STR-324, a Stable Analog of Opiorphin, Causes Analgesia in Postoperative Pain by Activating Endogenous Opioid Receptor–dependent Pathways

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ABSTRACT

Background: Opiorphin is a naturally occurring potent analgesic human peptide. It protects enkephalins from degradation and inhibits pain perception in various acute pain models via activation of endogenous opioid pathways. However, the efficacy of opiorphin continuous infusion and its chemically stable form, STR-324, in postoperative pain is unknown.

Methods: Using the Brennan model of plantar incision–induced hypersensitivity, the authors examined the postsurgical analgesic response to mechanical and thermal stimuli of 7-day continuously intravenously infused drugs (8 to 10 rats per group). Antinociception from opiorphin with reference to morphine and STR-324 was assessed. Spinal c-Fos expression and the involvement of opioid receptor–dependent pathways were investigated. The occurrence of respiratory and hemodynamic adverse effects of opiorphin was also tested.

Results: Intravenous infusion of opiorphin significantly reduced responses to mechanical stimuli from days 1 to 4 post surgical period at 143 to 175-kPa mean ranges compared with 23 to 30-kPa mean ranges for vehicle (P < 0.05). During the 3-day postoperative period, no respiratory rate, oxygen saturation, arterial pressure, or heart rate adverse effects were induced by opiorphin. STR-324 consistently inhibited mechanical and thermal hyperalgesia with similar potency as that of opiorphin. Mechanistic analyses demonstrated that the STR-324 antinociceptive effect was reversed by the opioid antagonist, naloxone. Also, STR-324 significantly reduced the number of pain-evoked spinal cFos-immunoreactive nuclei.

Conclusion: Intravenous infusion of opiorphin and STR-324 produced significant antinociceptive effect in a postoperative pain model. This study demonstrates that STR-324 is effective in postoperative pain management due to its strong antihyperalgesic effects mediated via opioid-dependent antinociceptive pathways. Opiorphin analog should represent a new class of potent and safe analgesics. (ANESTHESIOLOGY 2016; 125:1017-29)

OPIOIDS are the most powerful analgesic drugs for acute and chronic pain treatment; however, adverse side effects and potential misuse issues restrict their widespread prescription. Research on pain therapy has been driven by the desire to enhance the potency of natural human opioids, such as enkephalins, and thus treat pain without serious adverse effects.1,2 Opiorphin is the name given to the Gln-Arg-Phe-Ser-Arg peptide, an endogenous human regulator that was discovered using a functional biochemical approach.3,4 Its characterization demonstrates that it is a physiologic dual inhibitor of both Zn-dependent metalloendopeptidases: neutral endopeptidase (EC3.4.21.11) and aminopeptidase-N (EC3.4.11.2).1 These enzymes are implicated in the rapid inactivation of endogenous circulating opioid agonists, namely enkephalins. As a result, in vitro opiorphin totally
protects Met-enkephalin from degradation by these membrane-anchored peptidases and thus significantly improves the specific binding and affinity of enkephalin-related peptides to membrane opioid receptors without directly interacting with opioid receptors. Physiologic opioid pathways are a dominant part of an endogenous nociceptive-modulating system that counterbalances the activity of pain transmission pathways. The Met-enkephalin, one of the most efficient endogenous opioid peptides, plays a major role in the dynamic control of pain perception. In behavioral studies, opiorphin, at systemically or centrally active doses (1 to 2 mg/kg, intravenous bolus or 5 to 10 μg/kg intracerebroventricular bolus), has strong analgesic effects in various standard murine models of pain with a maximal effect over 15 to 30 min post injection period. These models include supraspinally controlled mechanically induced acute nociception in a rat model, spinally controlled thermally induced acute nociception in both rat and mouse models, and peripheral chemically induced inflammation underlying chronic pain in a rat model. In addition, opiorphin’s analgesic effects are specifically mediated via endogenous enkephalin-dependent activation of μ-opioid receptor (MOR) and δ-opioid receptor (DOR) pathways (fig. 1).

Because of its exciting in vivo properties, we wished to assess whether opiorphin might have therapeutic implications in the field of analgesia. Here, we hypothesized that continuous intravenous opiorphin infusion would induce analgesia in a clinically relevant pain model that mimics clinical postoperative pain. In this model, rats present spontaneous pain, mechanical and thermal hyperalgesia, from 4 to 7 days after incisional surgery, similar to human postoperative pain. The time course and the potency of the antihyperalgesic behavioral responses of opiorphin were analyzed with reference to morphine. The potential respiratory and hemodynamic adverse effects of opiorphin over a 3-day postoperative period were also tested.

