Pain is a unique, conscious experience with sensory-discriminative, cognitive-evaluative and affective-emotional components. 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. However, chronic pain can occur after several pathophysiological processes, as well as without any identifiable cause (such as in fibromyalgia). One example is neuropathic pain — a frequent complication of shingles, diabetes, antiviral or antitumour chemotherapy, as well as surgery or lower-back disorders — which is unsatisfactorily treated with morphine.

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, sodium channel blockers and NMDA (N-methyl-d-aspartate) receptor antagonists have failed in clinical trials despite showing signs of efficacy in preclinical studies. Other compounds targeting acid-sensitive channels or vanilloid receptors 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.

One innovative approach for the development of analgesics is based on the fact that the painkillers found in Papaver somniferum (morphine) or Cannabis sativa (Δ9-tetrahydrocannabinol; Δ9-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, or dysphoria, changes in motor coordination and memory disorders in the case of cannabinoids. 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$^{2+}$-dependent mechanism to interact specifically with two G protein-coupled...
receptors (GPCRs): the μ-opioid receptors (MORs) and the δ-opioid receptors (DORs)14. The affinity of enkephalins for MORs is similar to that of morphine, whereas their affinity for DORs is about tenfold higher14.

The crucial role of enkephalins in physiological pain control is supported by the increase in sensitivity to noxious stimuli elicited by PENK gene ablation17,18. Furthermore, when Met-enkephalin is injected into the rodent brain, it produces a morphine-like transient antinociceptive effect19. The limited duration of this effect is due to the rapid interruption of endogenous opioidergic signalling by the concomitant action of two zinc metalloendopeptidases — the neutral endopeptidase nephrilysin (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, respectively20,21 (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 receptors22. 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 stimulation23–24. When administered systemically, selective NEP inhibitors have no significant analgesic effects in rodents25 or in humans26 because the level of protected enkephalins is too low27 and the stimulation of opioid receptors is therefore insufficient. This finding led to the proposal that dual inhibitors targeting both NEP and APN28 might be more effective, and promising results were obtained with some of these compounds in various animal models of pain21,25,27–32. Accordingly, in a non-controlled open-label study, intrathecal administration of the combination of a selective NEP inhibitor12 and a nonspecific APN inhibitor elicited marked and lasting pain relief in terminally ill patients with cancer who were unresponsive to morphine33.

**Pain reduction by endogenous cannabinoids.** The major endogenous substances recognizing the same receptors as Δ9-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)34. In brain homogenates, the affinity of AEA for cannabinoid receptors is about 100 times weaker than that of Δ9-THC (2–6 nM)35. The signal conveyed by AEA is rapidly interrupted mainly by the intracellular fatty acid amide hydrolase (FAAH)36,37, 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 oleoylthanolamide, 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-α (PPARα).

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 secretion39. 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 co-administered with a compound that inhibits its catabolism40. Nevertheless, even after stressful stimulation41, endogenous cannabinoid-mediated analgesia19,42 is never as efficacious as the morphine-like analgesia induced by enkephalins or dual enkephalase (DENK) inhibitors43,44 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 observed43–49.

**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 inhibitors45,27,30,34,43,47,48. To this end, the structure of the metabolizing enzymes in complex with an inhibitor45–48, their central49,50 and peripheral distribution51–53 as well as their molecular mechanisms of hydrolysis52,53,55 have been taken into account.

Interestingly, both endogenous opioids and endogenous cannabinoids are present in primary sensory neurons32,59–63, offering the possibility to relieve, or at least reduce, the noxious inputs at their initial stage44,49,58–63,66–68. Indeed, more than 50% of the effects of morphine are attributable to the stimulation of peripheral neurons32,69,70. 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 effects30,49,58,63,66–69,71).

Results with one of the first synthetic orally active DENK inhibitors — PL37 (REF. 72) — in various animal models of pain have revealed interesting analgesic effects32,73,74; PL37 is the first DENK inhibitor to reach clinical trials. The first orally active FAAH inhibitor, URB597, was developed following a structure–activity study42 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 inactive43.

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...
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; they are negatively coupled via G_1 and G_0 proteines to similar intracellular signal-ling 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. The stimulation of opioid receptors and cannabinoid receptors blocks the conversion of noxious stimuli into electrochemical signals by inhibiting voltage-gated Ca\(^{2+}\) channels, stimulating K\(^{-}\) inward channels and subsequently inhibiting the Ca\(^{2+}\)-dependent release of pro-nociceptive effectors such as substance P, calcitonin gene-related peptide and bradykinin. 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. Both MORs and DORs are associated with the regulation of chronic pain.

