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THEMED ISSUE REVIEW

The inhibition of enkephalin catabolism by dual enkephalinase inhibitor: A novel possible therapeutic approach for opioid use disorders

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Despite the increasing impact of opioid use disorders on society, there is a disturbing lack of effective medications for their clinical management. An interesting innovative strategy to treat these disorders consists in the protection of endogenous opioid peptides to activate opioid receptors, avoiding the classical opioid-like side effects. Dual enkephalinase inhibitors (DENKIs) physiologically activate the endogenous opioid system by inhibiting the enzymes responsible for the breakdown of enkephalins, protecting endogenous enkephalins and increasing their half-lives and physiological actions. The activation of opioid receptors by the increased enkephalin levels, and their well-demonstrated safety, suggests that DENKIs could represent a novel analgesic therapy and a possible effective treatment for acute opioid withdrawal, as well as a promising alternative to opioid substitution therapy minimizing side effects. This new pharmacological class of compounds could bring effective and safe medications avoiding the major limitations of exogenous opioids, representing a novel approach to overcome the problem of opioid use disorders.

KEYWORDS
aminopeptidase N, dual enkephalinase inhibitor, neprilysin, opioid, opioid use disorders, δ-opioid receptor, μ-opioid receptor

1 | INTRODUCTION

Opioids have been used for centuries in the treatment of pain, although their misuse presents serious risks, including overdose and opioid use disorders (OUDs) (Benyamin et al., 2008). The diagnosis of OUD is based on the Diagnostic and Statistical Manuel of Mental Disorders (ed. 5; DSM-5) including a persistent desire to obtain and take opioids despite negative health, social and/or professional consequences (John et al., 2018). In the United States, opioid prescriptions increased dramatically from the 1990s, when lobbyists and companies succeeded in broadening the range of conditions the drugs could be used for, from specific conditions (pain due to surgery and late-stage terminal cancer) to more general conditions with poorer analgesic benefit (the potential adverse effects fail to outweigh the benefits), such as lower back pain, gastrointestinal pain, irritable bowel syndrome pain or minor odontology interventions (United Nations Office on Drugs and Crime, 2017). In 2015, Americans consumed about 50,000 prescribed doses of opioid painkillers per million people each day, almost doubling those handed out in Canada, and costing the nation half a trillion dollars (United Nations, 2020). This situation

Abbreviations: APN, aminopeptidase N; BBB, blood–brain barrier; DENKI, dual enkephalinase inhibitor; NEP, neprilysin/neutral endopeptidase; OUDs, opioid use disorders.

Beltran Alvarez-Perez and Hervé Poras participated equally to the manuscript.

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led to declaration that the opioid crisis is a national public health emergency in October 2017 (United Nations Office on Drugs and Crime, 2017; Wilson-Poe & Morón, 2018). Even though the Food and Drug Administration (FDA) released guidelines to steer drug companies towards opioid painkillers that are harder to abuse, the opioid crisis still remains a slow-motion emergency unfolding in real time, with no easy solution (U.S. Department of Health and Human Services, 2020).

In Europe, this opioid crisis is not of the size and nature seen in the United States (Seyler et al., 2021), although there are marked differences between countries in trends of opioid prescribing and of proxies for opioid-related harms (Häuser et al., 2021). This crisis has moved to Europe with a significant increase starting from 2015, involving mainly northern and eastern countries, and the Mediterranean Area (di Gaudio et al., 2021). Specifically, the levels of opioid consumption and their increase differed between countries, but there was a parallel increase in opioid prescriptions and some proxies of opioid-related harms in France, Finland and the Netherlands between 2004 and 2016 (Häuser et al., 2021). Recent studies in the United Kingdom have shown an increase in opioid use and attributed deaths, particularly in areas with higher deprivation (Alenezi et al., 2021). Although opioid overdose deaths increased between 2016 and 2018 in the United Kingdom, opioid prescriptions remain constant (Häuser et al., 2021).

In 2018, 57.8 million people globally were estimated to have used opioids in the previous year, including those who had used illegal opioids (30.4 million) and those who had misused pharmaceutical opioids (Editorial, 2017; United Nations, 2020). From the over 10 million people currently misusing prescription opioids worldwide, it was estimated that 2 million had an opioid use disorder (OUD; Editorial, 2018). In 2017, the European Monitoring Centre for Drugs and Drug Addiction estimated that the European Union had about 1.3 million high-risk opioid users and that about 81% of the fatal drug overdoses in Europe involved opioids (Ayoo et al., 2020). In addition, 67,367 drug overdose deaths occurred in the United States in 2018 and opioids were involved in 69.5% of these (U.S. Department of Health and Human Services, 2018). The two main drugs responsible for the opioid overdose crisis in the United States were **heroin** and **fentanyl**. Indeed, 808,000 people used heroin in 2018 and 15,349 deaths are attributed to overdosing on heroin that year (U.S. Department of Health and Human Services, 2018). Fentanyl is a synthetic opioid medication used for severe pain management, which is 100 times more potent than morphine. The rate of drug overdose deaths involving synthetic opioids other than **methadone**, including drugs such as fentanyl and fentanyl analogues, increased from 0.3 per 100,000 in 1999 to 9.0 in 2017 (Hedegaard et al., 2017). Fentanyl and pharmacologically similar synthetic opioids are illicitly manufactured and smuggled into the United States (United States Drug Enforcement Administration, 2018) contributing to the rapid increase in opioid overdose deaths in recent years with dramatic consequences particularly in United States (Hedegaard et al., 2017; Jones et al., 2018; Centers for Disease Control and Prevention, 2016).

2 | CURRENT LIMITATIONS IN THE PHARMACOLOGICAL TREATMENT OF OPIOID USE DISORDER (OUD)

According to the World Drug Report 2020, only one out of eight people who need drug use disorder treatment receives it (United Nations Office on Drugs and Crime, 2017). Medications currently available to treat OUD are methadone, **buprenorphine** and **naltrexone**, as well as **lofexidine** for acute withdrawal (National Institute on Aging, 2015), all treatment options having their caveats. Lofexidine, an α_2 -adrenocepter agonist, was approved in May 2018 by the FDA as the first non-opioid medication only restricted to short-term treatment of acute opioid withdrawal and has, unlike **clonidine**, fewer adverse effects, specifically absence of hypotension, anergy, weakness and tiredness (Kuszmaul et al., 2020). Methadone, a full **μ opioid agonist**, is the gold standard for OUD, but it is tied to misuse and deadly overdoses (Rudd et al., 2010). Methadone is thus only administered in a treatment facility and patients have to attend the clinic to get their treatment (National Institute on Aging, 2015). Buprenorphine is a partial μ agonist, which has a ceiling effect and it is given as a take-home treatment (Bell & Strang, 2020; National Institute on Aging, 2015). This home access also means it is easier to misuse. Thus, it was reported in 2010 that over 190 million dosage units of buprenorphine were distributed to pharmacies, which is over four times higher than the almost 40 million dosage units distributed just 4 years prior in 2006. Interestingly, only 1.1 million dosage units were distributed to licensed opioid treatment programmes and almost 800,000 individuals received prescriptions for buprenorphine from physicians with a waiver. In addition, an Australian study reported that the specific source of diverted buprenorphine was obtained from friends in about 81% of the cases and in the remaining 19% from acquaintances and dealers (Lofwall & Walsh, 2014). Naltrexone is a μ antagonist blocking the effects of opioid agonists and only needs to be injected once a month in extended release preparations, but it requires full detoxification before it can be used (Bell & Strang, 2020). Methadone and buprenorphine produce 'drug-liking' responses, which contributes to maintaining treatment compliance, although patients who miss doses experience opioid withdrawal (Kosten & George, 2002). In contrast, naltrexone produces no positive opioid effects and this may contribute to its erratic compliance, early dropout and increased risk of fatal opioid overdose when stopping treatment (Degenhardt et al., 2015).

