

RB101-mediated Protection of Endogenous Opioids: Potential Therapeutic Utility?

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ABSTRACT

The endogenous opioids met- and leu-enkephalin are inactivated by peptidases preventing the activation of opioid receptors. Inhibition of enkephalin-degrading enzymes increases endogenous enkephalin levels and stimulates robust behavioral effects. RB101, an inhibitor of enkephalin-degrading enzymes, produces antinociceptive, antidepressant, and anxiolytic effects in rodents, without typical opioid-related negative side effects. Although enkephalins are not selective endogenous ligands, RB101 induces these behaviors through receptor-selective activity. The antinociceptive effects of RB101 are produced through either the mu-opioid receptor alone or through activation of both mu- and delta-opioid receptors; the antidepressant-like and anxiolytic effects of RB101 are mediated only through the delta-opioid receptor. Although little is known about the effects of RB101 on other physiologically and behaviorally relevant peptides, these findings suggest that RB101 and other inhibitors of enkephalin-degrading enzymes may have potential as novel therapeutic compounds for the treatment of pain, depression, and anxiety.

INTRODUCTION

There are three types of opioid receptors in the central nervous system: mu, kappa, and delta (OP3, OP2, OP1 receptors, respectively). Currently, drugs that act predominantly as mu-opioid receptor agonists are used clinically and are utilized mainly for the treatment of

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pain. Exogenously administered mu-opioid agonists, such as morphine, are considered to be limited by their respiratory depressant effects, gastrointestinal and pruritic effects, abuse potential, and the development of tolerance and dependence. Endogenous opioid peptides can produce many of the same effects as selective opioid alkaloids; however, these peptides are rapidly destroyed by enzymes, minimizing any detectable effects. Therefore, prolonging the action of endogenous opioid peptides, such as enkephalins, could produce significant behavioral and potential clinical effects. Although these enkephalins may not selectively activate a single opioid receptor, the selective participation of multiple opioid receptors may produce interesting and beneficial profiles of therapeutic action.

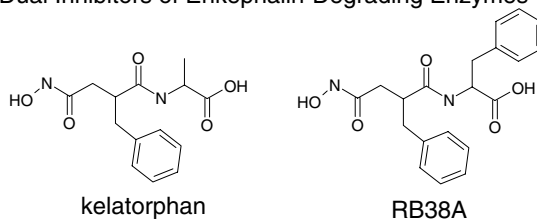
CHEMISTRY, METABOLISM, AND MECHANISMS OF ACTION

The endogenous opioid peptides, met- and leu-enkephalins, are inactivated by peptidases, primarily two zinc metallopeptidases: neutral endopeptidase (NEP) and aminopeptidase N (APN). Zinc metallopeptidases are membrane-bound enzymes with a short transmembrane domain and an extensive extracellular domain containing the active site. APN cleaves enkephalin at the N terminus between the Tyr¹ and Gly² bond, and NEP cleaves between Gly³ and Phe⁴; the cleavage of either bond eliminates binding to opioid receptors. These peptidases are densely localized to the brain regions containing enkephalins and opioid receptors, specifically in the globus pallidus, caudate putamen, substantia nigra, olfactory bulb, choroid plexus, and spinal cord in the rat (Waksman et al. 1986). Although enkephalins have only a 10-fold greater affinity for delta-opioid receptors than mu-opioid receptors, brain regions with dense population of mu-opioid receptors have low levels of these peptidases.

