

## Prevention and Reversal of Morphine-Induced Tolerance by Novel Muscarinic Receptor Agonist in Rats with Neuropathic Pain

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### Abstract

**Objective:** To investigate the effectiveness of a novel M2 muscarinic receptor agonist, the pyrazolo[3,4-b]pyrrolo[3,4-d]pyridine derivative LASSBio-981, on the prevention and reversal of morphine-induced antinociceptive tolerance in a rat model of neuropathic pain.

**Methods:** Thermal hyperalgesia and mechanical allodynia were induced in rats by spinal nerve ligation in L5. After rats displayed signs of sustained pain, continuous infusions of morphine were delivered in the peritoneum through osmotic mini-pumps. LASSBio-981 was daily administered by oral gavage starting at the same day of morphine infusion (prevention protocol) or after development of morphine-induced tolerance (reversion protocol). To access the mechanism of LASSBio-981 action on the M2 muscarinic receptor, a single dose of the specific antagonist, methoctramine, was injected through intrathecal route. In addition, the crystallographic structure of human M2 muscarinic receptor and the computer program GOLD 5.2 (genetic algorithm software: CDDC) simulated the docking of LASSBio-981 into the orthosteric binding site of the receptor.

**Results:** Morphine inhibited the thermal and mechanical hyperalgesia induced by spinal nerve ligation and this effect decreased time-dependently and totally disappeared (tolerance) 16 days of infusion. LASSBio-981 prevented and reversed morphine-induced antinociceptive tolerance. Treatment with methoctramine inhibited LASSBio-981 effect in reversing the morphine-induced tolerance suggesting the involvement of M2 muscarinic receptor activation. This hypothesis was strengthened because the molecular docking analyses determined that LASSBio-981 interacts with the orthosteric binding site of the M2 muscarinic receptor.

**Conclusion:** This study provides evidence that a novel M2 muscarinic receptor agonist (LASSBio-981) may prevent and reverse morphine-induced tolerance in rat model of neuropathic pain.

**Keywords:** Morphine tolerance; Hyperalgesia; Allodynia; LASSBio-981; Opioid receptors; M2 muscarinic receptor; Methoctramine; Chronic pain; Osmotic mini-pump

### Introduction

Recent studies estimated that 7–8% of the general population suffers from chronic pain with neuropathic features [1]. Unfortunately, current pharmacotherapies used to treat the main symptoms of this disorder, hyperalgesia and allodynia, are not completely effective [2]. Current treatments utilize several classes of chemically distinct drugs, including opioids, tricyclic antidepressants (TCAs), alpha-2 adrenergic receptor agonists and anticonvulsants [3-5]. Of these treatments, opioids are the most effective at managing chronic pain. There are three known opioid receptor subtypes,  $\mu$ ,  $\delta$ , and  $\kappa$ . Among them, the  $\mu$ -receptor has been the target of drug development for pain medication [6]. Morphine is a  $\mu$ -receptor agonist that is very effective in the treatment of chronic pain; however, its prolonged use may be followed by severe side effects and physical

dependence. Several studies have shown that chronic morphine treatment results in tolerance and hyperalgesia [7-9].

The development of opioid tolerance is typically measured as a change in antinociceptive or analgesic responses and likely involves several mechanisms. There is evidence that the analgesic effects of  $\mu$ -opioid agonist are reduced in a nerve ligation injury animal model of chronic pain [10]. Chronic morphine exposure triggers an intracellular signaling cascade that results in opioid receptors desensitization and endocytosis. Following endocytosis, receptors can be recycled and moved to cell membrane, stored in an intracellular compartment, or degraded in the cell cytoplasm [11,12]. Morphine-induced tolerance is also associated with activation of glia in the spinal cord, which could produce several neuroexcitatory molecules as nitric oxide, prostaglandins, adenosine triphosphate, excitatory amino acids and reactive oxygen species (ROS) [13]. The neuroexcitatory molecules and ROS enhance pain and may directly contribute to the mechanism of morphine-induced tolerance [14].