Using the postoperative pain model, we assessed, for the first time, the analgesic properties of the chemically stable opiorphin analog, named sTR-324. The sTR-324 is the cyclized form of the N-terminal glutamine 1-opiorphin, resulting in the pyroglutamate 1 peptide (fig. 2). Furthermore, in order to identify its mechanism of action, we...

Fig. 1. Schematic diagram showing the mechanism of opiorphin action in enkephalin-related opioid pathways: (A) nociceptive information (red lightning) is transmitted from the periphery to the spinal dorsal horn by primary sensory neurons. At the spinal level, these neurons transmit nociceptive signals to second order neurons in the brain. Enkephalins (purple dots) are present at the different levels of signal transmission from the periphery to the brain. (B) Enkephalins are released in response to nociceptive information. They can either bind to opioid receptors, thus reducing the nociceptive transmission, or they can be catabolized by membrane aminopeptidase-N (APN) and neutral endopeptidase (NEP) enkephalinases, localized in close proximity to the opioid receptors. If the trauma induces significant danger, the nociceptive signal (red lightning) is transmitted to upper brain levels (solid red arrow), due to insufficient levels of enkephalins capable of binding to opioid receptors. The presence of opiorphin helps modulate nociceptive signal transmission at peripheral, spinal, and central levels. (C) In the case of painful stimuli, the administration of opiorphin leads to an increase in the bioavailability of opioid receptor–binding enkephalins by inhibiting enkephalin degradation and thus blocking the transmission (slashed red arrow) of nociceptive signals (red lightning).
investigated the effects of STR-324 administration on spinal c-Fos protein expression induced in nociceptive pathways in response to postoperative pain stimuli. Finally, to further understand the endogenous events triggered by STR-324, we explored the involvement of endogenous opioid receptor–dependent pathways by the use of the broad-spectrum opioid receptor antagonist, naloxone.

**Materials and Methods**

**Animals**

Experiments were performed on healthy male Sprague-Dawley rats weighing 250 to 275 g (Charles River, IFFA-CREDO, France). Animals were housed in groups of three per cage, under a 12-h light/12-h dark cycle (lights on at 8:00 AM), at a constant room temperature of 22°± 2°C, and had access to food and water ad libitum. All experiments were performed in accordance with guidelines from the International Association for the Study of Pain Committee for Research and Ethical Issues and according to the official edict presented by the French Ministry of Agriculture (Paris, France). Animal use procedures were approved by the Institution’s Animal Care and Use Committee (CEEA 26; agreement number, 2012-097, France).

**General Procedure**

Animals were allowed 10 days to become accustomed to the colony room, were gently handled daily for 5 min, and left in the test room for 2 h (from 11:00 AM to 1:00 PM). All experiments began at 11:00 AM and were performed in a quiet room on groups of 6 to 8 animals during the light part of the light/dark cycle by the same investigator who was blinded to the treatment groups.

All experiments were performed by an investigator blinded to the drugs administered and animals acclimatized to the experimental environment before measurements. Basal value for each test was the mean of three consecutive measurements performed at day 0, before surgery, with 10-min intervals.

**Surgical Procedures**

An osmotic minipump (ALZET® Osmotic Pumps, Durect Corporation, USA), model 2ML1 (2-ml capacity), was subcutaneously implanted during general anesthesia (intraperitoneal, pentobarbital, 30 mg/kg body weight) in the back of the neck at the level of the right scapula of each rat and connected to the right jugular vein. Osmotic minipumps were set to deliver 10 μl/h solution for 7 days. The pumps were previously filled with a vehicle solution, i.e., 0.1 M citrate saline buffer (pH = 5.5), opioidhin, or STR-324 (50 or 250 μg/10 μl, BCN peptides, Spain) or morphine (500 μg/10 μl, Aguettant®, France). Subsequently, in order to induce circulating enkephalin release in response to nociceptive stimuli, a plantar incision was performed as previously described. After surgery, the animals were allowed to recover in their cages.

**Data Recording**

Rats were randomly allocated to experimental groups (8 to 10 rats) either of vehicle, opioidhin, or STR-324 (50 or 250 μg/h) or morphine (500 μg/h). The data (nociceptive

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**Fig. 2.** Representative primary structures of opioidhin and STR-324. The boxes highlight the distinct N-terminal amino acid of each structure: glutamine 1 for opioidhin and pyroglutamate 1 for STR-324.
Mechanical sensitivity of the hind paw was determined by positive paw withdrawal in response to von Frey stimuli. Light mechanical stimuli were applied from underneath to the midplantar area of the hind paw using von Frey filaments (Somedic, Höörby, Sweden) with incremental stiffness as follows: pressure at buckling of 17, 22, 28, 33, 45, 64, 91, 134, 173, 245, 314, and 383 kPa. As reported, a single trial of stimuli consists of 10 applications with the same von Frey filament to the plantar surface of the hind paw for approximately 4 to 6 s each time. We allowed a 3- to 4-min period before applying the next filament. Brisk foot withdrawals (at least six times per 10 applications) were considered positive for the von Frey filament. All animals received training sessions 10 days before surgery for acclimatization, as well as contact with the experimenter, installation on the mesh floor, and von Frey hair tests for 1 h each day.