The main concerns with all these treatments are the difficulties in starting and maintain the medications together with the high prevalence of craving and relapse to the illicit consumption of opioids (Strang et al., 2020). Actually, despite the positive impact of these treatments, many OUD patients continue to suffer from craving with negative affect and dysphoria (Kakko et al., 2019). Indeed, all participants under methadone treatment for 1 year had a relapse rate of 76.6%, with no significant gender differences (Moradinazar et al., 2020). Similarly, the proportion of opioid-relapse events was 57% of participants in buprenorphine maintenance treatment during only 6 months (Lee et al., 2018). Therefore, new alternative therapeutic approaches are required to identify compounds targeting novel

mechanisms of action in order to provide more safer and effective medications (Mongi-Bragato et al., 2018). Despite this urgency, there are few new chemical entities with novel mechanisms of action (Spahn et al., 2017) currently under development (Emery et al., 2016) and there is a clear unmet need to bring novel therapeutic strategies to help improving OUD treatment.

In particular, it is necessary to develop new alternative treatments to avoid and treat opioid withdrawal syndrome as well as craving and relapse. A novel and more physiological approach to address these unmet needs for the treatment of OUD may consist of enhancing the effects of the endogenous opioid system by protecting the natural opioid peptides, enkephalins, from their catabolism. Indeed, this approach has demonstrated high effectiveness avoiding the side effects of opioids and their potential abuse liability as a possible novel analgesic treatment (Roques et al., 2012). In this review, we summarize the current evidence that suggest the possibilities of inhibiting enkephalin catabolism for the treatment of OUD. Multiple animal studies and previous clinical trials targeting other indications have provided scientific data that supports the safety and possible efficacy of this approach, which supports the development of clinical trials to finally demonstrate the efficacy of the inhibition of enkephalin catabolism in OUD treatment.

3 | THE INHIBITION OF ENKEPHALIN CATABOLISM: A NOVEL AND UNEXPLOITED THERAPEUTIC TARGET

Enkephalins (**Met-enkephalin** and **Leu-enkephalin**) are pentapeptides produced in the CNS and peripheral tissues, and are the most abundant endogenous opioids acting on both μ- and **δ**-opioid receptors, providing the body with its own pain management system (Poras et al., 2015). In addition to their analgesic properties, enkephalins are highly implicated in motivational behaviours and stress responses, playing a key role in regulating depression-like behaviours and behavioural responses to stress (Nam et al., 2019). Regarding their analgesic properties, enkephalins bind, with high affinities, to opioid receptors to induce transient analgesia (Maldonado et al., 2018). Although their efficacy is the same as exogenous opioids, enkephalins are degraded within minutes of their release, which makes their development as therapeutic applications impossible. Once released, enkephalins are rapidly degraded by two membrane-bound exo-metalloproteases, termed **neprilysin (neutral endopeptidase; NEP)** and **aminopeptidase N (APN)**. The physiological inactivation of these peptides allows the maintenance of a balance to maintain an appropriate endogenous opioid tone, which is disrupted in pathophysiological conditions (Corder et al., 2018; Poras et al., 2015; Raffa et al., 2018; Roques et al., 2012). The inhibition of both NEP and APN using selective dual inhibitors increases the half-life of the released pro-analgesic enkephalin peptides causing a targeted physiological action on the endogenous opioid system. Interestingly, it has recently been demonstrated in humans increased levels of enkephalins induce complete analgesia, devoid of opioid-like side effects in patients suffering from congenital insensitivity to pain, thereby supporting the hypothesis that leveraging the physiological effects of endogenous enkephalins will provide effective and safe activation of the endogenous opioid system (Minett et al., 2015).

The ultimate candidates for such therapeutic purposes are the dual enkephalinase inhibitors (DENKIs), acting simultaneously on both NEP and APN. By inhibiting the extracellular enzymes responsible for the breakdown of enkephalins, DENKIs protect endogenous enkephalins and increase their half-lives and their physiological actions (Meynadier et al., 1988) (Figure 1). Increased levels of enkephalins (Schreiter et al., 2012) with preferential μ-opioid receptor occupancy (Ruiz-Gayo et al., 1992) have been demonstrated by radioimmunoassays after DENKI administration. Interestingly, μ receptors that are directly involved in opioid rewarding effects and abuse liability (Matthes et al., 1996) are also directly involved in the analgesic effects induced by endogenous enkephalins protected from the degradation by DENKIs. Multiple studies have shown that selective μ antagonists (**β-funaltrexamine /β-FNA** and **DAMGO**) (Le Guen et al., 1999, 2003; Noble, Soleilhac, et al., 1992), but not selective **δ receptor** (Le Guen et al., 2003; Noble, Soleilhac, et al., 1992; Noble, Turcaud, et al., 1992), nor **κ receptor** (Le Guen et al., 2003) antagonists blocked the analgesic effects of **RB101**, the first systemically active DENKI. Similarly, the analgesic effects of the systemically active DENKI **PL37** were reversed by the selective μ antagonist cyprodime, but not by δ or κ selective antagonists (Menendez et al., 2008). However, the locomotor, antidepressant and anxiolytic effects, as well as the lack of drug abuse liability, indicate the participation of δ receptors in the

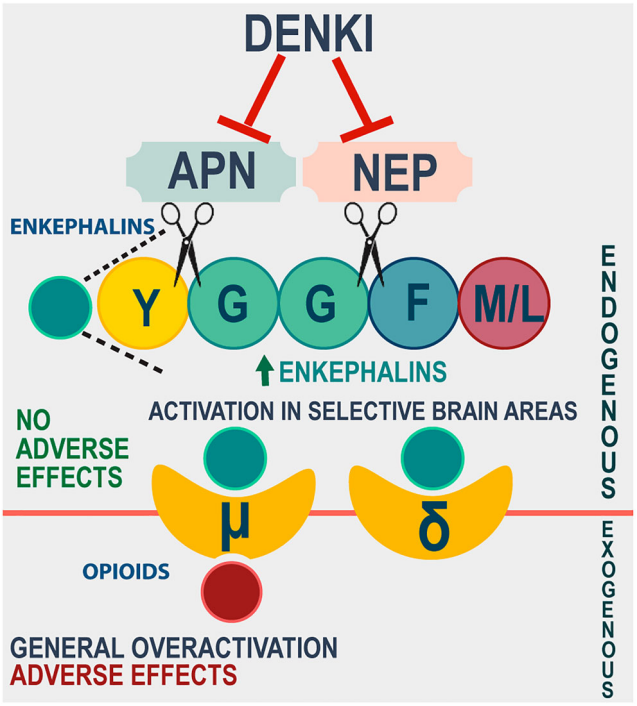

FIGURE 1 Mechanism of action of dual enkephalinase inhibitors (DENKIs). The inhibition of the extracellular enzymes aminopeptidase N (APN) and neprilysin (NEP) responsible for the breakdown of enkephalins by the DENKIs contributes to the increase in the half-lives and the physiological actions of endogenous enkephalins

action of DENKIs (Jutkiewicz et al., 2006; Maldonado et al., 1990; Roques, 2018). These well demonstrated interactions of endogenous enkephalins with μ receptors support the interest of targeting acute opioid withdrawal with DENKI treatment, as well as a possible substitutive treatment in OUD using these compounds. This new pharmacological class of compounds could bring to patients efficient and safe medications without the side effects of exogenous opioids and provide a possible novel therapeutic strategy for OUD.