Clinical and pre-clinical studies suggest that cholinergic mechanisms in the spinal cord and primary afferents fibers are also involved to analgesic effects. Acetylcholinesterase inhibitors and muscarinic acetylcholine receptor agonists produce analgesia in humans and animals in part, by activating M2 muscarinic receptors that inhibit nociceptive input to primary sensory neurons [15]. M2 muscarinic receptors are up-regulated in animals with diabetic neuropathy and peripheral nerve injury [15,16]. Acetylcholine modulates pain by increasing endogenous opiate peptides in the rat spinal cord [17]. Morphine dependence and withdrawal can produce changes in cholinergic signaling [18].

Based on the chemical structure of zolpidem, [a gamma-aminobutyric acid (GABA)A receptor agonist], novel pyrazolo[3,4-b]pyrrolo[3,4-d]pyridine derivatives have been synthesized for use in pain modulation. Among these new derivatives, the antinociceptive effect of LASSBio-872, LASSBio-873, LASSBio-980 and LASSBio-981 has been characterized in hot plate, formalin, carrageenan and neuropathic pain models [19-21].

In model of neuropathic pain, the analgesic effect of LASSBio-873 were inhibited by intrathecal injection of methocitramine, suggesting that M2 muscarinic receptors are involved in LASSBio-873-induced analgesia [20]. The muscarinic receptor-mediated analgesia has been demonstrated to occur in the spinal cord [22].

Of the pyrazolo[3,4-b]pyrrolo[3,4-d]pyridine derivatives, we recently demonstrated that the oral route administration of LASSBio-981 compound produced greater analgesia than the LASSBio-873 in a rat model of chronic pain (unpublished observations). Thus, in the present study, we evaluated the antinociceptive effects of LASSBio-981, using morphine-induced tolerance in a rat model of neuropathic pain. In addition, we also used molecular docking analysis to characterize the interactions of LASSBio-981 with the M2 muscarinic acetylcholine receptor.

## Methods

### Animals and housing

All protocols used were blinded and approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro, following the guidance of the Brazilian National Council of Experimental Animal Use Control (2014). Adult male Wistar rats (180-220 g) were housed on a 12 h light/dark cycle (light on at 6:00 a.m.) with ad libitum access to food and water. Room temperature and relative humidity were maintained at  $22 \pm 1^\circ\text{C}$  and  $60 \pm 5\%$ , respectively. Animals were acclimated to the laboratory for at least 30 min prior to the experimental testing and were randomly divided into different groups ( $n=4$  per group calculated using statistical power analysis) [23].

### Drugs

Morphine sulfate and amitriptyline hydrochloride were donated by Cristália Produtos Químicos e Farmacêuticos Ltda (Itapira, SP, Brazil). Pyrazolo [3,4-b] pyrrolo [3,4-d] pyridine (LASSBio-981) was synthesised and provided by the Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio) at UFRJ. Methocitramine tetrahydrochloride hydrate was purchased from Sigma-Aldrich (St Louis, MO, USA). All chemicals were dissolved in 0.9% saline, except for LASSBio-981, which was dissolved in dimethyl sulfoxide (DMSO; Gaylord Chemical Company L.L.C, USA).

### Surgical procedures

Neuropathic pain was induced by spinal nerve ligation (SNL), according to previously published methods [21]. Briefly, rats were anesthetized with ketamine (100 mg/kg, intraperitoneal [i.p.]) and xylazine (5 mg/kg, i.p.). Skin around the surgical site was sterilized with 0.5% chlorhexidine. An incision (~2.5 cm) was made over the L5-S1 spinal segment and the paravertebral musculature was retracted from the vertebral transverse processes. The L6 transverse process was partially removed, exposing the L4 and L5 spinal nerves. The L5 spinal nerve was ligated with 6-0 silk suture. Animals were individually housed after surgery during all time of study.