Variations in the endogenous levels of opioids and cannabinoids in specific nociceptive pathways are likely to account for the variability in the efficacy of DENK inhibitors and FAAH inhibitors. Opioid receptors bind to a limited number of endogenous opioids (for example, enkephalins, β-endorphin and, to a much lesser extent, small fragments of β-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, and the binding of endogenous cannabinoids is not limited to cannabinoid receptors. Depending on its concentration, AEA can interact with the transient receptor potential subfamily V member 1 receptor to induce pro-nociceptive responses and/or with PPARα to reduce inflammatory pain. At high concentrations, AEA also behaves as a substrate of cyclooxygenase 2, resulting in the generation of biologically active oxygenated derivatives of AEA.

Difficulties 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\(^{2+}\)-dependent manner, by exocytosis. 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. Their affinity for their targets is in the nanomolar range, about 1,000 times stronger than that of classical neurotransmitters, which is in the micromolar range.

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 \(^{3}H\)AEA and the transporter FLAT. 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. Interruption of AEA signalling is ensured by a two-step mechanism that involves AEA reuptake by FLAT and cleavage by cytosolic FAAH, which is probably located in membranes near the AEA synthesis site. Inhibition of intracellular FAAH or selective blockade of FLAT 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. 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 tissues. 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. 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, and allows them to potentially exert long-lasting 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, neuromedin C, CCK and bradykinin, which are cleaved in vivo by their own peptidases. The other endogenous opioid peptides dynorphin and β-endorphin — which are often regarded as NEP and/or APN substrates — also have their own metabolizing enzymes. In vivo, NEP modulates the activity of atrial natriuretic peptide and endothelin, whereas APN contributes to angiotensin metabolism in the brain and fluid homeostasis in the kidney. Importantly, DENK inhibitors enhanced the analgesic effect of enkephalins without increasing neurokinin 1 receptor activation by endogenous substance P.

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, oleoyl ethanolamide and PEA 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.

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
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. At the periphery, endogenous opioids and endogenous cannabinoids are present in epithelial cells of the intestine and kidney, in the joints, lung, testis and skin, as well as in various types of immune cells, including dendrocytes and Schwann cells surrounding nerve fibres. Cannabinoid receptors and fatty acid amide hydrolase (FAAH) are found at the nociceptor level and in immune cells where inhibitors can block the noxious inputs. Noxiously stimulated cutaneous fibres converge to the dorsal horn, sometimes along with non-stimulated fibres from distant cutaneous, muscular or visceral areas. μ-opioid receptors (MORs) and δ-opioid receptors (DORs) are mainly located at the presynaptic end of afferent fibres in the spinal dorsal horn, whereas neuropeptide (NEP) is found in interneurons. 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. FAAH is also highly expressed near neurons enriched in cannabinoid receptor 1 (CB1R), including structures involved in fear and emotions but also in non-neural cells. 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 spinal nociceptive neurons, where they block the transfer of nociceptive inputs to the brain via an increase in K+ conductance and subsequent hyperpolarization. Regarding the endogenous cannabinoids, it is hypothesized that, like enkephalins, N-arylalkylaminoalkanolamide (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 spinohalamic neurons by inhibiting their glutamate-dependent excitation. Glutamate may also be released from interneurons or from glial cells.

Putative limitations of nonspecific endogenous signaling 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. DENK-knockout mice 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 inRefs 30,112) or PL37 in humans did not result in tolerance, sedation, respiratory depression, emesis, constipation or dependence.

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 (reviewed in Refs 109,113) or PL37 in humans did not result in tolerance, sedation, respiratory depression, emesis, constipation or dependence.

© 2012 Macmillan Publishers Limited. All rights reserved
www.nature.com/reviews/drugdisc
### Table 1 | Pharmacological activity of various classes of DENK inhibitors

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

APN, aminopeptidase N; CCI, chronic constrictive injury; CFA, complete Freund’s adjuvant; DENK, dual enkephalinase; ED\(_{50}\), 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; TTF, tail-flick test.