**Randall–Selitto Test.** Mechanical hyperalgesia was measured in gently restrained rats as the threshold response to an increasing pressure, using an analgesimeter (Ugo Basile, Biosed, Italy) as described by Randall and Selitto. The right hind paw of the animal was positioned on a flat surface under a pressure pad, with the blunt probe tip being applied under a pressure pad. A constantly increasing pressure was applied to the paw by means of an automated gauge. A sharp paw withdrawal occurred when the nociceptive threshold was reached. At that time, the pressure value (in grams) was noted, and 600-g cutoff value was applied to avoid any tissue damage. Three to four consecutive trials were performed at 10-s intervals, and the average value was used for analysis.

**Hargreaves Test.** Hargreaves test was also used to measure thermal hyperalgesia with a plantar analgesia meter/instrument (Ugo Basile, Biosed, Italy) and according to manufacturer's instructions. Briefly, each rat was placed in a transparent plexiglass chamber above a transparent glass floor. A mobile radiant heat source was focused beneath the midplantar surface of the hind paw proximal to the injury site with a 20-s cut-off value to avoid tissue damage. The latency for the animal to withdraw its paw was automatically measured and recorded. Three to four consecutive trials were performed at 10-min intervals, and the average value was used for analysis.

**Noninvasive Carotid Artery Plethysmography**

RR, SpO₂, and HR were monitored using a noninvasive arterial plethysmographic CollarClip sensor (Mouse OX Plus, Starr Life Sciences Corp, USA). The CollarClip sensor was put around the neck of the animal and connected to a Mouse OX Plus unit, the core element of a robust measurement system, and connected itself to a computer for collection of instant data. The connector between the CollarClip sensor and the Mouse OX Plus unit stayed vertical by means of a counterbalance hanger assembly, avoiding impediment of the animal's movements. Animals were allowed to move freely in a conscious measurement enclosure. Three independent measures were collected daily at 10-min intervals, and values were reported as the mean of the three values.

**Blood Pressure Monitoring System**

MAP and SAP were monitored using a tail cuff sphygmomanometer coupled to a student oscillograph (Harvard Apparatus Inc, USA) that let to measure both parameters simultaneously.

Three independent measures were collected daily for each rat at 10-min intervals, and values were reported as the mean of the three measurements.

**Immunohistochemistry for c-Fos Protein**

Twelve rats were randomly allocated to receive either STR-324 or vehicle (n = 6 per group) followed by a plantar incision performed as reported (see Surgical Procedures). Three hours after surgery, rats were euthanized under pentobarbital sodium (100 to 150 mg/kg intraperitoneal) anesthesia and were transcardially perfused with saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). The spinal cord was dissected out and prepared for c-Fos immunohistochemical staining as previously described. Briefly, tissue sections were successively washed and incubated, according to manufacturer's instructions, with primary (anti-c-Fos antibody SC-52, Santa Cruz Biotechnology, USA) and secondary antibodies (biotinylated goat anti-rabbit IgG, Vector laboratories, United Kingdom). The slides were then incubated in a streptavidin–horseradish peroxidase complex (Kit ABC, Fisher Scientific, France), and immunostaining was developed using a diaminobenzidine chromogen substrate (DAB peroxidase substrate kit, Vector Laboratories, USA). Immunostaining c-Fos nuclei were plotted and counted using a camera attachment at 10 × 20 magnification under a bright-field microscope. The total number of c-Fos-immunoreactive neurons per section was counted. At least three sections per animal were averaged, so that mean numbers of regional and total c-Fos immunoreactive neurons were obtained for each rat. Results are expressed as mean ± SD.
Antagonism Study Using Naloxone

A set of experiments was conducted in order to examine the potential implication of endogenous opioid pathways in the antinociceptive effects of STR-324 using the postoperative pain model described (see General Procedure and Surgical Procedures). Briefly, after baseline nociceptive threshold measurements, the osmotic pumps prefilled with vehicle solution, i.e., 0.1 M citrate saline buffer (pH 5.5), or STR-324 (50 μg/10 μl, BCN peptides) were subcutaneously implanted during general anesthesia. In parallel, a plantar incision was performed and the animals were allowed to recover in their cages. One day after surgery, nociceptive thresholds were assessed by von Frey hairs and Hargreaves methods and repeated 1 h after intraperitoneal injection of 2 mg/kg naloxone hydrochloride (Mylan S.A., France) or saline. Nociceptive threshold assessments were repeated at days 2 and 3 thereafter. Three to four consecutive trials were performed at 10-s intervals, and the average value was used for analysis.