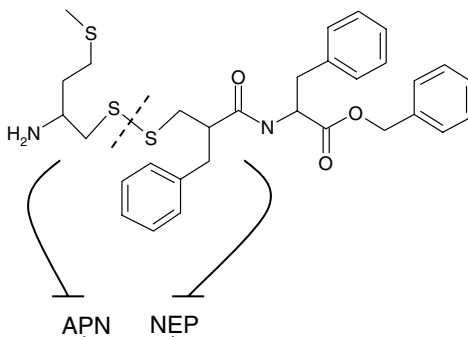
Inhibition of only one of these peptidases produces minimal enkephalin rescue and limited antinociceptive-related responses (Carenzi et al. 1983; Bourgoin et al. 1986; Dickenson et al. 1987). However, simultaneous inhibition of both enzymes is required to significantly increase enkephalin levels and to observe robust behavioral effects. Dual inhibitors of NEP and APN, such as kelatorphan and RB38A (Fig. 1a), completely prevented enkephalin degradation and produced significant antinociceptive effects *in vivo*; however, these compounds are highly hydrophilic and do not readily cross the blood-brain barrier, so that they are administered centrally to produce behavioral effects (Maldonado et al. 1989). Based on these findings, a number of selective peptidase inhibitors with improved lipophilic profiles were developed to enhance penetration of the blood-brain barrier by the addition of mercapto- and carboxyl-protecting groups (Roques and Fournié-Zaluski 1989; Fournié-Zaluski et al. 1992a). RB101 (N-[(R,S)-2-benzyl-3] (S)(2-amino-4-methylthio)butyldithio)-1-oxopropyl)-L-phenylalanine benzyl ester) is a compound that combines one APN and one NEP inhibitor linked by their mercapto groups (Fig. 1b) (Noble et al. 1992a). These mercapto groups linked with a disulfide bond are unavailable for coordinating with the zinc-binding motif in the catalytic domain of the enzymes, rendering RB101 itself inactive; however, cleaving the disulfide bond produces these highly effective inhibitors of APN and NEP (Fig. 1b,c). Therefore, RB101 is considered a prodrug because the parent compound is inactive, but it is metabolized to active components.

Interestingly, cleavage of the disulfide bond does not occur in blood, but only in the brain (stability in CSF is not known). Incubation of RB101 in rat serum produced a compound that was not an effective inhibitor, but the two inhibitors were formed only following incubation with rat brain membranes (Noble et al. 1992a). Furthermore, intravenous administration of

a. Early Dual Inhibitors of Enkephalin-Degrading Enzymes



b. RB101



c. Enkephalin Tyr-Gly-Gly-Phe-Met (or Leu)

d. Opioid Receptors mu delta

FIG. 1. Scheme for the inhibition for enkephalin degradation by aminopeptidase N (APN) and neutral endopeptidase (NEP) and consequent stimulation of opioid receptors by enkephalins. (a) Structures of kelatorphan and RB38A, dual inhibitors of enkephalin-degrading enzymes. (b) The structure of RB101. The dashed line signifies the disulfide bond that is cleaved in the brain to produce two inhibitors, one for APN and one for NEP. (c) Enkephalin structure and indication of APN and NEP cleavage sites. (d) Enkephalins bind to mu- and delta-opioid receptors.

RB101 (9 mg/kg) completely inhibited mouse brain NEP within 10 min of administration and remained inhibited by approximately 71%, even 60 min following intravenous injection; however, it was demonstrated that some of the antinociceptive effects of 10 mg/kg RB101 (i.v.) lasted only 30–40 min (Noble et al. 1992a; Le Guen et al. 2003b).

The activity of the NEP inhibitor component of RB101 is influenced by its stereochemistry, such that the (S,S,S)-isomer of RB101 was more active than the (S,R,S)-isomer at one dose tested (Fournié-Zaluski et al. 1992b), but further studies would be required to determine if this was an actual shift in the RB101 dose-effect curve. Because of its lipophilic profile, RB101 has limited solubility. In most studies this compound is dissolved in a mixture of ethanol (10%), cremophor EL (10%), and water (80%), although RB101 has also been dissolved in β -cyclodextrin or administered in a sustained-release emulsion made of water, liquid paraffin oil, and mannide monooleate (Mas Nieto et al. 2001; Le Guen et al. 2002, respectively). Considering that ethanol can alter behavior and influence the behavioral effects of drugs, vehicle solutions in control experiments should always be considered.

Consequently, the active components of RB101 increase levels of enkephalins that bind to mu- and delta-opioid receptors (Fig. 1d). RB101 administration *in vivo* produces long-lasting increases in the met-enkephalin-like material as measured by *in vivo* microdialysis experiments (Daugé et al. 1996) and displaced [³H]diprenorphine in rat brain homogenates (Ruiz-Gayo et al. 1992). In conclusion, the dual inhibitor RB101 is expected to be metabolized in the brain, but not blood serum, to form inhibitors of enkephalin-degrading enzymes causing elevations in endogenous enkephalins that bind to opioid receptors to produce behavioral changes.