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). Owing to the relatively low basal concentrations of released Met-enkephalin—which are in the femtomolar range—and the large, rapidly renewable intracellular pool of enkephalins, repetitive stimulation of enkephalin-containing neurons is unable to exhaust the intracellular content of opioid peptides that can be mobilized. 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. 5) 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, euphorogenic) effects of opiates. During noxious stimulation, the aminophosphonic DENK inhibitor PI.253 (REF. 125) (FIG. 5) increased the basal enkephalin concentration by 88% in the periaqueductal grey (PAG), an area of the brain that is involved in pain modulation. 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. Kelatorphan (FIG. 5) almost completely prevented the spinal degradation of exogenous [3H]Met-enkephalin in the superfused spinal cord of halothane-anaesthetized rats. The recovery of the spontaneous outflow of endogenous Met-enkephalin 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.

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, which may explain why DENK inhibitors do not induce respiratory depression—a severe side effect of morphine. Likewise, there is little or no tonic endogenous opioid receptor activation in the locus coeruleus—an area involved in physical dependence to morphine. Unlike morphine, DENK inhibitors do not induce constipation, even at high and repeated doses. This is probably due to a restricted release of enkephalins in intestinal plexi, where MOR stimulation influences transit and may elicit constipation. 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. The 15-fold increase in AEA levels in the brain of FAAH-knockout mice is not reflected in the effects of FAAH inhibitors on acute pain, which are generally absent or weak 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. This may be related to the rapid degradation of AEA, not only by FAAH but also by other mechanisms. 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 Ch1r- mice, suggesting that the action of AEA is mediated by other receptors in the brain (very few CB2Rs are found in the brain). 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. 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.

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. 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. 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. 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.

Rational design of various DENK inhibitors

NEP and APN are membrane-bound zinc metalloendopeptidases 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. Site-directed mutagenesis of rabbit NEP, computer modelling and crystallographic studies have shown that the catalytic sites of NEP and APN are very similar but there are subtle structural differences.
differences in their S₁, S₁′ and S₂′ subsites\textsuperscript{141}. In pig APN, the Glu350 residue in the S₁ site is essential for the exoaminopeptidase activity\textsuperscript{38} of the enzyme, whereas the Arg102 residue in the S₃ site of NEP is essential for the carboxydipeptidase activity of NEP (FIG. 1a).

Taking into account the substantial similarities in the active sites of zinc metallopeptidases\textsuperscript{52,53,139,140}, the rational design of potent selective or dual inhibitors of NEP and APN\textsuperscript{[21,30,58,142]} 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 S₁–S₂′ subsites surrounding the catalytic site, as evidenced by inhibitor co-crystallization\textsuperscript{[52,53]} (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 zinc-chelating 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\textsuperscript{21} that are obviously not identical\textsuperscript{52,53}. Accordingly, kelatorphan strongly inhibits NEP (IC\textsubscript{50} = 1.8 nM) and less efficiently inhibits APN (IC\textsubscript{50} = 380 nM).

Kelatorphan was the first compound that completely inhibited enkephalin catabolism\textsuperscript{5}. It had antinociceptive effects in numerous acute nociceptive animal models\textsuperscript{20,142} and, after intrathecal administration, it induced longer-lasting analgesia in patients with cancer (M.C.F.Z. and J. Meynadier, unpublished observations) than the combination of both bestatin and thiorphan\textsuperscript{24}. Kelatorphan was also active in complete Freund’s adjuvant-induced arthritis in rats — a widely used model of chronic pain\textsuperscript{26,144} — and it reduced nociception by 60% in mono-neuropathic rats\textsuperscript{26,145}. 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\textsuperscript{46}.

DENK inhibitors with improved brain penetration were developed from 1992 onwards by linking two highly potent inhibitors (K < 10 nM) for each peptidase\textsuperscript{26,146} by a disulphide bridge (co-drug) (FIG. 3), which is rapidly cleaved \textit{in vivo} by an enzymatic process\textsuperscript{46}. The pharmacokinetic properties of these disulphides were modulated by introducing hydrophobic ester groups\textsuperscript{28,144}.

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\textsuperscript{25,30,146}. The pain-allieving 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\textsuperscript{52,65}.