Despite the increasing impact of OUD on society, there is a disturbing lack of effective medications for clinical management of this disorder. DENKIs selectively inhibit NEP and APN rapidly and with high selectivity (Rose et al., 2002), and could represent a novel potential medication for acute opioid withdrawal treatment. Having a new mechanism of action based on the enhancement of the endogenous levels of enkephalins and that this mechanism may also have potential as a novel maintenance treatment of OUD. Indeed, previous studies have shown that the activation of μ and δ opioid receptors minimizes opioid craving and relapse (Befort et al., 2008; Le Merrer et al., 2009). This suggests that the activation of these opioid receptors by "protected" endogenous enkephalins could represent an interesting approach for opioid maintenance therapy avoiding the classical side effects, such as respiratory depression, tolerance or abuse liability. Further research is needed to confirm this potential use of DENKIs in avoiding opioid craving and relapse.

4 | ANTINOCICEPTIVE ACTIVITY OF DUAL ENKEPHALINASE INHIBITORS (DENKIS) IN ANIMAL PAIN MODELS

Analgesia is the most well-characterized therapeutic effect derived from the pharmacological activation of the endogenous opioid system. Thus analgesic measurements have been mainly used to characterize the pharmacological profile of DENKIs in different experimental models in both animals and humans. The analgesic effects of systemically active DENKIs administered by intravenous or oral route can be, in some cases, of lower intensity than the effects of morphine. Indeed, the effectiveness of peptidases inhibitors towards different pronociceptive stimuli is directly dependent on the concentrations of enkephalins released in the extracellular space. This correlates with the efficacy of peptidase-protected endogenous opioid peptides and with the subsequent stimulation of opioid receptors across the areas involved in pain control (Basbaum & Fields, 1984; Besson & Chaouch, 1987). Furthermore, as enkephalins harbour high affinities for μ and δ receptors, their protection by DENKI could add the specific antinociceptive responses mediated by δ receptors to that associated with μ receptors activation, leading to a modified response different to the one obtained by μ receptors activation alone (Gomes et al., 2000).

DENKIs have been extensively evaluated and compared in several models of pain in rodents (Roques et al., 2012). In the hotplate test (O'Callaghan & Holzman, 1975), a model of centrally controlled and integrated acute pain mostly used in mice (Carter, 1991; Knoll et al., 1955), potent time- and dose-dependent antinociceptive effects were observed. Intravenous administration of several systemically active DENKIs (RB101, **RB120** and PL37) showed antinociceptive effects in the hotplate test after 10 min and were statistically significant for 30 min (Poras et al., 2014). The dose–response curves after intravenous administration showed the dose that produces 50% maximum effect (ED_{50}) at 3 $mg{\cdot}kg^{-1}$ for RB120 and 9 $mg{\cdot}kg^{-1}$ for RB101, whereas the ED_{50} for PL37 was from 4.5 to 16 $mg{\cdot}kg^{-1}$, depending on the composition of the solvent (Fournié-Zaluski et al., 1992; Noble et al., 1997; Poras et al., 2014). The dose–response curves showed ED_{50} of 410 $mg{\cdot}kg^{-1}$ for RB120 and 133 $mg{\cdot}kg^{-1}$ for PL37 by oral route (Noble et al., 1997; Poras et al., 2014). Their duration of action remained relatively short after oral administration with a significant antinociceptive effect having a duration of less than 1 h.

The tail flick is a predominant spinal reflex (Bonnycastle et al., 1953; Irwin et al., 1951; Sinclair et al., 1988), which can be modulated by the activity of supraspinal structures (Mitchell & Hellon, 1977). The application of thermal radiation to the tail of a rat or a mouse provokes the withdrawal of the tail by a vigorous movement (D'Amour & Smith, 1941; Smith et al., 1943). There is a consensus that this test is very accurate in revealing the activity of opioid analgesics and is adequate for predicting their analgesic effects in humans (Archer & Harris, 1965; Grumbach, 1966). RB101, RB120 and PL37 produced dose-dependent antinociceptive responses in the tail-flick test after intravenous administration with the maximal effects 10 min after administration and were significant during 40 min. The ED_{50}s were 80, 50 and 20 $mg{\cdot}kg^{-1}$ respectively for these three DENKIs (Fournié-Zaluski et al., 1992; Noble et al., 1997; Poras et al., 2014). Novel DENKIs, such as **RB3007**, were also evaluated with this test after intravenous and intraperitoneal administration. At 50 $mg{\cdot}kg^{-1}$, 25–30% analgesia was obtained with a maximal effect at 60 min (Chen et al., 2001). In all these tail-flick experiments, the antinociceptive responses were completely reverse by **naloxone** and partially by **naltrindole**, a δ receptor, demonstrating the involvement of μ and δ receptors.

PL37 and **PL265** have also shown antinociceptive responses in peripheral pain models, such as the formalin test, inflammatory pain models, such as λ-carrageenan and complete Freund's adjuvant, neuropathic pain, such as the chronic constrictive injury in rats or the partial nerve ligation in mice (Bonnard et al., 2015; Poras et al., 2014), and bone cancer-induced pain (Gonzalez-Rodriguez et al., 2017; Menendez et al., 2008), as well as in a corneal pain animal model (Reaux-Le Goazigo et al., 2019).

Altogether, these results suggest that DENKIs constitute a valid alternative to opioids in terms of their antinociceptive effects for the treatment of several chronic pain conditions.

5 | DENKIs ARE DEVOID OF THE MAJOR DRAWBACKS OF MORPHINE AND ITS SYNTHETIC DERIVATIVES

The serious shortcomings of morphine and opioid synthetic derivatives limit their use in chronic pain management. These unwanted

effects are caused by the ubiquitous interaction of these exogenous compounds with all opioid receptors in the body, whether or not involved in pain modulation. The main side effects of opioids are tolerance, physical dependence, abuse liability, respiratory depression, nausea and constipation (Baldini et al., 2012). The mechanism of action of DENKIs being based on the protection of enkephalins released by nociceptive stimuli is unlikely to trigger the same side effects as exogenous opioids. This has actually been demonstrated experimentally by comparing at the same time and on the same experimental models the occurrence of these unwanted effects by morphine and DENKI-protected enkephalins.

5.1 | Absence of antinociceptive tolerance

Tolerance, defined as the need for escalating doses to maintain the same pharmacological effect, is a well-known side effect of morphine and exogenous opioids. Analgesic tolerance makes difficult to many patients to manage an appropriate control of their chronic pain with opioids (Attal et al., 2002; Tassain et al., 2003). We assessed the degree of tolerance to DENKI using the hotplate test in mice (Figure 2a,b; Noble et al., 1993). Dose–response curves were established for morphine and RB101 to determine their respective ED_{50}. Then, mice received chronic treatment for 4 days of either morphine (3 $mg \cdot kg^{-1}$, i.p., twice daily) or RB101 (80 $mg \cdot kg^{-1}$, i.p., twice daily), at equipotent doses regarding their antinociceptive effects, or saline. One day after the last administration, new dose–effect curves of morphine and RB101 were established for each group. In mice treated chronically with saline or RB101, the ED_{50} remained unchanged, whereas the ED_{50} was significantly increased in mice treated chronically with morphine. These results confirm that, as expected, morphine induced tolerance, but RB101 was devoid of this drawback. Interestingly, in mice treated chronically with RB101, morphine produced the same analgesia as in mice treated with morphine alone, but without the generation of tolerance, indicating also that there was no cross-tolerance between morphine and RB101. Similar results were obtained with PL265 (Bonnard et al., 2016), demonstrating that DENKIs do not develop antinociceptive tolerance after repeated administration and could be a substitute to opioids in patients who have become tolerant.