PHARMACOLOGY

Antinociceptive Properties of RB101

RB101 was originally developed as a potential alternative to morphine for analgesia. It was proposed that without extensive receptor activation as observed with morphine, the side effects, such as respiratory depression, would not occur. Many studies have demonstrated antinociceptive effects of systemic RB101 in a variety of assays: the hot plate test in mice (Noble et al. 1992a, 1992b, 1995, 1996; Jayaram et al. 1997; Mas Nieto et al. 2001), the mouse writhing assay (Noble et al. 1992a, 1997), the rat tail flick test (Noble et al. 1992a, 1997; Ortega-Alvaro et al. 1998; Mas Nieto et al. 2001), the rat tail electrical stimulation (Noble et al. 1992a; 1997; Valderde et al. 1996), and peripheral inflammation tests (Maldonado et al. 1994; Mas Nieto et al. 2001). RB101 produced an effect similar in magnitude to the mu-opioid agonists DAMGO (Noble et al. 1992a) or morphine (Noble et al. 1992b, 1995; Maldonado et al. 1994) in antinociceptive assays; however, these compounds differed in terms of potency and duration of action. Morphine was approximately 6–10 times more potent than RB101, depending on the assay used (Maldonado et al. 1994; Noble et al. 1995; Jayaram et al. 1997). A time-course comparison between RB101 and morphine is difficult because there are no direct comparisons of the duration of action of these compounds across different doses in the literature. Both RB101 and morphine appear to have similar onsets of drug action (within 5 min) following a bolus intravenous administration. The antinociceptive effects of intermediate doses of intravenous morphine persisted for 45 to 60 min (Ouellet and Pollack 1997), whereas a dose of 10 mg/kg RB101 (*i.v.*) lasted less than 30 min (Noble et al. 1992a; Le Guen et al. 2003a). An intraperitoneal injection of a large RB101 dose (150 mg/kg) lasted at least 60 min, but the full time-course was not evaluated (Jayaram et al. 1997). Interestingly, RB101 produced elevations in met-enkephalin levels for more than 3 h; however, it is unknown if any of the behavioral effects of RB101 persist for the same period (Daugé et al. 1996). It would be beneficial to compare the duration and magnitude of RB101-induced behavioral effects with increases in enkephalin levels (or levels of other peptides) to better understand the therapeutic potential of endogenous opioids and to create novel inhibitors with improved actions.

Although RB101-induced antinociception is robust and, in some ways, similar to morphine, there are discrepancies in the literature as to the opioid receptor mediating its antinociceptive effects. Low doses of naloxone (0.1 mg/kg) and the selective mu-opioid antagonist β -funaltrexamine, but not the delta-opioid antagonist naltrindole (0.1 mg/kg), blocked the antinociceptive effects of RB101 in the mouse hot plate and writhing tests, suggesting the mu-opioid receptor mediates this response (Noble et al. 1992a, 1992b, 1995). However,

the dose of naltrindole used in these studies was very low. Although this dose of naltrindole was previously shown to block rearing activity of the selective delta-opioid peptides BUBU and BUBUC (Gacel et al. 1990), it was insufficient to prevent the effects of selective delta-opioid agonists in other studies (Broom et al. 2002; Jutkiewicz et al. 2004). A more recent study also demonstrated that the effects of RB101 in the mouse hot plate test were not blocked by a high dose of naltrindole (5 mg/kg) and were eliminated in mu-opioid receptor knockout mice, further supporting the role of mu-opioid receptors only in this response (Mas Nieto et al. 2005). Similarly, in the warm water tail withdrawal assay (tail immersion test) in mice, 5 mg/kg naltrindole only partially blocked the antinociceptive effects of RB101, and these effects of RB101 in mu-opioid receptor knockout mice were significantly reduced (Mas Nieto et al. 2005).