In the HPT, i.v. administration of RB-101 elicited a maximum 85% analgesia with an ED\textsubscript{50} (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\textsuperscript{110}. This is consistent with binding experiments demonstrating that RB-101 does not completely displace \textsuperscript{3}H]-diprenorphine bound to opioid receptors in the

---

### Figure 3 | Main selective dual NEP–APN inhibitors.

The knowledge of detailed mechanisms of substrate hydrolysis by zinc metallopeptidases\textsuperscript{51,139} was used for rationally designing dual enkephalase (DENK) inhibitors. Specific nephrin (NEP) or aminopeptidase N (APN) inhibitors (thiorphan and PC 18, 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 S₁–S₃′ of NEP and APN, as inferred from site-directed mutagenesis\textsuperscript{51,139}, docking and molecular modelling studies\textsuperscript{46}. Crystallographic data\textsuperscript{51,53} and K\textsubscript{ᵱ} values, Hydroxamate inhibitors (kelatorphan and RB-38) and aminophosphinic DENK inhibitors (PL 253, PL 254 and PL 265) have been designed to fit the active sites of both APN and NEP, whereas RB-101, RB-120 and PL 37 are produgs 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.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Model</th>
<th>Dose (route)</th>
<th>Tests (animal)</th>
<th>Result (% MPE)</th>
<th>Refs</th>
</tr>
</thead>
</table>
| OL-135              | Inflammatory pain (acute)    | 10 mg per kg (i.p.) | * Mild thermal injury  
* Paw pressure test (rats)  
* Von Frey test | 50% ↓ in allodynia (opioid receptor-dependent)  
30% ↑ in latency | 170 |
|                      | Neuropathic pain (SNL)       | 20 mg per kg (i.v.) | * Paw pressure test (rats)  
* Von Frey test | 80% ↓ in allodynia (opioid receptor-dependent)  
30% ↑ in latency |  |
| OL-135              | Acute pain                   | 10 mg per kg (i.p.) | * Tail immersion test (mice)  
* HPT (mice)  
* Paw lifting test | ~30% ↓ in allodynia (opioid receptor-dependent)  
30% ↑ in latency  
30% ↓ in response | 238 |
|                      | Viceral pain (formalin)      | 10 mg per kg (i.p.) | * Paw licking test (mice) | ~30% ↓ in response |  |
| OL-135              | Neuropathic pain (CCI model) | 10 mg per kg (i.p.) | * Paw pressure test (mice)  
* Paw lifting test | ~60% ↓ in allodynia  
30% ↓ in allodynia (CB1R- or CB2R-dependent) | 134 |
|                      |                             | 10 mg per kg (i.p.) | * Paw pressure test (mice)  
* Von Frey test  
* Cold acetone test | ~35% ↓ in allodynia  
~80% ↓ in allodynia (CB1R- or CB2R-dependent) | 134 |
| URB597              | Neuropathic pain (CCI model) | 0.3 mg per kg (i.p.) | * Paw pressure test (rats)  
* Cold acetone test  
* Plantar test (rats) | 90% ↓ in allodynia  
50% ↓ in allodynia | 132 |
| URB597              | Inflammatory pain (CFA; i.p.) | 0.3 mg per kg (i.p.) | * Paw pressure test (rats)  
* Von Frey test  
* Cold acetone test | 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  
* Electronic Von Frey test  
* Thermal hyperalgesia  
* Paw withdrawal latency | 35% ↓ in allodynia (10 mg per kg)  
70% ↓ in allodynia (50 mg per kg)  
75% ↓ in hyperalgesia | 133 |
| URB597              | Inflammatory pain; iodoacetic acid-induced osteoarthritis | 5 mg per kg (s.c.) | * Paw pressure test  
* Paw oedema measurement (mice) | 80% reduction in oedema (CB2R-dependent)  
19% MPE | 168 |
| URB597              | Bone cancer                  | 9 μg 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 FAAH inhibitor) | Neuropathic pain (SNL) | 1 mg per kg (single dose; i.p.) | * Paw pressure test  
* Withdrawal latency  
* Thermal stimulation  
* Thermal stimulation and withdrawal latency  
* Paw pressure and Von Frey tests | ~50% ↓ in hyperalgesia  
100% ↓ in hyperalgesia  
1000% ↓ in hyperalgesia | 49 |
| URB937              | Neuropathic pain (SNL)       | 1 mg per kg (i.p.) once a day for 7 days | * Paw pressure and withdrawal latency  
* Thermal stimulation and withdrawal latency  
* Paw pressure and Von Frey tests | Long-lasting similar effects (lack of tolerance) | 49 |
mouse brain\textsuperscript{12,148}. As DENK inhibitors do not modify enkephalin secretion, and only modify its extracellular concentrations\textsuperscript{23}, they may be of great interest for \textit{in vivo} studies of opioid receptor occupation in various situations (for example, pain, anger, stress or emotion) using positron emission tomography (PET) scans\textsuperscript{149}. In various animal models of inflammatory pain and neuropathic pain, RB-101 suppressed mechanical hyperalgesia and reduced allodynia, mainly by recruiting peripheral opioid receptors\textsuperscript{32,66,73}\textsuperscript{,112,144,150,151} (BOX 1; TABLE 1). The oral bioavailability of DENK inhibitors was improved by introducing cascade esters, which are known to enhance intestinal absorbance\textsuperscript{152}. This yielded PL37 (FIG. 3), the first orally active DENK inhibitor\textsuperscript{22}. 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\textsuperscript{32,72,74,112,153} (TABLE 1). Antinociception is observed at doses higher than those resulting in complete antihyperalgesia, suggesting spinal or central participation\textsuperscript{22}. As for RB-101 (REFS 30,111,112,154), repeated administration of PL37 does not induce tolerance or any cross-tolerance with morphine\textsuperscript{22}. Although DOR expression and functionality is increased during chronic pain\textsuperscript{148}, all antinociceptive responses\textsuperscript{32,72,153} 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\textsuperscript{66,67,69} (BOX 1). As tolerance to morphine may contribute to the transition to chronic pain, DENK inhibitors, which do not induce tolerance\textsuperscript{96,111,112}, are likely to be devoid of this risk\textsuperscript{130}.