5.2 | Absence of physical dependence

Physical dependence was investigated in rats by chronic intravenous infusion of morphine (0.17 mg per 120 $\mu l \cdot h^{-1}$) or the systemically active RB101 (1.2 mg per 120 $\mu l \cdot h^{-1}$) at equipotent doses in respect to their antinociceptive effects for 5 days (Noble et al., 1994), as well as with intracerebroventricular infusion of the selective μ agonist DAMGO (0.18 $\mu g \cdot \mu l^{-1} \cdot h^{-1}$), the selective δ agonist **DSTBULET** (66.5 $\mu g \cdot \mu l^{-1} \cdot h^{-1}$), and the DENKIs RB38B (40 $\mu g \cdot \mu l^{-1} \cdot h^{-1}$) and RB38A (40 $\mu g \cdot \mu l^{-1} \cdot h^{-1}$) (Maldonado et al., 1990). The withdrawal syndrome was precipitated in both cases by naloxone administration (5 $mg \cdot kg^{-1}$, s.c.). The major signs of opioid withdrawal that are widely used to evaluate physical dependence (weight loss, diarrhoea, writhing, wet dog shakes, teeth chattering and runny nose) were observed in rats chronically perfused by intravenous route with morphine but not in rats perfused with RB101. Only tremor appeared significant with RB101-treated rats as compared with control groups; also, ptosis was higher when comparing the RB101-administered animals with saline-treated mice but not vehicle-treated mice. This ptosis is reduced when compared with morphine-treated animals (Figure 3b). In agreement, these signs of opioid withdrawal were avoided in rats when

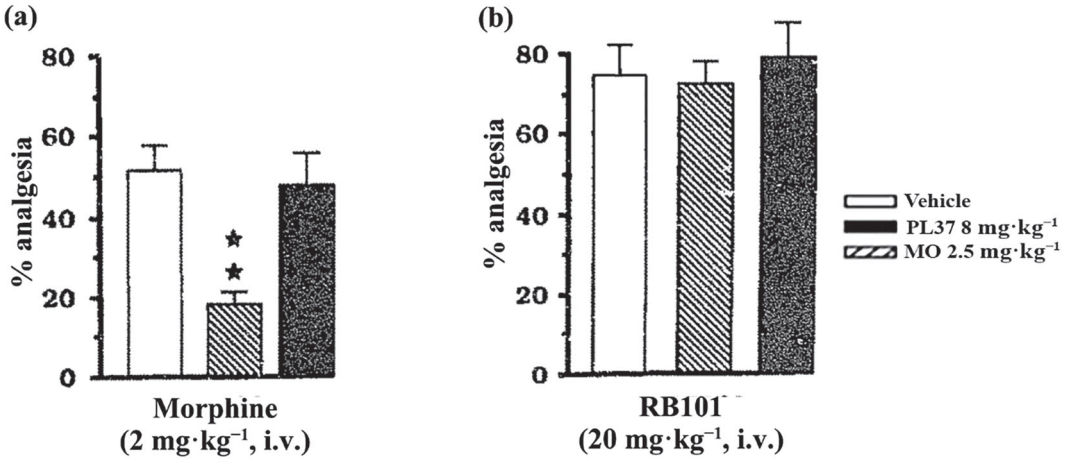

FIGURE 2 Lack of antinociceptive tolerance and cross-tolerance with morphine (MO) in mice treated with dual enkephalinase inhibitors (DENKIs) adapted from Figure 2 in Noble et al., 1992. Antinociception was evaluated in the hotplate test (jump response). (a) Antinociceptive responses 10 min after intravenous administration of 2 $mg \cdot kg^{-1}$ of MO to mice chronically pretreated with saline (white column), 80 $mg \cdot kg^{-1}$ of RB101 (black column) or 3 $mg \cdot kg^{-1}$ of MO (hatched column) intraperitoneally, twice daily for 8 days. (b) Antinociceptive responses 10 min after intravenous administration of 20 $mg \cdot kg^{-1}$ of RB101 to mice chronically pretreated with saline (white column), 80 $mg \cdot kg^{-1}$ of RB101 (black column) or 3 $mg \cdot kg^{-1}$ of MO (hatched column) intraperitoneally, twice daily for 8 days. $^{\star}P < 0.05$ versus saline (Newman–Keuls test))

