute nociceptive pain (alone or in combination with morphine) owing to their high affinity for opioid receptors and their high concentrations (basal or induced by a noxious stimulus) in brain structures (such as the thalamus, PAG and cortex) that are crucially involved in acute pain regulation. FAAH inhibitors may be more suitable for treating inflammatory pain by enhancing the synaptic concentration of AEA and PEA at the periphery. The antidepressant and anxiolytic properties of both endogenous opioid- and endogenous cannabinoid-enhancing agents look promising, but will require further investigation. Overall, the development of DENK inhibitors and FAAH inhibitors (and of their combinations) could lead to innovative, effective and safe additions to the armamentarium of painkillers, which have long been awaited<sup>195</sup>.

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#### Competing interests statement

The authors declare <u>competing financial interests</u>: see Web version for details.