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 α-aminophosphinic DENK inhibitors with $K_i$ values in the nanomolar range\textsuperscript{125} are transition-state analogues of substrates, as shown by the structures of their complexes with APN\textsuperscript{52} and NEP\textsuperscript{53}. 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\textsuperscript{65,81,121,123}. These (and all DENK inhibitors described above) have no affinity (>10 μM) for endogenous opioid receptors and other GPCRs.

Other DENK inhibitors that have been developed\textsuperscript{111} or purified from different sources include opioidin (QRF53), 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\textsuperscript{149}. All DENK inhibitors displayed in FIG. 3 are reversible inhibitors of NEP or APN, as shown by classical enzymatic methods or using radiolabelled inhibitors\textsuperscript{22,117}.

Compared with morphine, enkephalins have a lower propensity to induce tolerance and addiction\textsuperscript{113,118}. This may be related to their ability to stimulate the internalization and recycling of active opioid receptors at the cell surface\textsuperscript{105}, thus reducing receptor reactivation and preventing the widespread changes in neural plasticity that are associated with tolerance and addiction to opiates\textsuperscript{102}. Moreover, the limited occupation of opioid receptors in the brain by enkephalins that are protected by DENK inhibitors\textsuperscript{111}, at doses that completely block the \textit{in vivo} catabolism of enkephalins\textsuperscript{23}, prevents any risk of opioid receptor overstimulation\textsuperscript{148}. Finally, DENK inhibitors induce a weaker dopamine release in the reward system than morphine\textsuperscript{161} (BOX 2).
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. Noxious stimuli can be blocked or largely reduced at their source by enhancing the extracellular concentrations of enkephalins. This may result from a constant upregulation of opioid receptor expression in the dorsal root ganglia during inflammation, and their efficient transport to peripheral nerve endings, where protected enkephalins could act. In chronic constriction injury models of neuropathic pain, opioid receptors are also strongly augmented on both sides of the nerve injury, with μ-opioid receptor recycling preserving the antinociceptive effects of continuously available enkephalins and thus counteracting peripheral opioid tolerance.

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. Chemokines, corticotropin-releasing factor (CRF), interleukins, leukotrienes and protons are released by membrane disruption of the insulted tissue or nerve. They interact with lymphocyte receptors (for example, CRF receptors) or ion channels, leading to a release of enkephalins. Along with the enkephalins issuing from inflamed keratinocytes and the stimulated nerve fibre, they bind to opioid receptors and reduce or eliminate the transfer of noxious inputs to the spinal cord (peripheral desensitization).

Nephrilysin and aminopeptidase N, which break down enkephalins, are located on fibroblasts, keratinocytes, lymphocytes and neurons. 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 constriction injury models, even after a single oral administration. 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.

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 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 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 or inflammatory pain than the diffusion of endogenous opioids away from their storage and secretion site.