Experimental Design and Groups for Each Set of Experiments

Analgesic effects of opiorphin: 50 μg/h opiorphin versus 500 μg/h morphine versus vehicle (von Frey hairs), 8 rats per group. Ventilatory and hemodynamic effects of opiorphin: 50 μg/h opiorphin versus 250 μg/h opiorphin versus vehicle, 10 rats per group.

Analgesic effects of STR-324: 50 μg/h STR-324 versus 250 μg/h STR-324 versus vehicle (von Frey hairs, Randall–Selitto test, and Hargreaves test), eight rats per group.

c-Fos expression: STR-324 versus vehicle versus sham, 6 rats per group

Naloxone antagonism study: (STR-324 plus saline, intraperitoneal) versus (STR-324 + naloxone, intraperitoneal) versus (vehicle plus saline, intraperitoneal; von Frey hairs and Hargreaves test), eight rats per group.

Statistical Analyses

The number of animals needed to achieve a power of 90% in each experiment was based on previous results from our laboratory, considering a minimum effect size of 20%. Data were first tested for departure from normality using the Shapiro–Wilk test and qq plots on raw and transformed data. The homogeneity of variances was also tested using Levene’s piro–Wilk test and qq plots on raw and transformed data. The lack of between-group significant differences of baseline measurements was tested using the Kruskal–Wallis test or ANOVA. The effect of plantar incision was assessed using the Kruskal–Wallis test on the sum of scores across time, followed by the Dunn test with Holm correction as appropriate.

Results

Human Opiorphin and STR-324 Both Display Antiallodynic and Antihyperalgesic Effects in a Postoperative Pain Model

For animal antinociception testing, we used Brennan plantar incision model13 because it can be reliably translated clinically to postoperative pain, to assess for a potential antihyperalgesic effect of opiorphin and its stable naturally occurring derivative, STR-324. This pain model includes a skin, fascia, and muscle plantar incision in the hind paw of rats and causes consistent and quantifiable mechanical hyperalgesia, lasting for several days after surgery. In the current study, drugs were administered to adult rats for 7 days post surgery using ALZET osmotic pumps connected to the jugular vein. Pain was evaluated by von Frey hairs20 24h before (day 0, “presurgery”) and for 7 successive days after surgery (days 1–7). Drug administration was begun at the time of surgery.

Effect of Opiorphin on Plantar Incision–Induced Mechanical Hyperalgesia

The time course of opiorphin response to plantar incision–induced mechanical hypersensitivity was analyzed with reference to morphine (500 μg/h).18 Opiorphin (50 μg/h)—infused dose was used as estimated from previous behavioral studies.3,10,21 In control citrate buffer–treated rats (vehicle), the paw incision induced mechanical hyperalgesia around the scar (P = 0.011, corrected for ex-aequos; fig. 3A). As expected, intravenous infusion of morphine at 500 μg/h produced a strong antihyperalgesic effect (P = 0.0004 vs. vehicle), peaking at day 1 post surgery from 52.1 ± 9.8 kPa (day 0) to 389.1 ± 87.6 kPa (fig. 3A) and linearly decreasing day by day to return close to presurgical values by days 6 to 7. Intravenous infusion of opiorphin at 50 μg/h also produced significant antihyperalgesia (P = 0.008 vs. vehicle) but with a distinct response profile compared to the morphine profile. The antihyperalgesic effect of opiorphin appeared stable throughout days 1 to 4 post surgery, from 47.4 ± 6.7 kPa at day 0 pre surgery to 175.3 ± 85.3 kPa at day 1 and
to 167.6 ± 91.6 kPa at day 4 post surgery (fig. 3A) and then gradually decreased day by day to return close to presurgical values by day 6 to 7. The opiorphin analgesic response profile appears inversely correlated to the control vehicle response profile and is in accordance with its mechanism of action shown in figure 1.3,10