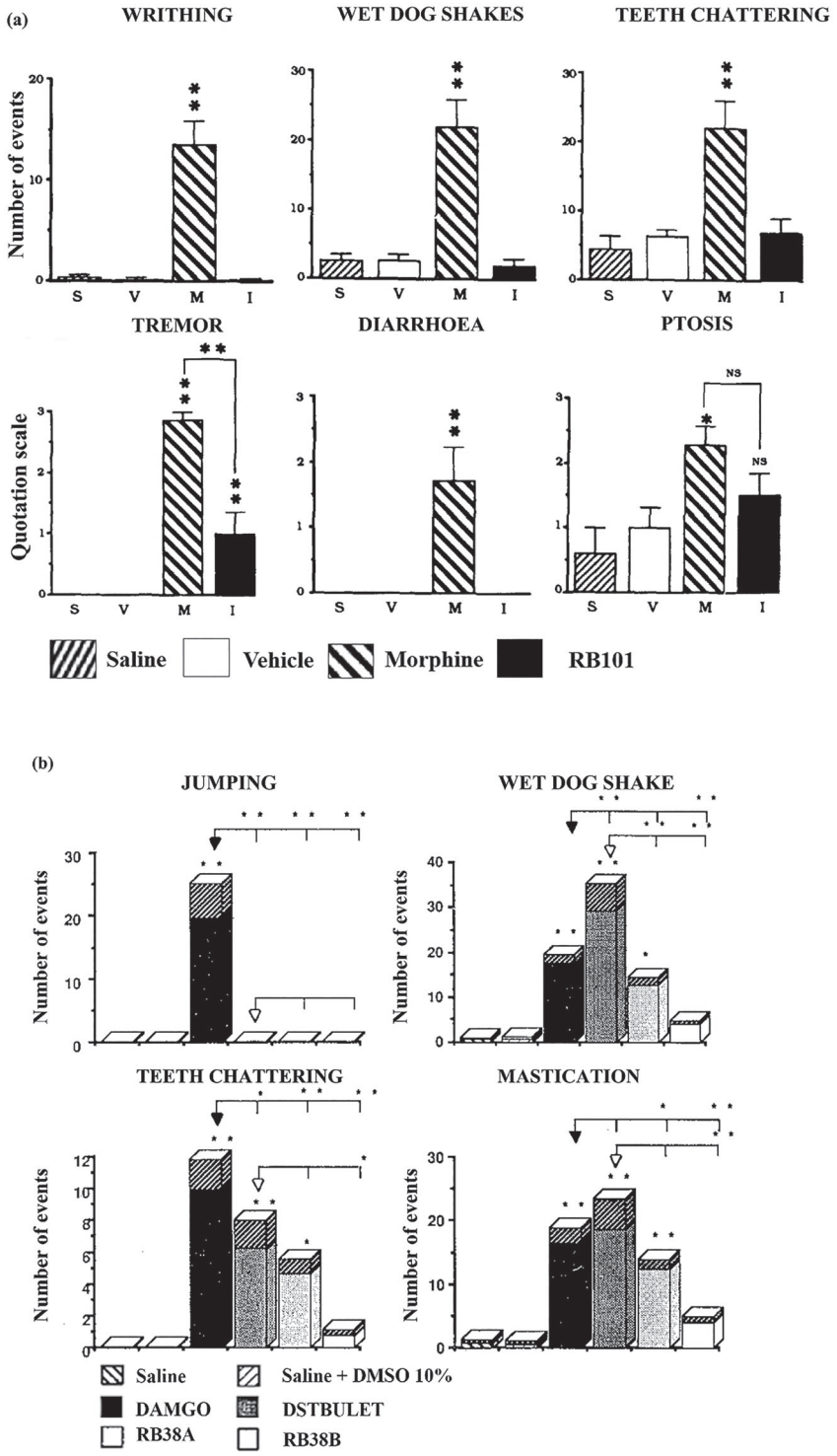

FIGURE 3 Lack of physical dependence after chronic administration of dual enkephalinase inhibitors (DENKIs), (a) adapted from Figure 1 in Noble et al., 1994 and (b) from Figure 3 from Maldonado et al., 1990. a) Effects of naloxone administration (5 mg·kg^{-1}, s.c.) on behaviour of rats chronically intravenously perfused for 5 days with morphine (M) (0.17 mg per 120 µl·h^{-1}), RB101 (I) (1.20 mg per 120 µl·h^{-1}) or control solutions (S, saline; V, vehicle) (120 µl·h^{-1}). The results are expressed as means + SEM of the number of events counted during the 30-min period of observation immediately after naloxone injection (writhing, wet dog shakes and teeth chattering), according to the quotation scale established: One point was given for the presence of each sign over 10-min periods during the 30 min of observation (maximum score: 3) (tremor, diarrhoea and ptosis). *P < 0.05 and **P < 0.01 versus control (Newman–Keuls test). (b) Effects of naloxone administration (5 mg·kg^{-1}, s.c.) on the behaviour of rats chronically intracerebroventricularly perfused for 5 days with DAMGO (0.18 µg·µl^{-1}·h^{-1}), DSTBULET (66.5 µg·µl^{-1}·h^{-1}), RB38A (40 µg·µl^{-1}·h^{-1}), RB38B (40 µg·µl^{-1}·h^{-1}) and control solutions. Values are means ± SEM. *P < 0.05 versus control groups, when they are placed on the columns, or versus DSTBULET group when they are placed on the opened arrow, or versus DAMGO group when they are placed on the closed arrow (Newman–Keuls test)

intracerebroventricularly perfused with RB38B, with the exception of tremor, as mentioned before in RB101. Wet dog shakes were also observed in RB38A intracerebroventricular-administered rats, but there were not significant when compared with vehicle administered animals (Figure 3a). Based on these results, jumping, considered one of the main signs of the withdrawal syndrome, seems to be associated with physical dependence to µ receptors, because δ agonists and inhibitors of enkephalin catabolism do not induce this action (Maldonado et al., 1990).

These results demonstrate that, unlike morphine, chronic administration of DENKIs does not cause the development of physical dependence.

5.3 | Absence of abuse liability

Morphine and µ agonists can act as discriminative cues in rats (Joharchi et al., 1993). Discrimination of different classes of opioid

drugs shows a close correlation to differences in subjective experiences of these drugs in humans and these responses are evaluated in abuse liability studies. Rats were trained in a two-level choice task to select an appropriate lever depending on whether they have been administered with morphine or saline before the test session. The systemically active PL37 was tested for its ability to replace or generalize the trained drug cue expressed by responding on the appropriate lever. Unlike the positive effects of morphine and **hydrocodone**, PL37 intraperitoneal, at a dosing range of up to 32 mg·kg^{-1}, did not provoke discriminative properties in rats. These data suggest that analgesic doses of PL37 do not produce morphine-like subjective effects and should not have the abuse potential of μ receptor agonists.

The ability of morphine and other μ-opioid agonists to induce rewarding properties or to directly promote operant responses revealing their reinforcing effects is well established. The rewarding properties of DENKIs were investigated using the conditioned place preference test (Figure 4a,b; Noble et al., 1993). In this model, mice were treated with morphine (3 mg·kg^{-1}, i.p.), RB101 (80 mg·kg^{-1}, i.p.) or saline for developing place conditioning. After this conditioning phase, a shift towards the drug-associated compartment was observed only in mice treated with morphine and this effect was increased after administration of naloxone.

DENKIs' reinforcing properties were first evaluated in the self-administration test, using animals trained in a two-lever choice task to select an appropriate lever depending on whether they have been administered morphine or saline before the test session. Novel drugs can be tested in this paradigm for their ability to replace the trained drug cue expressed by responding on the appropriate lever. In this model, rats treated with morphine (3 mg·kg^{-1}) presented discriminative responses, but not rats treated with RB120 (10 mg·kg^{-1}) (Hutcheson et al., 2000). Operant intravenous self-administration in animals is the most frequently used and reliable method to assess drugs' reinforcing effects. Animals almost exclusively intravenous self-administer compounds abused by humans and the specific pattern of intake for each drug is quite comparable (Panlilio & Goldberg, 2007).

Altogether, these results demonstrate that DENKIs do not present any abuse potential at analgesic doses and demonstrate the major difference compared with the effects of administration of exogenous opioid agonists.

5.4 | Absence of respiratory depression

One of the major side effects of opioids is respiratory depression. Endogenous opioid peptides, such as enkephalins, are potent analgesics, but they depress ventilation due to an effect on the CNS (Denavit-Saubié & Foutz, 1997; Yeadon & Kitchen, 1989). To study the consequences of the increase of these endogenous opioids by the action of DENKIs in the respiratory control, ventilation was measured in cats and rodents in both awake and anaesthetized states (Boudinot et al., 2001). RB101 tested at antinociceptive doses (40–160 mg·kg^{-1}, i.p.) did not affect ventilation evaluated using a plethysmograph chamber by the barometric method, indicating that DENKIs are devoid of respiratory-depressant effects.

5.5 | Absence of constipation

Constipation is a major side effect of morphine and exogenous opioids. The effects of DENKIs on gastrointestinal transit have been evaluated in rodents. In a model of castor oil-induced diarrhoea in mice (Noble et al., 2008), RB101 showed antidiarrheal properties in wild-type animals, as previously shown with the NEP-specific enkephalinase inhibitor **acetorphan (racecadotril)** (Roge et al., 1993), but only a slight effect in knockout mice for the preproenkephalin gene (*Penk1*$^{-/-}$ mice). These results suggest a differential effect of exogenous opioids and DENKIs.

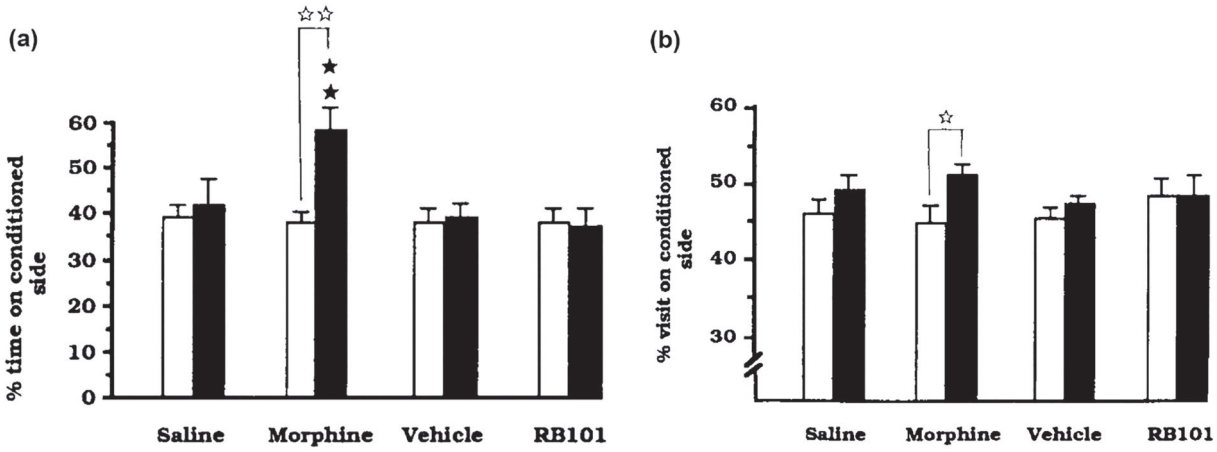

FIGURE 4 Absence of drug abuse liability in animals treated with dual enkephalinase inhibitors (DENKIs) adapted from figure 1 in Noble et al., 1993. (a) Percentage of time spent in the conditioned compartment (drug-paired compartment) and (b) percentage of time of visit to the conditioned compartment on Day 2 (preconditioning, white bars) and Day 11 (postconditioning, black bars). Mice were conditioned with morphine (3 mg·kg^{-1}, i.p.) or RB101 (80 mg·kg^{-1}, i.p.) using four place pairings. ★$P < 0.05$ compared with the control group on Day 11; ☆$P < 0.05$ compared with values on Day 2 (Noble et al., 1993)

6 | POTENTIAL INTEREST OF THE INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES IN OPIOID USE DISORDER (OUD)

The main concerns with current opioid maintenance treatments are the difficulties to start and maintain the medications together with the high prevalence of craving and relapse to illicit consumption of opioids (Strang et al., 2020). Indeed, despite the positive impact of these treatments, many OUD patients continue to suffer from craving with negative affect and dysphoria (Kakko et al., 2019). The most difficult aspect in the treatment of addiction is the protracted abstinence syndrome, one of the main factors contributing to relapse. Indeed, the first days after cessation of prolonged drug use leads to acute withdrawal syndrome, which consists of physiological changes (i.e. agitation, hyperalgesia, tachycardia, hypertension, diarrhoea, vomiting cardiovascular and thermoregulatory) and emotional subjective changes that may persist for months or even longer after the last opioid administration (Galinkin & Koh, 2014; Sigmon et al., 2012). Therefore, new alternative therapeutic approaches are required to identify compounds targeting novel mechanisms of action in order to provide more safe and effective medications (Mongi-Bragato et al., 2018).