Pain-reducing effects of FAAH inhibitors

Many reviews have been devoted to the enzymatic and pharmacological properties of FAAH inhibitors. FAAH belongs to a large group of enzymes characterized by a Ser217-Ser241-Lys252 catalytic triad (also known as an amidae signature) that is different from the classical Ser-His-Asp triad found in serine proteases. 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 the binding of irreversible or reversible inhibitors. The planar carbamate (URB597) or urea groups, which have a crucial role in AEA amide bond hydrolysis and in the alleviation of neuropathic pain or inflammatory pain.

The early FAAH inhibitors (developed in 1994–1999) were designed to mimic the arachidonic part of AEA by introducing a trifluoroketone (CF3-CO) group in place of the AEA amide or other fatty acid amide groups to inhibit the catalytic process. A first breakthrough in FAAH inhibition was the discovery in 2000 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. In 2003, the substitution of the amide group with a carbamate group was assessed based on the inhibition of FAAH by serine hydrolase inhibitors. 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 but other FAAH inhibitors stemming from URB597 have been developed.

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. 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.

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. Possible reasons for these discrepancies include the possible involvement of receptors that are different from CB1R and CB2R, as well as the need for prior endogenous cannabinoid mobilization by slight stressful or noxious stimuli to trigger endogenous cannabinoid signalling before testing.

Experiments with URB937, a derivative of URB597, have shed light on the debated involvement of cannabinoids receptors in peripheral versus central or spinal pain reduction. 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. As URB937 does not enter the CNS, these effects take place unambiguously at the peripheral level (BOX 1).

Except for URB937, 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 constriction injury. Unlike DENK inhibitors, 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. URB597 seems to be devoid of the main unwanted behavioural and reinforcing effects of Δ9-THC but was reported to facilitate alcohol consumption in animals. Such a central effect is not to be feared with the strictly...
synergy is present. If a statistically significant
This theoretical ED
isobolographic plot
Potential antidepressant effects of DENK inhibitors
Synergistic effects of DENK and FAAH inhibitors
Synergistic effects of morphine or DENK inhibitors with exogenous or endogenous cannabinoids.

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

This theoretical Ed value is compared with the experimental Ed value. If a statistically significant difference is observed, synergy is present.

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

Thermal noiceception induced in a rat TFT and mouse
HPT is synergistically reduced by the combination of D9-THC with morphine or DENK inhibitors, and a similar facilitation is observed in models of inflammatory pain and neuropathic pain. In the mouse HPT, a single co-administration of subanalgentic doses of RB-101 (2.5 mg per kg; i.v. administration) or PL37 (0.4 mg per kg; i.p. administration) with D9-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. The synergistic effects are reversed by a MOR antagonist but not by a DOR or δ-opioid receptor antagonist. The replacement of D9-THC with AEA in combination with the FAAH inhibitor URB597 elicits a similar antiinflammatory potentiation, which is also reversed by MOR but not DOR antagonists.

Several explanations have been proposed to account for the synergistic facilitation of endogenous opioid and endogenous cannabinoid signalling; reciprocal enhancement of extracellular levels of endogenous opioids and endogenous cannabinoids (as demonstrated by microdialysis) or of PENK gene expression; creation — by MORs and CB1R — of membrane-bound heterodimers with increased pharmacological efficiency, and amplification of transduction pathways downstream of opioid receptors and cannabinoid receptors when both receptors are colocalized on the same cell, resulting in greater antiinflammatory responses. 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.

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. 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 and increases by 43% the levels of released Met-enkephalin measured by microdialysis. This contributes to the spinal control of pain by afferent neurons from the PAG. Moreover, the activation of opioid receptors by the protected enkephalins enhances receptor trafficking and increases the amounts of active opioid receptors at the cell surface. By elevating enkephalin

© 2012 Macmillan Publishers Limited. All rights reserved
levels, DENK inhibitors may facilitate MOR–DOR heterodimerization\(^{46}\), hence inducing a greater pharmacological response than individual stimulation of each opioid receptor\(^{46}\). 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\(^{23}\). 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\(^{46}\) — enhances the PL37-induced alleviation of thermal hyperalgesia\(^{32}\). 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 co-administration of an anti-enkephalin antibody with either PL37 or A-317491 completely blocks their anti-hyperalgesic effects, proving that the action of PL37 selectively involves endogenous enkephalins\(^{32}\). 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 periphery and the subsequent reduction of noxious inputs. Consistently, NO from various biochemical donors reduces noxious transmission and potentiates the analgesic properties of morphine in neuropathic pain symptoms caused by cancer in humans. 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. Reduction of these processes by endogenous opioids decreases the pain threshold and thereby the doses of PL37 necessary to elicit analgesia.