**Effects of STR-324, a Stable Opiorphin Derivative, on Mechanical and Thermal Hyperalgesia**

In-house stability studies using ALZET pumps revealed that opiorphin is not stable in solution. Indeed, under our experimental conditions, the N-terminal glutamine of the opiorphin native peptide undergoes transformation to the pyroglutamate form, up to 60% transformation over a 54-h period at 32°C (unpublished results of the opiorphin stability studies, C. Rougeot and V. Juarez-perez, March 2012). Furthermore, under International Conference on Harmonisation storage conditions, the freeze-dried powder of both the hydrochloride and acetate opiorphin forms also undergoes rapid transformation to the N-terminal pyroglutamate form (unpublished results of the opiorphin stability studies, V. Juarez-Perez, September 2012). In our experiments, a mixture of glutamine 1- and pyroglutamate 1-opiorphin forms was present; therefore, we decided to synthesize and study the pyroglutamate 1 stable form of opiorphin, named STR-324, to evaluate its analgesic efficacy (fig. 2). It is important to point out here that STR-324 is also a natural product present in human fluids, as demonstrated by Rougeot et al.3,22

STR-324 has the same in vitro affinity for human neutral endopeptidase but a lower affinity for human aminopeptidase N compared to the parent molecule; therefore, a second series of experiments was undertaken to determine whether or not STR-324 is equally effective in the postoperative pain model. Pure STR-324 was infused continuously by intravenous route at doses of 50 and 250 μg/h. In addition, nociceptive thresholds, measured 24 h before and for 7 successive days after surgery, were assessed not only by tactile stimuli with von Frey filaments but also by the Randall–Selitto paw pressure method and by the Hargreaves thermal method.18

Intravenous administration of STR-324 significantly reduced the mechanical hyperalgesia induced by paw incision observed in vehicle-treated rats, with a similar time course at 50 or 250 μg/h doses (P = 0.40; 50 vs. 250 μg/h; fig. 3B). We were not able to observe a significant dose-dependent response
under these conditions; therefore, we may conclude that 50 μg/h is the apparent maximal effective dose in rats. The von Frey nociceptive thresholds of STR-324-treated rats increased significantly compared with vehicle-treated rats ($P = 0.01$ and 0.006; 50 and 250 μg/h vs. vehicle, respectively; fig. 3B). Similarly to opiorphin, the antihyperalgesic effect of STR-324 appeared stable at least up to day 7 post surgery. Mechanical stimulation induced by the Randall–Selitto method led to similar hyperalgesia in the vehicle-treated group. STR-324 infusion significantly abolished this hyperalgesia ($P = 0.034$ and 0.0052; 50 and 250 μg/h vs. vehicle, respectively; fig. 4A). As with von Frey hairs, the effect of 50 and 250 μg/h was similar ($P = 0.20$). Strikingly, the plantar incision–induced thermal hyperalgesia observed among vehicle-treated rats by the Hargreaves method was abolished in STR-324–treated rats at 50- and 250-μg/h doses ($P = 0.0064$ and 0.0052; 50 and 250 μg/h vs. vehicle-treated rats, respectively; fig. 4B). Once more, no difference was observed between the two doses ($P = 0.47$).

**Effects of STR-324 on c-FOSExpression**

Immunohistochemical expression of c-Fos protein has been widely used to identify the nociceptive stimuli–activated neurons. Three hours after paw injury, the number of c-Fos immunoreactive nuclei in the dorsal horn was significantly greater in the ipsilateral as compared to the contralateral side of the vehicle control group ($P = 0.002$; fig. 5, A to C). The total number of surgery-induced c-Fos nuclei was significantly reduced in the ipsilateral side of STR-324–treated group as compared to the vehicle-treated group ($P = 0.0006$; fig. 5, A to C) and was not different between ipsi- and contralateral sides in the STR-324 group ($P = 0.149$; fig. 5, E to C). Thus, we demonstrated the ability of STR-324 to reduce c-Fos expression in the rat spinal cord and therefore to confirm its action on the nociceptive pathways and consequently its intrinsic analgesic properties.

Together these data clearly indicate that both infused opiorphin and STR-324, at 50-μg/h dose, reliably inhibit mechanical allodynia and thermal hyperalgesia induced by plantar incision for at least 4 days post surgery in agreement with its mode of action on pain pathways.

**Opiorphin Infusion Does Not Result in Opioid-like Adverse Effects**

The potential respiratory adverse effects of opiorphin were tested by measuring RR and Spo2 using a noninvasive arterial plethysmographic CollarClip sensor, which measures

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**Fig. 4.** STR-324 shows similar antinociceptive response profiles using two distinct stimuli. (A) Withdrawal thresholds to thermal stimuli determined by the Hargreaves test in rats before and after paw incision, eight rats in each group. Thresholds are expressed as the mean ± SD per second. Open circle = vehicle control group, black square = 50 μg/h STR-324, and black triangle = 250 μg/h STR-324. (B) Withdrawal thresholds to mechanical stimuli determined by the Randall–Selitto paw pressure test in rats before and after paw incision, eight rats per group. Thresholds are expressed as the mean ± SD per gram (gr). Open circle = vehicle control group, black square = 50 μg/h STR-324, and black triangle = 250 μg/h STR-324. Statistical differences between groups are noted in each figure.
HR, RR, and Spo2. MAP and SAP were measured using plethysmography at the tail level. The two doses of opiorphin (50 and 250 μg/h) did not induce any statistically significant changes in these respiratory and cardiovascular variables (Table 1).