In other conditions, however, both mu- and delta-opioid receptor antagonists blocked the effects of RB101. For example, in the rat tail flick test (radiating thermal stimulus), a selective dose of naltrindole (1.0 mg/kg) and naloxone (0.5 mg/kg) blocked the antinociceptive effects of RB101 (Noble et al. 1992a). In the same study, both antagonists at the same doses prevented the motor response elicited by electrical stimulation, but only naloxone antagonized the vocalization, and vocalization post-discharge induced by electrical stimulation. Similarly, intrathecal naltrindole and systemic naloxone blocked the antinociceptive effects of RB101 in the paw pressure-induced vocalization test in diabetic rats (Coudoré-Civiale et al. 2001). Naltrindole (0.5 mg/kg) also attenuated the RB101-induced decrease in licking in the early and late phases of the formalin test in mice (Noble et al. 1995).

Based on the original studies, it was proposed that RB101 increased endogenous enkephalins and produced antinociception either through mu- and/or delta-opioid receptors depending on the paradigm used. Mu-opioid receptors alone were proposed to be preferentially involved in supraspinal antinociception (hot plate and writhing tests), but both mu- and delta-opioid receptor receptors were implicated in spinal antinociception (tail flick and motor responses to electrical stimulation). However, other studies demonstrated that delta-opioid receptors play a role in the antinociceptive effects of RB101 in models of neuropathic and inflammatory pain, suggesting a more complex profile of delta-opioid receptor involvement. Overall, the dual inhibitor RB101 has antinociceptive properties in morphine-sensitive assays, demonstrating that this prodrug may have therapeutic potential. Depending on the type of nociceptive stimuli involved, the effects of RB101 are mediated through the mu- and/or delta-opioid, but not the kappa-opioid, receptors.

c-Fos protein expression is considered an indirect marker of cell populations, especially in the spinal cord, involved in the transmission of nociceptive stimuli and is thought to reflect long-term intracellular changes associated with pain. Clinically used analgesics decrease c-Fos expression in rodent models. A number of studies demonstrated that RB101 decreased spinal c-Fos expression following carrageenin-induced inflammatory and heat stimulation. In these studies, RB101 was not as effective as morphine, but was more effective than aspirin or acetaminophen (Abbadie et al. 1994). The RB101-evoked decrease in c-Fos activation was mediated by the mu- and delta-opioid receptors, since *in vivo* administration of either the mu-opioid antagonist β -funaltrexamine or naltrindole, but not the kappa-opioid antagonist, nor-binaltorphimine, attenuated the effects of RB101 (Le Guen et al. 1999, 2003a). These data further support the behavioral studies demonstrating that RB101 produces antinociceptive effects that are mediated through the mu-opioid and, most likely, the delta-opioid receptor as well.

Other Effects Related to Morphine Analgesia

In humans and animals, morphine and other mu-opioid analgesics used to treat and manage pain produce respiratory depression, a major limiting factor in their clinical use. Considering the actions of RB101 at mu-opioid receptors, the effects of RB101 on respiration were evaluated. RB101, over a range of antinociceptive doses, administered peripherally in awake and anesthetized mice did not alter ventilation (minute volume) even at high doses, 160 mg/kg (i.p.) (Boudinot et al. 2001). In the same study, central administration of a large dose of RB101 into the fourth ventricle of awake cats also did not decrease ventilation as compared with vehicle controls. Another study evaluated equieffective antinociceptive doses of morphine and RB101 and found that morphine, but not RB101, decreased respiratory rate 30 min after administration in restrained, pregnant mice (Jayaram et al. 1997). These studies demonstrated that RB101 produced significant, morphine-like antinociception without serious respiratory depressant effects, suggesting that RB101 might be an effective analgesic with a larger margin of safety than morphine-like agonists.