Opioid and CCK systems are counteracting, which explains the antagonism between enkephalins and CCK8 — the amino terminal fragment of CCK. This was unambiguously established using DENK inhibitors. Selective activation of the CCK2 receptor reduces the analgesic effects of RB-101, whereas CCK2 receptor antagonists strongly potentiate them in models of acute and chronic pain. 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. 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.

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.

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, 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. 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. Several recent studies indicate that FAAH inhibitors may preferably find their clinical indication as anti-inflammatory agents for reducing both oedema and nociception and/or as anxiolytic drugs. 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. 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.

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.

5. This is an interesting clinical study showing that the activation of the endogenous pain inhibition system in patients with fibromyalgia.
14. This seminal study introduced the idea that ‘physiological’ and ‘analogical’ can be elicited by increasing extracellular levels of enkephalins via inhibition of their inactivating enzymes.
20. This study reported the first knockout of the PENK gene, and demonstrated the importance of enkephalins in crucial physiological functions such as pain control, reward and adaptation. See also reference 32.
22. This study demonstrates that the antinociceptive effects of DENK inhibitors at the supraspinal and peripheral levels are absent in PENK-knockout mice, thus underscoring an essential specificity of these inhibitors for the in vivo protection of endogenous enkephalin. See also reference 32.
25. This electrophysiological study demonstrated for the first time the presence of a low tonic release of enkephalins in some structures rich in opioid receptors. This release, which is unique to morphine—DENK inhibitors have no depressive respiratory effects, and also explains why the actions of DENK inhibitors are primarily related to the extracellular concentrations of enkephalins that activate opioid receptors.
27. This was the first in vivo demonstration, using a superfused rat spinal cord, of the relationship between a pain-induced increase in the concentration of endogenous DENK inhibitor-protected enkephalins and the magnitude of the antinociceptive effects. This study demonstrated that the inhibitors increase extracellular levels of enkephalins but do not modify their release.
32. This was a comparative analysis of the affinities of exogenous and endogenous cannabinoids for cannabinoid receptors.
34. This was the first molecular description of FAAH and of its mechanism of action.
37. This was the first molecular characterization of a FAAH isoform that is devoid of enzymatic activity and able to act as a shuttle transporter for the uptake and possible release of AEA. This is a major discovery that explains the extracellular actions of AEA.
39. This was the first in-depth review of the mechanisms and roles of endogenous cannabinoids.
This study reported the rational design of the first active FAAH inhibitor containing a carbamate group. This irreversible inhibitor is currently used as a standard in biochemical and pharmacological studies (see reference 54 as well). This study also described the detailed use of [H]AEA to study FAAH enzyme mechanisms. I will provide a detailed review of the role of FAAH in nociception and endocannabinoid levels in neuropathic rats. These were the first FAAH inhibitors that were effective in treating pain.

Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. This study reported the rational design of the first active FAAH inhibitor containing a carbamate group. This irreversible inhibitor is currently used as a standard in biochemical and pharmacological studies (see reference 54 as well). This study also described the detailed use of [H]AEA to study FAAH enzyme mechanisms. I will provide a detailed review of the role of FAAH in nociception and endocannabinoid levels in neuropathic rats. These were the first FAAH inhibitors that were effective in treating pain.


57. This paper describes the discovery of an orally administered FAAH inhibitor, which was tested in patients with knee osteoarthritis, but was found to lack efficacy.


62. This was the first demonstration of the crucial role of MORs in pain control and dependence mechanisms.


64. This study shows that knockout of the gene encoding MOR does not alter the emotional responses of mice subjected to stressful conditions, thus demonstrating the predominance of ORs in the control of these physiological functions.


76. This study shows that knockout of Cbr does not block the pharmacological action of AEA and S9-TLC (spontaneous activity) and suggests the presence of other unknown targets for endogenous cannabinoids; this may be a potential issue associated with the use of FAAH inhibitors.

NATURE REVIEWS | DRUG DISCOVERY
© 2012 Macmillan Publishers Limited. All rights reserved
The narcotic antagonist naloxone enhances clinical anti-inflammatory actions of palmitoylethanolamide. Extracellular biotransformation of M. a cholecystokinin-inactivating serine peptidase. The dual peptidase inhibitor acting at a short distance with micromolar receptor—terminals of dorsal root ganglion neurons via vanilloid receptor-


This study shows that aminopeptidase — and not NEP — is the main enzyme involved in bradykinin catabolism, thus reducing the risk of bradykinin-mediated adverse effects caused by DENK inhibitors.