### Nociceptive behavioral testing: thermal hyperalgesia and mechanical allodynia

Paw withdrawal latency was measured after application of radiant heat to the hind paw of animals [24,25]. Hind paw withdrawal was detected as an interruption in the light beam heat source. Latency was defined as the duration of time until an interruption occurred as determined by a Dynamic Plantar Esthesiometer (model 37450, Ugo Basile SRL, ITALY). To prevent tissue damage, maximal latency was set at 30 s. Latency values were calculated as the average response of three measurements. Thermal hyperalgesia was defined as significantly shorter withdrawal latencies in SNL animals compared to control.

To measure mechanical allodynia, a Digital Analgesimeter pressure transducer (model EFF301, Insight, SP, Brazil) was used to apply increasing pressure of a trough plastic tip (1 mm external diameter) to the paw [26-28]. The mechanical threshold was defined as the pressure applied when the animal lifted its paw. The average value of five trials was taken as the mechanical threshold. To avoid tissue damage, maximal pressure was set to 120 g. Mechanical allodynia was defined as a statistically significant reduction in the mechanical threshold of SNL-treated animals compared to controls.

### Morphine-induced tolerance

After thermal hyperalgesia and mechanical allodynia had developed and stabilized 7 days SNL surgery osmotic minipumps (Alzet, Cupertino, CA) were implanted for infusion of morphine (2.5 mg/kg/d) for 16, 23 or 30 days at a constant delivery rate of 2.5  $\mu\text{l/h}$ . The pump size was selected to allow at least 30 days of continuous morphine infusion. In SNL animals, tolerance to morphine-induced analgesia emerged after as few as 3 days of continuous morphine treatment. The tolerance effect was completely developed by 16 days, when the pain reactions of SNL-treated animals returned to control level. LASSBio-981 (10 mg/kg/d), amitriptyline (10 mg/kg/d, positive control), or vehicle (DMSO, 100  $\mu\text{l/d}$ ) was administered orally for the remaining 14 days (therapeutic or reversion protocol). For the prophylactic or prevention protocol, LASSBio-981 (10 mg/kg/d), amitriptyline (10 mg/kg/d) or vehicle (DMSO, 100  $\mu\text{l/d}$ ) was administered from 7 to 16 days after surgery.

### Mechanism of action of LASSBio-981

To investigate the potential involvement of M2 muscarinic receptors as targets for LASSBio-981, methocitramine (10  $\mu\text{g}$  in 40  $\mu\text{l}$ ) was delivered by intrathecal administration into the L4-L5 intervertebral space under light anesthesia (sevoflurane 3 vol% gas/gas) [24]. Methocitramine treatment occurred after 7 days of oral treatment with LASSBio-981 (10 mg/kg/d). Nociceptive test started 15

min after animals recovered from anesthesia. Paw withdrawal latency was measured before and 15, 30, 60, 90, 120, and 150 min after methocramine administration.

### Docking of LASSBio-981 to the human M2 muscarinic receptor

Molecular docking studies were performed with the crystallographic structure of human M2 muscarinic receptor (Protein Data Bank, PDB ID: 3UNO) [25]. GOLD 5.2 (genetic algorithm software: CCDC) was used to simulate docking of LASSBio-981 into the orthosteric binding site of the receptor. A distance of 10 Å from the Tyr104 residue was used to define the set of amino acid residues selected as the binding site. To validate the methodology, the GoldScore fitness function was used to perform a re-docking study using the structure of the M2 muscarinic receptor antagonist 3-quinuclidinyl-benzilate (QBN).

The structure of LASSBio-981 was energy-minimized by the ab initio HF/3-21G method and Spartan'08 software (Wavefunction Inc.). Docking runs with the optimized LASSBio-981 structure were conducted in triplicate; each run generated 10 poses. The pose with the highest score of all runs was chosen for analysis. The scale of the score gives an indication of pose fit, with higher scores indicating a docking result.