**Antinociceptive Effect of STR-324 Requires Endogenous Opioid Pathways**

The antihyperalgesic effect displayed by STR-324 in the postoperative pain model was abolished after intraperitoneal injection of the nonselective opioid receptor antagonist, naloxone (fig. 6).

At day 0, the intergroup difference was not significant ($P = 0.432$). At day 1, after plantar incision, only rats receiving vehicle showed significant mechanical hyperalgesia ($P = 0.00034$; all STR-324 groups vs. vehicle group; fig. 6A). One hour after intraperitoneal injection of naloxone, the STR-324–treated group returned to vehicle control values ($P = 0.421$), whereas the difference remained significant for STR-324 plus intraperitoneal NaCl injection ($P = 0.0008$ and 0.003 by Dunn test; STR-324 vs. vehicle and STR-324 vs. STR-324 plus naloxone, respectively; fig. 6A). No significant difference between groups was observed thereafter at days 2 to 3.

The reversibility by naloxone of the STR-324 potency to exert antihyperalgesic effects clearly proves the involvement of endogenous opioid pathways in its mechanism of action.

The blocking action of naloxone on the antihyperalgesic effect of STR-324 was similar when we measured the postsurgical pain response to thermal stimuli by the Hargreaves method. Thus, at baseline (day 0), the groups did not show any significant difference, whereas at day 1, only rats receiving vehicle showed significant thermal hyperalgesia ($P < 0.00001$; all STR-324 groups vs. vehicle group; fig. 6B). One hour after intraperitoneal injection of naloxone, the STR-324 group returned to vehicle values ($P = 0.262$), whereas the difference remained significant for STR-324 plus intraperitoneal NaCl injection ($P = 0.0002$ and 0.0024 by Dunn test; STR-324 vs. vehicle and STR-324 vs. STR-324 plus naloxone, respectively; fig. 6B). The difference was significantly different thereafter at days 2 to 3 when the STR-324 groups were compared with vehicle ($P = 0.0038$).
Vehicle
Day 0 102 ± 22 96 ± 0 524 ± 44 115 ± 10 88 ± 17
Day 1 111 ± 21 96 ± 1 520 ± 47 116 ± 11 95 ± 12
Day 2 120 ± 10 96 ± 1 515 ± 50 120 ± 6 98 ± 13
Day 3 122 ± 13 96 ± 1 516 ± 42 119 ± 3 95 ± 14
50 μg/h
Day 0 105 ± 12 96 ± 0 517 ± 67 113 ± 12 84 ± 11
Day 1 117 ± 19 97 ± 1 507 ± 39 112 ± 18 81 ± 16
Day 2 117 ± 11 97 ± 0 493 ± 50 123 ± 8 96 ± 13
Day 3 110 ± 11 96 ± 1 506 ± 47 120 ± 12 95 ± 17
250 μg/h
Day 0 101 ± 31 96 ± 1 531 ± 36 111 ± 11 84 ± 19
Day 1 119 ± 5 96 ± 1 534 ± 31 112 ± 12 87 ± 10
Day 2 122 ± 12 96 ± 1 517 ± 43 119 ± 12 92 ± 16
Day 3 120 ± 7 96 ± 1 515 ± 44 124 ± 9 102 ± 15

SpO₂, HR, and RR were evaluated using carotid artery plethysmography and expressed as the mean ± SD of % (SpO₂), beats/min (HR), and breaths/min (RR) for 10 rats per group. MAP and SAP were evaluated using pulse oscillography and expressed as the mean ± SD of mm Hg for 10 rats per group. Continuous intravenous infusion of vehicle or opiorphin (50 or 250 μg/h) was performed for three consecutive days. The two doses of opiorphin (50 and 250 μg/h) did not induce any statistically significant changes, in the respiratory and cardiovascular variables (by two-way ANOVA).

HR = heart rate; MAP = mean arterial pressure; RR = respiratory rate; SAP = systolic arterial pressure; SpO₂ = arterial pulse oxymetry.