Another limiting factor of morphine-like analgesics is the development of tolerance to the therapeutic effects and physical dependence following chronic use. It was demonstrated that the antinociceptive effects of RB101 were not greatly altered following repeated RB101 administration as compared with repeated vehicle injection; this was demonstrated for different chronic RB101 administration paradigms: twice daily administration of 80 mg/kg RB101 i.p. for 4 or 8 days (Noble et al. 1992b) or twice daily administration of 20 mg/kg RB101 i.v. for 5 days (Valverde et al. 1995). To evaluate the development of cross-tolerance with morphine, rats were treated twice daily with 3 mg/kg morphine (i.p.) (the ED₅₀ values following i.p. administration) and antinociceptive responses for morphine and RB101 were determined. In morphine-tolerant rats, the morphine-induced antinociceptive dose response curve was shifted approximately 2-fold to the right, demonstrating tolerance development; however, RB101 produced similar antinociceptive effects in morphine-tolerant rats as compared with nontolerant rats (Noble et al. 1992b). Also, in agreement with these behavioral studies, tolerance did not develop to the effects of RB101 on spinal c-Fos activation following chronic administration of morphine or RB101 (Le Guen et al. 2002). In general, these data demonstrated that repeated RB101 administration failed to produce tolerance to its antinociceptive effects and that cross-tolerance did not occur between morphine and RB101. This is an interesting finding that could suggest RB101 and morphine have different mechanisms of action in terms of antinociception or differential localization of receptor or enzymes/receptors involved in this effect; however, other characteristics of these compounds should be considered, for example, duration of action. Following peripheral administration, morphine has a longer duration of action; therefore, morphine may be able to produce greater tolerance than RB101 due to prolonged receptor activation. It might be beneficial for future studies to use more extreme measures of RB101 exposure to determine if tolerance and/or dependence develop.

In addition to tolerance, repeated administration of morphine-like drugs can produce physical dependence, such that spontaneous drug deprivation-induced or antagonist-induced precipitated withdrawal can produce large behavioral changes. Administration of mu-opioid antagonists to morphine-dependent mice produces jumping behaviors, wet dog shakes, tremor, teeth chattering, body weight loss, diarrhea, and other changes. In mice chronically treated with morphine (6 mg/kg i.p.) or RB101 (160 mg/kg i.p.) for 5 days, administration of naloxone (5 mg/kg s.c.) only produced robust weight loss, jumping

behavior, and wet dog shakes in morphine-treated mice, but not in RB101-treated mice (Noble et al. 1992c). RB101 also did not produce much dependence following chronic infusion. In RB101-infused animals naloxone did not produce weight loss, wet dog shakes, or diarrhea; however, a small increase in tremor was observed following naloxone precipitated withdrawal, suggesting more RB101 exposure may be required to produce dependence (Noble et al. 1994). In these studies, repeated administration of RB101 failed to produce a morphine-like withdrawal syndrome, demonstrating that the physical dependence to RB101 did not occur. This lack of a withdrawal syndrome could also be related to activation of multiple opioid receptors or the type of withdrawal symptoms evaluated. For example, RB101-induced increase in enkephalins activate mu- and delta-opioid receptors, but tolerance has not been observed following delta-opioid receptor activation, and delta-opioid agonists have been shown to decrease morphine-related withdrawal symptoms (Lee et al. 1993). In addition, withdrawal from a mixed-action opioid ligand may not manifest the same behaviors as withdrawal from morphine or other selective mu-opioid agonists, therefore, other types of withdrawal symptoms should be considered (Covington and Miczek 2003; Koeltzow and White 2003). Overall, RB101 does not produce morphine-like tolerance and dependence, but these behavioral effects cannot be completely excluded yet.

Another significant problem with mu-opioid analgesics is their abuse potential. Mu-opioid agonists, such as heroin, have reinforcing effects in animal models, promoting self-administration behaviors and place preference in drug-associated environments. In conditioned place preference paradigms, a range of high antinociceptive doses of RB101 (80, 160, and 250 mg/kg) did not produce conditioned place preference in mice (Noble et al. 1993), suggesting that RB101 does not have reinforcing effects. Although RB101 has not been evaluated in self-administration paradigms, the dual inhibitor of enkephalin-degrading enzymes acetorphan had minimal reinforcing effects in rhesus monkeys over a 100-fold range of doses, suggesting that other dual inhibitors, like RB101, may also have a low abuse potential (Knisely et al. 1989). Considering RB101 has actions at both mu- and delta-opioid receptors, it is interesting that RB101 does not have reinforcing effects, as mu- and delta-opioid receptor peptides produced conditioned place preference (Shippenberg et al. 1987; Bals-Kubik et al. 1990; Suzuki et al. 1996) and were self-administered into the ventral tegmental area of rats (Devine and Wise 1994).