### Statistical analysis

Data were expressed as the mean  $\pm$  standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) with Newman-Keuls post-test was used for multiple group comparison.  $P < 0.05$  was considered statistically significant.

## Results

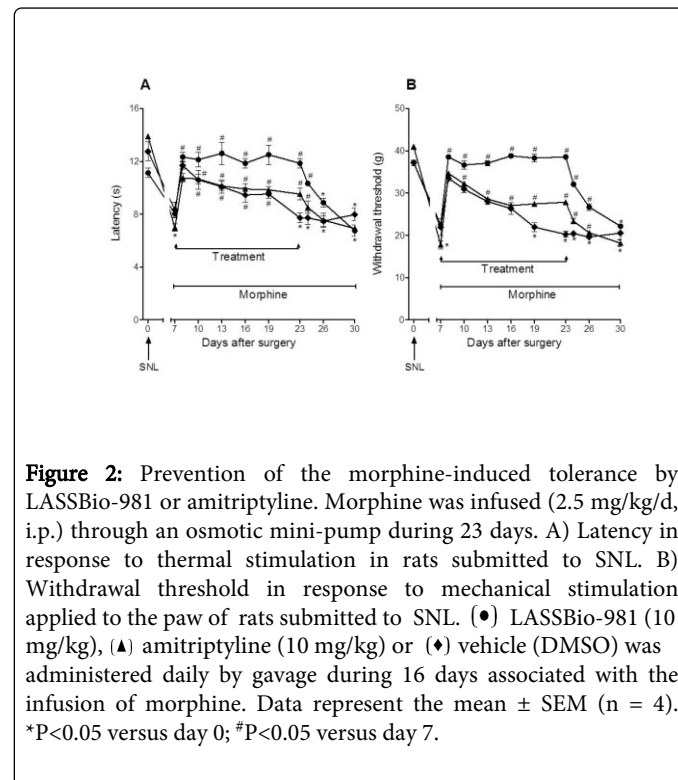
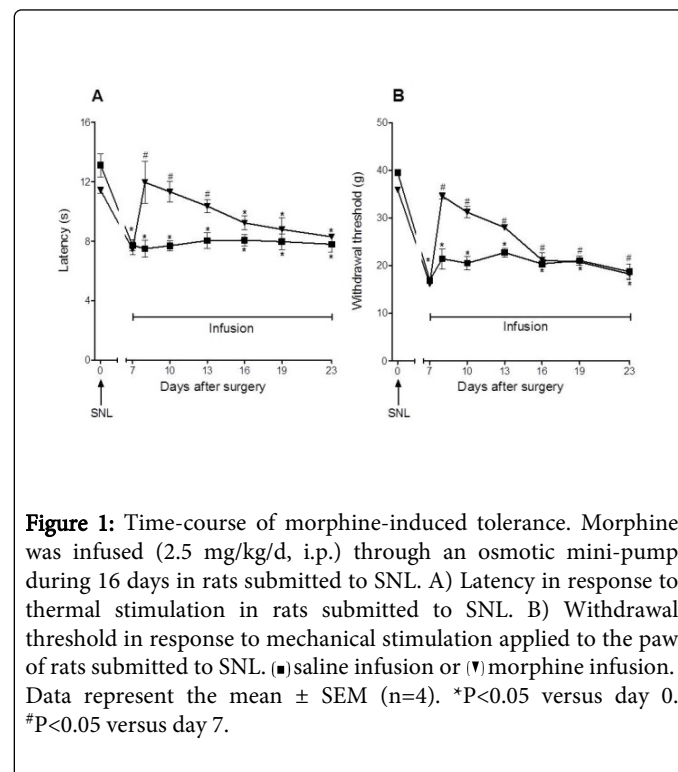
### Morphine-induced antinociceptive tolerance