Thus, a still uncovered challenge in OUD is to develop an effective treatment to minimize the short-term withdrawal syndrome and to obtain a substitution treatment that avoids protracted opioid abstinence.

The use of a more 'physiological' maintenance treatment by increasing the level of endogenous opioid peptides represents an interesting new approach for the treatment of acute opioid withdrawal, and a potential maintenance treatment for OUD that could prevent craving and relapse, as suggested in multiple previous studies. In contrast to exogenous opioid agonists or antagonists, chronic administration of mixed enkephalin-degrading enzyme inhibitors does not induce changes in the synthesis of the clearing peptidases and in the synthesis of its target peptide precursors, as well as in the release of the endogenous peptides (Roques, 1988). Accordingly, animal studies revealed that a transgene-mediating enkephalin expression in rats also efficiently attenuated opioid withdrawal (Hao et al., 2009). Interestingly, the brain extracellular levels of enkephalins were increased in rats chronically treated for 5 days with morphine (Fukunaga & Kishioka, 2000; Nieto et al., 2002), which underlies the potential interest of inhibiting their catabolism as a therapeutic alternative.

Withdrawal syndromes are expected to be avoided or reduced by means of peripheral administration of peptidase inhibitors, as observed in rodents (Dzoljic et al., 1986; Dzoljic et al., 1992; Haffmans & Dzoljic, 1987). Early studies have shown that the NEP inhibitors **phosphoramidon**, **thiorphan** and acetorphan and the mixed inhibitor **phelorphan** minimize the severity of the naloxone-precipitated morphine withdrawal syndrome in rats and mice (Dzoljic et al., 1986; Haffmans & Dzoljic, 1987; Livingston et al., 1988). The first DENKIs, **kelatorphan** and RB38A, were also evaluated on the somatic manifestations of naloxone-precipitated morphine withdrawal. These early DENKIs were unable to cross the blood–brain barrier (BBB) and were consequently evaluated after intracerebroventricular administration (Maldonado et al., 1989). Kelatorphan and RB38A showed a higher effectiveness in the attenuation of the behavioural and somatic manifestations of naloxone-precipitated morphine withdrawal than the selective NEP inhibitor thiorphan (Figure 5). The greater efficacy of the mixed inhibitors presumably is due to the resulting greater increase in enkephalins in certain brain regions, especially those enriched in μ receptors, such as the periaqueductal grey matter which also contains high levels of NEP (Waksman et al., 1986) and could be an important site of action for the manifestations of the behavioural symptoms of physical morphine dependence (Maldonado et al., 1995). Accordingly, local administration of kelatorphan or RB 38A into the periaqueductal grey matter produces a severe attenuation of the severity of the withdrawal syndrome in rats. This result indicates that during morphine

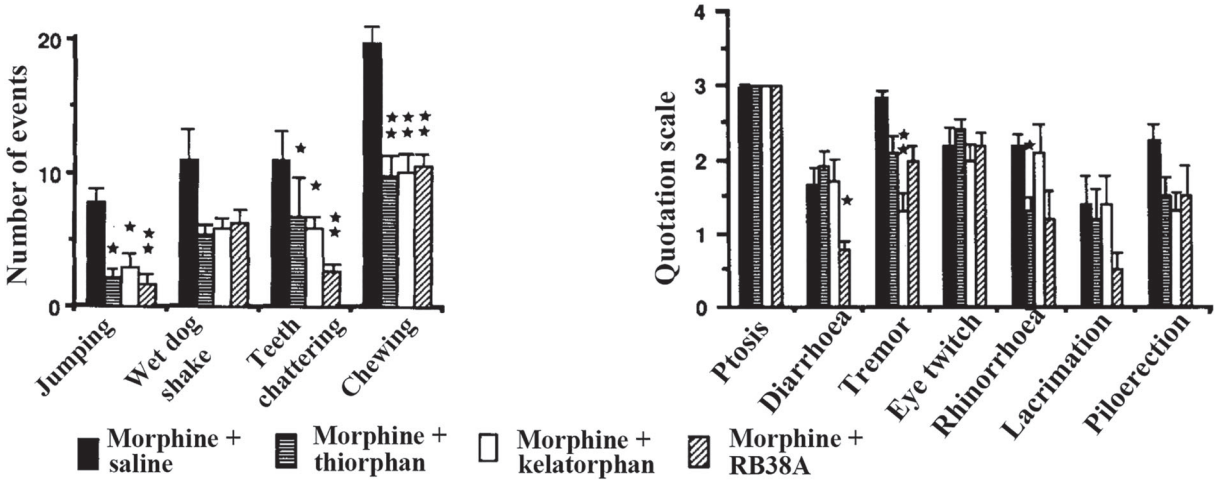

FIGURE 5 Acute administration of dual enkephalinase inhibitors (DENKIs) attenuates the behavioural manifestations of morphine withdrawal adapted from Figure 1 and 2 in Maldonado et al., 1989. Effect of saline, thiorphan (100 μg), kelatorphan (32 μg) or RB38A (12 μg), 30 min before naloxone (5 $mg \cdot kg^{-1}$) on the behavioural manifestations of naloxone-precipitated morphine withdrawal syndrome. Values are means ± SEM. ⋆ $P < 0.05$ versus morphine + saline group (Mann–Whitney *U*-test)

withdrawal syndrome, there is a tonic release of opioid peptides, presumably enkephalins, into this structure and that local inhibition of their degradation strongly decreases the severity of the withdrawal syndrome (Maldonado et al., 1995).

The inability to cross BBB of the first inhibitors precluded investigations of their possible effects using a clinically relevant route of administration. New series of compounds able to cross the BBB and to inhibit NEP and APN were developed. RB101 easily crossed the BBB following intravenous injection in mice but was poorly soluble in vehicles suitable for administration in human. The intravenous administration of this systemically active DENKI significantly and dose-dependently attenuated the behavioural manifestations of naloxone-precipitated morphine withdrawal syndrome. Interestingly, this beneficial effect of RB101 was potentiated by the co-administration with a cholecystokinin octapeptide antagonist (PD-134.308/CI-988) (Figure 6a; Maldonado et al., 1995). To evaluate the responses triggered by a spontaneous morphine abstinence at different times, an experimental model of spontaneous opioid abstinence and substitutive maintenance treatment in rats was developed (Figure 6b; Ruiz et al., 1996). This experimental model was used to evaluate the effects induced by RB101 and to compare the responses to those produced by compounds used to reduce opioid dependence in humans, such as clonidine and methadone. In this model, clonidine decreased the severity of spontaneous abstinence only after its acute administration and this effect was significant only during this initial observation session when compared with the saline group, in agreement with the acute moderate responses revealed in clinical studies with α_2-adrenoceptor agonists (Van der Laan & De Groot, 1988). The responses induced by RB101 in this model of spontaneous withdrawal were similar to those induced by methadone (Figure 6b). Indeed, RB101 decreased opioid abstinence in all the observation sessions and this effect was particularly strong in the sessions performed at the end of the substitutive administration, suggesting a long-term effectiveness of DENKIs in OUD substitutive treatment. Indeed, the responses induced by RB101 in these late sessions could be due to an effective interruption of the adaptive changes underlying the expression of opioid abstinence as a consequence of the potentiation of the endogenous opioid system induced by the peptidase inhibitor. Interestingly, the chronic maintenance with RB101 or methadone not only avoided the manifestation of the spontaneous withdrawal but