This quantitative assessment of the pain—reduction in the spinal model with a dual inhibitor at the spinal level showed that repeated administration of the DENK inhibitor RB101 does not induce tolerance; furthermore, both morphine and DENK inhibitors are crucially dependent on the phasic release of enkephalin.


This study shows that extracellular levels of DENK inhibitors—produced (measured by microdialysis in the PAG matter) are parallel to antinociceptive responses.


This is a detailed presentation of the pharmacology results with oral UR597, the most studied FAH inhibitor in neuropathic pain models.


This was the initial demonstration of the relationship between endogenous cannabinoid pathways and antinociception that triggered interest in FAH as a therapeutic target.


This study reported the first cloning and sequencing of NEP.


This study, along with references 52 and 53, provides a description of the first compounds that were able to differentiate the tic sites of NEP and APN with a similar nonlinear affinity.


This PET neuroimaging study shows that neuropathic pain increases an increase in circulating levels of enkephalins, which displace exogenous opioids bound to opioid receptors.
the recycling of active receptors (unlike morphine), enkephalins to opioid receptors does not alter receptor internalization in dorsal horn neurons by

This study demonstrates that — unlike morphine — enkephalins induce a recycling of active MORs at the cell surface, thus accounting partly for the lack of DENK inhibitor

Dauge, V., Kalivas, P. W., Duffy, T. & Roques, B. P. Effect of inhibiting enkephalin catabolism in the VTA on motor activity and extracellular dopamine. 


One explanation for the lack of dependence elicited by the opioid agonist is that the reward system is more strongly stimulated by morphine than by enkephalins protected by DENK inhibitors.


This study demonstrates a synergistic analgesic effect of the combination of very low (infra-active) doses of the DENK inhibitors in mice. This opens up the possibility of using low-dose DENK inhibitor–morphine combinations to reduce the unwanted effects associated with morphine.


This paper shows that, unlike enkephalin, morphine does not induce MOR endocytosis — a process that may be partly a cause of tolerance to morphine.


This is a review on the cross-tolerance of cannabinoids and cholecystokininergic systems, and their roles in pain and depression.


This review highlights the painful situations in which the response to endogenous cannabinoids protected by FAAH inhibitors is due to the subsequent release of enkephalins.


This important study unequivocally demonstrates that immune cells secrete endogenous opioids that accumulate in injured nerves in animal models of neuropathic pain; this supports the mechanism of peripheral neuropathic pain alleviation by DENV inhibitors.

This study shows that there is a continuous availability of endogenous opioids in inflamed tissues, which increases the recycling of MOR and preserves its signaling in sensory neurons, thereby counteracting the development of peripheral opioid tolerance.

This is the most complete and convincing study on inflammatory pain reduction by endorphins that are released from immune cells and act on peripheral opioid receptors.

This is the first demonstration that DENV inhibitor-induced neuregulation of endogenous opioids increases resistance to fear, suggesting a possible use for DENV inhibitors in treating anxiety disorders.

This is the first study providing evidence of the immune system: potential for the treatment of inflammatory diseases?

This study provides an early demonstration — using ketorolac, an inhibitor of COX2 involved in mood regulation via activation of the dopaminergic mesocorticolimbic pathways, whereas MORs govern fear, anxiety and drug dependence.

This study unequivocally demonstrates that immune cells secrete endogenous opioids that accumulate in injured nerves in animal models of neuropathic pain; this supports the mechanism of peripheral neuropathic pain alleviation by DENV inhibitors.

This is the most complete and convincing study on inflammatory pain reduction by endorphins that are released from immune cells and act on peripheral opioid receptors.

This study provides evidence that MOR in the effects induced by endogenous enkephalins protected by systemic administration of an inhibitor of enkephalin-degrading enzymes, thereby counteracting the development of peripheral opioid tolerance.

This study shows that there is a continuous availability of endogenous opioids in inflamed tissues, which increases the recycling of MOR and preserves its signaling in sensory neurons, thereby counteracting the development of peripheral opioid tolerance.

This is the most complete and convincing study on inflammatory pain reduction by endorphins that are released from immune cells and act on peripheral opioid receptors.