Emotion-Related Behavioral Effects

Endogenous and exogenous opioids influence mood and emotional states, for example, mu-opioid receptor stimulation produces euphoria and kappa-opioid receptor stimulation produces dysphoria. Although delta-opioid agonists have not been evaluated in humans, it is thought that delta-opioid receptor activation may enhance mood and emotional state based on preclinical studies. For example, delta-opioid receptor agonists produce antidepressant-like effects in various rodent models of depression and antidepressant assays (Broom et al. 2002; Saitoh et al. 2004; Jutkiewicz et al. 2004; Cordonnier et al. 2005; Vergura et al. 2006). The delta-opioid receptor may even be required for positive mood, since it was demonstrated that delta-opioid receptor knockout mice demonstrate anxiety and depressive-like behaviors (Filliol et al. 2000). Therefore, it is interesting to consider the effects of the dual inhibitor of enkephalin-degrading enzymes RB101 on mood and emotion-related behaviors.

RB101 indirectly increases enkephalins that act at mu- and delta-opioid receptors. As stated previously delta-opioid receptor activation produces antidepressant-like activity, but mu-opioid receptor activation does not (Broom et al. 2002). Therefore, RB101 may not have any effects on mood/emotion because combined activity at mu- and delta-opioid receptors could work in opposite directions. Interestingly, it was demonstrated that RB101 could improve mood or emotional state by producing antidepressant-like effects in a number of rodent paradigms sensitive to antidepressant drugs. For example, RB101 attenuated the conditioned suppression of motility in mice at doses but did not stimulate locomotor activity alone (Mas Nieto et al. 2005). RB101 also decreased immobility in the forced swim test in rats (Jutkiewicz et al. 2006) and in mice (Jardinaud et al. 2005; Cordonnier et al. 2005; Mas Nieto et al. 2005). Antidepressant-like effects were also observed in learned helplessness model of depression, such that RB101 decreased the number of escape failures (Tejedor-Real et al. 1998). These antidepressant-like effects of RB101 were attenuated by the delta-opioid antagonist naltrindole, but not by mu-opioid receptor-selective doses of naltrexone or the kappa-opioid receptor antagonist nor-binaltorphimine (Tejedor-Real et al. 1998; Cordonnier et al. 2005; Mas Nieto et al. 2005; Jutkiewicz et al. 2006). In addition, the effects of RB101 in the mouse forced swim test and the conditioned suppression of motility were not altered in mu-opioid receptor knockout mice, demonstrating that the mu-opioid receptor does not play a role in these behavioral effects (Mas Nieto et al. 2005). Overall, these data demonstrate that the antidepressant-like properties of RB101 are mediated exclusively through the delta-opioid receptor.

In addition to antidepressant-like effects, RB101 also has anti-anxiety properties in predictive behavioral paradigms. For example, RB101 increased the number of visits in the open sectors of the elevated O-maze in mice (Mas Nieto et al. 2005) and decreased the time spent in the dark compartment of the light-dark box in mice (Jardinaud et al. 2005). The anxiolytic effects of RB101 were not altered in mu-opioid receptor knockout mice, further demonstrating that, in general, mood-related behaviors influenced by endogenous opioids are mediated through delta-opioid receptors (Mas Nieto et al. 2005).

Considering that the emotion-related behaviors of RB101 are mediated through the delta-opioid receptors, it is worthwhile to consider the possible convulsant effects of RB101. Delta-opioid receptor activation with high efficacy agonists, such as SNC80 and BW373U86, produce convulsions in mice, rats, and monkeys (Comer et al. 1993; Broom et al. 2002; Jutkiewicz et al. 2004; Danielsson et al. 2006). However, RB101 failed to induce convulsions or any EEG changes representative of seizure or preconvulsive activity (Jutkiewicz et al. 2006). These data demonstrate that mood-enhancing effects of the pro-drug RB101 are potentially safer compared to those produced by direct-acting delta-opioid agonists.