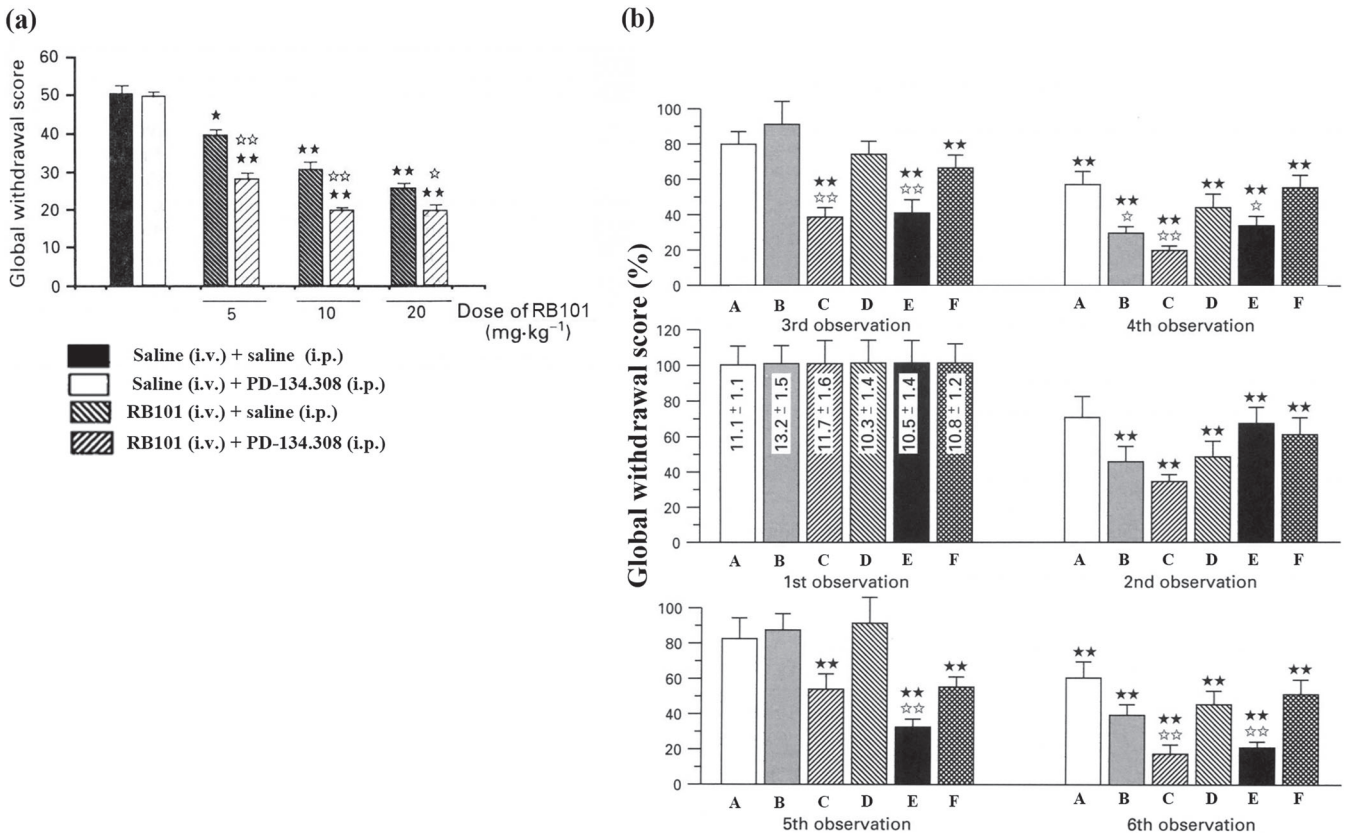

FIGURE 6 Acute administration of dual enkephalinase inhibitors (DENKIs) attenuates the behavioural manifestations of morphine withdrawal adapted (a) from Figure 5 in Maldonado et al., 1995 and (b) from Figure 6 in Ruiz et al., 1996. (a) PD-134.308 (3 mg·kg^{-1}), RB101 (5, 10 and 20 mg·kg^{-1}), PD-134.308 + RB101 and saline effect on the global withdrawal score after naloxone administration in morphine dependent rats. Values are means ± SEM. ★P < 0.05 versus saline + saline group; ☆P < 0.05 versus RB101 + saline group (Newman–Keuls test) (Maldonado et al., 1995). (b) Effects of clonidine (0.025 mg·kg^{-1}, B), methadone (2 mg·kg^{-1}, C), PD-134.308 (3 mg·kg^{-1}, D), RB101 (40 mg·kg^{-1}, E), RB101 + PD-134.308 (F) and saline (A) on the global withdrawal score of spontaneous morphine abstinence in rats. Values are means ± SEM. ★P < 0.05 versus value of the same group in the first session (Newman–Keuls test); ☆P < 0.05 versus value of saline in the same session (Dunnett's test)

also removed the previous state of opioid dependence, because no signs of withdrawal were observed when naloxone was injected 2 h after the last substitute injection. This effectiveness in decreasing withdrawal symptoms, not only after its acute administration but also under maintenance circumstances, points to the possible usefulness of RB 101 as substitutive treatment for the maintenance of patients suffering OUD. Moreover, preliminary recent results demonstrate that the intravenous administration of PL37 also attenuates naloxone-precipitated morphine withdrawal in mice. In this last study, increasing doses of morphine (from 10 to 40 $mg \cdot kg^{-1}$, s.c., twice daily) were administered for 5 days and the withdrawal syndrome was precipitated by a naloxone challenge (1 $mg \cdot kg^{-1}$, s.c.). A low dose of PL37 (5 $mg \cdot kg^{-1}$, i.v.) or saline was administered 10 min before naloxone and PL37 was found to significantly attenuate the global withdrawal score in these mice when compared with saline-treated mice. In support of these data, the peptidase-resistant enkephalin analogues FK 33-824 and metkephamid (LY-127,623) completely suppress opioid withdrawal in monkeys (Gmerek et al., 1983). Furthermore, a herpes simplex virus vector that promotes local release of enkephalins in animals with persistent pain attenuated the manifestations of naloxone-precipitated morphine withdrawal syndrome (Hao et al., 2009). Probably, increased concentration of enkephalin in the spinal dorsal horn produced by vector-mediated release competes with naloxone, leading to a more intensive saturation of the opioid receptor and corresponding attenuation of the withdrawal syndrome.

It has been hypothesized that enkephalin release is increased during morphine withdrawal as a compensatory mechanism ameliorating the effects of withdrawal (Fukunaga & Kishioka, 2000). These previous results suggest that enkephalins or other proenkephalin-derived peptides, released during morphine withdrawal may induce a wave of constitutive activity, which could attenuate naloxone withdrawal. The persistence of such endogenous opioid activity, produced by enkephalin release, may be a homeostatic mechanism ameliorating opioid withdrawal and should be improved by the protection of enkephalins from metabolism by DENKIs.

All these data reveal that an increase in opioid receptor occupancy by endogenous enkephalins protected from catabolism by dual inhibitors significantly reduced morphine abstinence in rodents.