Discussion

The discovery of opiorphin, a human endogenous dual inhibitor of enkephalin-inactivating peptidases, was an exciting challenge for the development of a strong analgesic drug without opioid side effects. We are now at the cusp of this development, and here, in vivo pain models are presented that strongly indicate that opiorphin and its derivative STR-324 will represent a new generation of safe, yet potent, analgesics. In animal and clinical studies so far with opiorphin corresponds to a mixture of opiorphin/STR-324 in different ratios according to the experimental conditions used.

The most efficient drugs to date that alleviate severe pain are opioid receptor agonists, such as morphine or its surrogates that interact with MOR. However, their clinical usefulness is limited due to the development of tolerance and dependence that occurs after long-term treatment, while constipation, hypotension, and respiratory depression remain dose-limiting adverse effects after systemic administration. Endogenous enkephalins have a similar affinity than morphine for MOR but much higher affinity for the DOR, and therefore, they need to occupy a smaller proportion of opioid receptors to elicit previous behavioral studies and not by dose-response analyses. In dose-response experiments, morphine analgesic action decreases rapidly and linearly as it is also observed in the current study for 500-μg/h dose. In contrast, the antihypersensitivity effect of opiorphin is stable throughout 4 days post surgery.

In preliminary studies, under our experimental conditions, the N-terminal glutamine of the opiorphin native peptide undergoes transformation to the pyroglutamate form. This pyroglutamate-opiorphin form has been shown to be chemically stable, and recent data demonstrate that it is the natural opiorphin form that would be able to cross the blood–brain barrier (BBB). This highly stable form of opiorphin (STR-324) was chosen for further study and found to have a similar analgesic profile, using the same postoperative rodent model. We confirmed these antinociceptive properties using other pain tests (Randall–Selitto test and Hargreaves test) and obtained similar positive results. We can conclude too that all the previous work performed so far with opiorphin corresponds to a mixture of opiorphin/STR-324 in different ratios according to the experimental conditions used.

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Opiorphin avoids, by its targeted action, excessive stimulation of the ubiquitousy distributed MOR that is, at least in part, responsible for major morphine-induced adverse effects. Previous reports demonstrated that opiorphin does not develop significant abuse liability or antinociceptive drug tolerance when subchronically administered at morphine equieffective analgesic doses. In addition, morphine-induced antiperistalsis was not observed for opiorphin after acute administration. In the current study, for the first time, the potential effects of opiorphin on the occurrence of respiratory and hemodynamic disturbances were evaluated. In the absence of external painful stimuli, the tonic release of enkephalins is very weak. Therefore, the safety tests were carried out under conditions of nociception-induced increase in enkephalin secretion, leading to significant opiorphin pharmacologic action. The potential respiratory and hemodynamic adverse effects of opiorphin were tested, and no effects were found. Opiorphin consistently failed to induce respiratory depression and major hemodynamic variations, such as HR, SAP, and MAP, after sustained systemic infusion even at high doses (five-fold the antinociceptive effective dose). A recent study reported that intravenous bolus administration of opiorphin could induce transient increase in the blood pressure of conscious rats, through the renin–angiotensin system; due to methodological differences, this effect was not observed in the current study.

We investigated the STR-324 mechanism of action, using the same plantar incision–induced pain rat model. We first tested the c-Fos expression change in neurons of the dorsal horn of spinal cord using an immunohistochemical approach. c-Fos has been described as a marker of neuronal populations involved in nociception, in particular at the spinal nociceptive
inputs. Nociceptive stimulation produces an increase of early gene c-Fos expression and thus its c-Fos protein product. There is a correlation between the abilities of analgesics to inhibit nociceptive behaviors and to decrease or abolish c-Fos expression in the spinal cord dorsal horn. In the current study, the infusion of STR-324 decreased the number of c-Fos-immunoreactive neurons compared to vehicle, especially neurons located in the superficial laminae (I and II) of the spinal cord dorsal horn, which contains numerous neurons responding to nociceptive stimuli. This finding reinforces the behavioral data described (fig. 4) and confirms the tissue-specific site of action of STR-324 on nociceptive processes and consequently its intrinsic analgesic properties.