Interactions with Cholecystokinin (CCK)

CCK, a peptide found in the central nervous system (predominantly CCK-8), acts as a modulator of the opioid system in rodents. This peptide binds to two receptor subtypes, CCK1 and CCK2, also known as CCK_A and CCK_B respectively, and CCK2 is preferentially localized in the brain, whereas CCK1 is found mostly in the gastrointestinal tract. CCK-induced activation of CCK2 receptors produces a functional antagonism of the opioid

system, whereas blockade of CCK2 receptors enhances opioid signaling. Although the mechanism of the opposing actions of opioid and CCK is unknown, it has been proposed that CCK receptor activation may alter ligand binding to the opioid receptor or that CCK may counteract the effects of opioid agonists on calcium regulation in cells (Wiesenfeld-Hallin et al. 1999). CCK ligands have been shown to modulate the behavioral effects of endogenous opioids. For example, it has been demonstrated that CCK2 receptor antagonists enhanced the antinociceptive responses produced by RB101 administration in multiple animal paradigms (Noble et al. 1995; Valverde et al. 1994, 1995; Xu et al. 1997; Coudoré-Civiale et al. 2001). In addition, the CCK2 receptor antagonist CI988, but not the CCK1 antagonist devazepide, augmented the RB101-induced reduction of carrageenin-stimulated spinal c-Fos expression (Honoré et al. 1997).

Similar interactions have been observed with the antidepressant actions of RB101. Administration of the CCK2 receptor antagonist PD-134,308 facilitated the antidepressant-like effects of RB101 in the forced swim test in mice and in the conditioned suppression of motility test in mice and rats (Hernando et al. 1996; Smadja et al. 1995, 1997). To demonstrate the modulatory role of this receptor, it was also shown that the CCK2 receptor agonist BC264 blocked the antidepressant-like effects of RB101 (Smadja et al. 1995). Unlike antinociceptive experiments, CCK1 receptor antagonists also attenuated the antidepressant properties of RB101 (Hernando et al. 1996; Smadja et al. 1995). Interestingly, the CCK2 receptor antagonist L-365,260 alone had antidepressant-like effects in the mouse forced swim test, and these effects were blocked by selective doses of the delta-opioid antagonist naltrindole (Hernando et al. 1996). Therefore, administration of the CCK2 receptor antagonist may eliminate the functional antagonism of the delta-opioid receptor system, such that activation of delta-opioid receptors by endogenous ligands can produce antidepressant-like effects. In general, these data suggest that the CCK system has a modulatory role on the opioid system by regulating levels of system activation. This modulation can be exploited to enhance the effects of endogenous enkephalins elevated by RB101 to produce more robust opioid-related behaviors through different receptors.

Future Studies with RB101

As discussed previously, RB101 produces robust behavioral responses indicative of therapeutic actions in preclinical assays. Although there is a wealth of knowledge available about this compound, some critical information remains unexplored. For example, the duration and magnitude of RB101-induced behavioral effects should be directly compared with the RB101-mediated alterations in enkephalin levels in the brain. Likewise, potency and efficacy comparisons for multiple dual inhibitors across a variety of measurements, such as degree of enzyme inhibition, antinociceptive effects, and changes in enkephalin levels, could identify critical actions of behaviorally active dual inhibitors. Measuring enkephalin levels by *in vivo* microdialysis is a challenging task; however, this information could lead to improved understanding of the endogenous opioid system as well as assist the development of new dual inhibitors of enkephalin-degrading enzymes.

RB101 and related compounds also need to be evaluated in terms of their possible non-opioid effects. For example, the peptidase inhibition produced by RB101 may alter levels

of other central and peripheral peptides that might be susceptible to APN- or NEP-induced degradation: angiotensin III, atrial natriuretic factor, substance P, bradykinin, oxytocin, neurotensin, endothelin-1, bombesin, and bombesin-like peptides (Albiston et al. 2004; Sumitomo et al. 2005). Alterations in multiple systems could change the therapeutic effects of endogenous opioids or produce deleterious effects. Along these lines, RB101 appears to have a safe profile of action, unlike other mu-opioid agonists, such that it does not produce respiratory depression or dependence. Future studies should test more extreme measures of RB101 exposure or higher doses to determine if these opioid-related effects or other toxic/lethal effects are produced. If very high doses of RB101 produce respiratory depression, then RB101 must have a large margin of safety (e.g., a large difference between doses producing antinociception and doses producing respiratory depression); but if these effects are not elicited, it would be interesting to consider why and how morphine and RB101 produce different profiles of activity. Studies evaluating the margin of safety and its non-opioid-related effects (including toxicology and carcinogenic effects) would be required prior to administering RB101 to humans.