7 | DENKIs AS ALTERNATIVE FOR AVOIDING OPIOID WITHDRAWAL

In agreement with animal studies, withdrawal syndrome in heroin addicts can be reduced by the administration of endogenous opioid peptides (Wen & Ho, 1982; Wen & Ho, 1984). In patients on heroin withdrawal, **dynorphin (1-13)** has shown to relief the manifestations of the withdrawal syndrome (Wen & Ho, 1982. It was also observed that the duration of this relief lasts longer than other endogenous opioid peptides such as **β-endorphin**, **[D-Ala^2, D-Leu^5]-enkephalin** or dynorphin (Wen & Ho, 1984). In addition, human studies have also compared the effectiveness of different opioid peptides in suppressing the withdrawal syndrome in heroin addict patients and the most effective results were obtained with the intravenous administration of [D-Ala^2, D-Leu^5]-enkephalin analogues (Wen & Ho, 1984).

These withdrawal syndromes are expected to be avoided or reduced by means of peripheral administration of peptidase inhibitors. In fact, the enkephalinase inhibitor acetorphan has shown to attenuate some aspects of the opioid withdrawal syndrome, such as lacrimation, weight loss and diarrhoea in humans (Hartmann et al., 1991). These results make enkephalinase inhibition a novel and safe therapeutic approach for the treatment of opioid withdrawal, despite the need of more human studies.

8 | CLINICAL STUDIES WITH DENKIS

Pharmacological studies in animals have demonstrated the safety and potential therapeutic interest of DENKIs in the treatment of OUD. These data are supported by early studies in humans using enkephalin analogues and inhibitors of the enkephalin catabolism, as previously discussed. In addition, several clinical trials have now been carried out using the most advance DENKI in terms of research, PL37, which further demonstrate the safety of this novel therapeutic approach. Indeed, safety, tolerability and preliminary pharmacokinetics of PL37 were investigated in a 'first-in-human' clinical trial (PL37-2008-C01/EudraCT No. 2008-000863-41; Debio0827-102/EudraCT No. 2010-018271-18). A total of 80 healthy volunteers of both genders were exposed to single ascending oral doses of PL37. Subjects were randomized in a 6:2 ratio to receive active treatment (PL37) or placebo. Single doses of 6.25, 12.5, 25, 50, 100, 200, 400 and 800 mg of PL37 or matching placebo were administered. Two doses (50 and 200 mg) were tested in female subjects with low differences in pharmacokinetic time course profiles. Metabolites were also measured in plasma and urine. The most frequently reported adverse events in Phase 1 studies (EudraCT No. 2008-000863-41; EudraCT No. 2010-018271-18) were CNS disorders, mainly headaches rated as mild or moderate in intensity and orthostatic intolerance rated as moderate or severe in intensity. A number of episodes of postural hypotension also occurred in the single-dose study, most of them asymptomatic, but not at higher doses in the multiple-dose study. All doses were well tolerated and no severe adverse effects were observed in Phase 1 studies, supporting the safety of this therapeutic approach.

The endogenous NEP and APN activities and the inhibitory effects of ascending doses of PL37 were assessed in plasma samples from the first Phase 1 study. Maximal NEP inhibition was similar for subjects receiving 100 to 800 mg of PL37, with an almost complete inhibition during the first hours. The time needed to get back to basal activity tended to be longer for subjects receiving 400 and 800 mg of PL37. Mean APN inhibition results reveal a clear dose-dependent effect on enzyme inhibition for subjects receiving 100 to 400 mg of PL37, whereas 400 and 800 mg of PL37 produced similar inhibition profiles with an almost complete inhibition during the first hours (EudraCT No. 2008-000863-41).

Analgesic efficacy and safety of oral PL37 were assessed in a 4-week Phase 2a, multicentre, randomized, double-blind,

placebo-controlled add-on study (PL37-C03-2013/EudraCT No. 2013-004876-37) in diabetic subjects suffering from neuropathic pain with inadequate pain relief despite being on stable dose of **pregabalin** or **gabapentin**. Randomized subjects received, in addition to their therapy, either oral PL37 (200 mg, t.i.d.) or matching placebo t.i.d. for 4 weeks (28 days). Subjects were assigned to a treatment group (PL37 or placebo) in a 1:1 ratio. In the pregabalin stratum, the primary endpoint was considerably improved in the PL37 group in comparison with placebo (although no significant). Importantly, no biological or vital signs changes nor laboratory alterations nor severe adverse events were reported, underlying the safety of this novel therapeutic approach.

It must be mentioned that NEP has been involved in the degradation of natriuretic peptides *in vitro*, and its inhibition has been explored to reduce BP, which may raise concerns about the safety of this approach in normotensive subjects. Indeed, **sacubitril** was the first NEP inhibitor approved by FDA in 2015 and marketed for heart failure in association with an angiotensin II receptor antagonist (**valsartan**) under the drug name LCZ696 or Entresto® and has demonstrated safety and efficacy over a period of 5 years (Campbell, 2017). Angioedema events were not recorded in either the 497- or 297-patient trials receiving sacubitril for 8 weeks (Srivastava et al., 2018). However, one patient out of 149 with heart failure that received sacubitril therapy for 36 weeks developed severe angioedema (Raheja et al., 2018), although angioedema incidence was acceptably low in heart failure patients receiving sacubitril therapy (0.45%), not different from that for **enalapril** (angiotensin-converting enzyme inhibitor) therapy (0.5%) (Campbell, 2018). The cardiovascular safety of PL37 was also reported in Phase 2a clinical trial. No clinically significant changes in ECG were reported. In sharp contrast with sacubitril, BP showed no changes between baseline and end of study (PL37 systolic BP change from baseline to end of treatment = −1.2 ± 13.1 mmHg; placebo systolic BP change from baseline to end of treatment = −0.0 ± 15.98 mmHg), and no differences between the placebo (135.0 ± 17.85 mmHg) and PL37-treated group (132.6 ± 17.32 mmHg), which underlines the differences between these two drugs in cardiovascular safety.

In summary, Phase 1 and Phase 2 clinical studies reveal that PL37 is well tolerated and safe in humans by oral route. Altogether, these studies show that DENKIs are well poised to serve as drugs with novel mechanisms of action for OUD treatment.

9 | CONCLUSIONS

After a long time of misuse, society now is becoming conscious about the danger of inappropriate use of opioids. The current situation in the United States due, at least in part, to initial improper medical use of opioids has led to a dramatic epidemic of opioid use disorder (OUD). Most of the patients affected by this epidemic do not receive an appropriate treatment and the therapeutic approaches now available has serious caveats. The use of physiologically produced endogenous opioid peptides could represent an excellent approach to open novel therapeutic perspectives for OUD. Taking into account that enkephalins are the most abundant endogenous opioids and they are degraded within minutes of their release, making impossible their therapeutic application, the inhibition of their catabolism could be an innovative method to maintain the activation of the endogenous opioid system avoiding the classical opioid-like side effects. To date, preclinical and clinical studies carried out confirm the safety of this novel therapeutic approach showing an absence of the classical harmful side effects caused by opioids. Preclinical studies have also provided a solid amount of data that demonstrate pharmacological effects of these inhibitors of the enkephalin catabolism that are of potential interest for OUD treatment. Early studies in humans have demonstrated that endogenous opioids are able to alleviate the severity of opioid withdrawal. Therefore, all of the current data highlight the usefulness of enkephalin catabolism inhibitors as a potential novel therapeutic strategy to minimize the current opioid crisis.

9.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos et al. 2021; Alexander, Fabbro et al. 2021).

AUTHOR CONTRIBUTION

B. A.-P. and H. P. equally contributed to the plannification and writing of the manuscript. R. M. helped in the plannification, writing and revision of the manuscript.

CONFLICT OF INTEREST

Hervé Poras is the Chief Scientific Officer and Director of CMC of Pharmaleads S.A. Rafael Maldonado and Beltrán Álvarez-Perez declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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