Second, by using the broad-spectrum opioid antagonist, naloxone, on the antinociceptive potency of STR-324, we found that naloxone when administered at a maximal efficacy period of STR-324 (day 1) abolishes its mechanical and thermal antihyperalgesic effects. This finding indicates that the antinociceptive effect of STR-324 on incisional pain is mediated by opioid receptors and confirms previous results obtained with opiorphin. Altogether, these data show the involvement of endogenous opioid receptor–dependent pathways in the mode of action of both glutamine 1- and pyroglutamate 1-opiorphin (STR-324) forms. Other experiments would be necessary to first evaluate the respective roles of MOR, DOR, and K- opioid receptor pathways in STR-324 effects and second to determine at which level, central and/or peripheral pain pathways, the efficient sites of action of opiorphin and STR-324 are preponderant. Indeed, in vitro and in vivo data demonstrated that opiorphin and STR-324 are able to cross the BBB regulatory interface. Thus, after intravenous bolus injection of tritiated opiorphin in rats, a selective uptake of the radioactive compound at the level of brain tissues is observed. Similarly, after intravenous infusion of tritiated STR-324 in monkeys, a selective sequestration of the radioactive peptide was observed in the spinal and cerebral tissues (unpublished data of rat and monkey pharmacokinetic analyses, C. Rougeot and V. Juarez-Perez, September 2013 and April 2015, respectively). Moreover, using a BBB culture model, it has been recently demonstrated that opiorphin is transferred through the cultured BBB and that the N-terminal glutamine form of opiorphin is converted into the pyroglutamate form (STR-324) during the transfer.

Our results provide strong evidence that opiorphin and STR-324 are able to induce a long-lasting analgesic effect without adverse effects, compatible with clinical development. The pharmaceutical development of enkephalinase inhibitors has in mind the paradigm “provide analgesia at the right time at the right moment.” Enkephalins are released by nervous tissues in response to time-localized nociceptive stimuli. In the postoperative pain model used here, the pharmacodynamic response profile of opiorphin-treated animals demonstrates that opiorphin acts only during the painful phase. In addition, the opiorphin efficacy kinetic profile is different from that of morphine; morphine induces a rapid antihyperalgesia with a rapid onset and rapid decrease of potency, whereas the antihyperalgesic potency induced by opiorphin remains stable for at least 4 days and is the mirror image of the algesic response of vehicle-treated animals. The decrease in the antihyperalgesic action of morphine could be explained by the development of acute tolerance and/or opioid-induced hyperalgesia; the stable antihyperalgesic action observed during opiorphin administration suggests that opiorphin does not have these adverse effects. Further experiments are needed to confirm this hypothesis.

The absence of adverse effects, such as respiratory depression, and of major hemodynamic disturbances after continuous systemic treatment with opiorphin may be due to the limited and targeted stimulation of enkephalin-dependent opioid receptors and is consistent with a lack of nonspecific activation of the opioid pathway innervating pulmonary and cardiovascular systems.

Despite the compelling advantages of opiorphin peptide, its pharmaceutical development is hampered by its low stability, typical of N-terminal glutamine peptides and caused by enthalpy instability. An exciting new possibility using the native opiorphin analog, STR-324, that retains the characteristics and efficacy of opiorphin analgesic potency yet is stable under physiologic conditions is now available. Due to the close structural similarities between the two compounds, we hypothesize that STR-324 administration, as demonstrated in the case of opiorphin, will not give rise to opioid agonist side effects. STR-324 could, therefore, become a major player in postoperative pain treatment in the near future.

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Competing Interests
Dr. Benhamou is the president of Mise Au Point en Anesthésie-Réanimation Foundation (Le Kremlin-Bicêtre, France), which cofounded the anesthesiology laboratory 30 yr ago. Dr. Juarez-Perez is employed by Stragen France.
SAS (Lyon, France) as a scientific advisor for the development of opiorphin and STR-324. Dr. Rougeot headed a research laboratory at Institut Pasteur (Paris, France), which has applied for a patent for the use of opiorphin and its derivatives in the treatment of pain. Dr. Sitbon received financial assistance (registration, transport, and hotel) from Stragen France to present a part of this work at the American Society of Anesthesiologists meeting in 2013 in San Francisco, California. The other authors declare no competing interests.

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References

From Chloroforming Snakes to Sculpting Marble: The Arts of Harriett Hosmer

A physician’s daughter, Harriett Hosmer (1830 to 1908, left) studied human anatomy at Missouri Medical College before returning to her native Massachusetts. From 1853 to 1860, she apprenticed in Rome, Italy, with master sculptor John Gibson of Wales. As Gibson’s “dear little Hatty,” Hosmer had sworn off marriage (and rearing children) in deference to mastering her art. As her “second daughter,” Hosmer sculpted a bust (right) of Medusa to complete her first original commission in Rome (1853 to 1854). While preparing to carve the snaking curls of Medusa, Hosmer chloroformed and then plaster-cast snakes before releasing them back to the wild. Popular in Rome with both American and British expatriate artists, Hosmer battled overtly against gender bias and covertly against gender-preference bias to gain public recognition as America’s most “Distinguished Sculptress” of the nineteenth century. Around 1900, Hosmer sailed from England back to New England, eventually dying eight years later in the town of her birth, Watertown, Massachusetts. (Copyright © the American Society of Anesthesiologists’ Wood Library-Museum of Anesthesiology.)

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