CONCLUSIONS

Opioid receptor activation has clinical use in the treatment of human disease. Mu-opioid receptor activation is utilized in the treatment and management of pain, and delta-opioid receptor activation is proposed to elevate or enhance mood and emotional states in depression and anxiety. Exogenously administered, selective agonists for these receptor types can produce side effects that limit their effectiveness, such as respiratory depression (mu-opioid) and convulsions (delta-opioid). These receptor systems can also be activated by endogenous enkephalins; however, these ligands are rapidly metabolized by peptidases, aminopeptidase N (APN) and neutral endopeptidase (NEP), reducing their therapeutic actions. Dual inhibition of these enzymes increases the availability of enkephalins to activate opioid receptors and produces robust behavioral effects. RB101 is the combination of an APN and an NEP inhibitor linked by a disulfide bond; cleavage of this disulfide bond in the brain produces two potent inhibitors that in turn prevent the breakdown of enkephalins. RB101 has antinociceptive, antidepressant-like, and anxiolytic properties in a number of rodent models. The antinociceptive properties of RB101 appear to be regulated through either the mu-opioid receptor alone or both the mu- and delta-opioid receptors, depending on the type of pain and assay evaluated. The effects of RB101 on mood and emotional state, as evaluated in terms of antidepressant-like and anxiolytic effects, are mediated through the delta-opioid receptor. Although enkephalins are not selective endogenous ligands, RB101-elevated enkephalins can produce selective, robust behavioral effects in preclinical models.

Similar to RB101, other dual inhibitors of enkephalin-degrading enzymes also demonstrate therapeutic profiles of action in animal paradigms, such as the orally active inhibitor RB120 (Noble et al. 1997) and the long-acting, systemically active inhibitor RB3007 (Le Guen et al. 2003b). The evaluation of RB101 and related compounds can be difficult considering the compounds are not commercially available, and the synthesis is not simple. However, the findings reviewed here suggest that RB101 and other dual inhibitors of enkephalin-degrading enzymes may have therapeutic potential for treating human disease.

ADDENDUM

- RB101: N-[(R,S)-2-benzyl-3] (S)(2-amino-4-methyl-thio)butyldithio)-1-oxopropyl)-L-phenylalanine benzyl ester
- DAMGO: Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol
- BC 264: Boc-Tyr(SO₃H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH₂
- BUBU: Tyr-D-Ser-(OtBu)-Gly-Phe-Leu-Thr-(OtBu)
- BUBUC: Tyr-D-Cys(StBu)-Gly-Phe-Leu-Thr(OtBu)
- SNC80: (+)-4-[(α R)- α -[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-3-methoxyphenyl)methyl]-N,N-diethylbenzamide
- BW373U86: α (R)- α -[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-3-hydroxyphenyl)methyl]-N,N-diethylbenzamide
- CI988: 4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4]oxo[R-(R,R)]-N-methyl-D-glucamine
- PD-134,308: 4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amin}-4-oxo-[R-(R*,R*)]butanoate-N-methyl-D-glucamine
- L-365,260: 3R-(+)-N-(2,3-dihydro-1methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3yl-N'-(3-methyl phenyl) urea
- RB120: (N-((S)-2-benzyl-3[(S)(2-amino-4-methylthio)butyldithio]-1-oxopropyl)-L-alanine benzyl ester
- RB3007: (⁺H₃N-CH(Ph)-P(O)(O-CH₂CH₂-SCOCH₃)-CH₂-CH(CH₂Ph(*p*-Ph))-CONH-CH(CH₃)COOCH₂Ph

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