In SNL-treated animals thermal stimulation-induced paw withdrawal latency decreased over 7 days after SNL ( $11.4 \pm 0.2$  to  $7.5 \pm 0.3$  s;  $P < 0.05$ ), but increased after 1 day of continuous morphine infusion ( $2.5$  mg/kg/d, i.p.;  $12.0 \pm 1.4$  s;  $P < 0.05$ ; Figure 1A). Treatment with vehicle did not cause any antinociceptive effect. Morphine tolerance developed over the first 16 days of continuous morphine exposure, with withdrawal latency returning to pre-morphine levels by day 16 ( $8.3 \pm 1.5$  s; Figure 1A). The mechanical stimulation threshold in SNL-treated animals decreased 7 days after SNL treatment ( $35.9 \pm 0.3$  to  $16.2 \pm 0.4$  g;  $P < 0.05$ ), but increased after 1 day of continuous morphine infusion ( $34.6 \pm 0.6$  g;  $P < 0.05$ ). The mechanical stimulation withdrawal threshold decreased over the first 16 days of continuous morphine infusion, returning to pre-morphine levels by day 16 ( $18.2 \pm 1.0$  g; Figure 1B).

### Effect of LASSBio-981 on morphine-induced tolerance

Morphine was infused for 23 days after the animals were submitted to SNL and after thermal hyperalgesia and mechanical allodynia were established. The thermal stimulation-induced paw withdrawal latency was decreased in all groups 7 days after SNL treatment (LASSBio-981, amitriptyline and DMSO;  $P < 0.05$ ,  $n = 4$  per group; Figure 2A), but increased back to control levels after 1 day of continuous morphine infusion. Morphine-induced tolerance developed in a time-dependent manner. Co-administration of oral amitriptyline ( $10$  mg/kg/d) did not

change the course of morphine-induced tolerance; however, oral LASSBio-981 ( $10$  mg/kg/d) completely blocked this effect (Figure 2A).



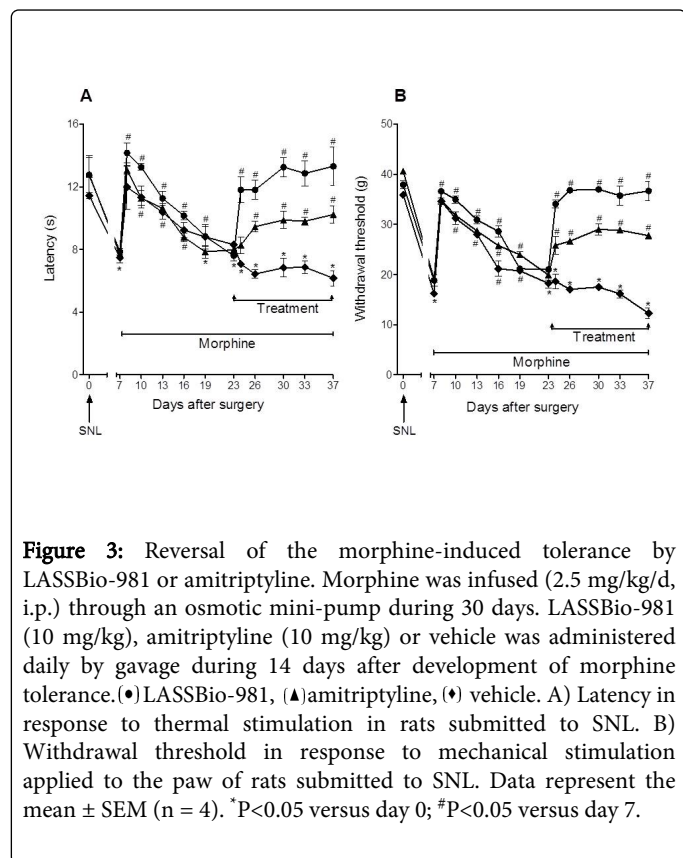
The mechanical stimulation withdrawal threshold was reduced into to all groups 7 days after SNL treatment (LASSBio-981, amitriptyline and DMSO;  $P < 0.05$ ;  $n = 4$ ; Figure 2B), but returned to control levels after 1 day of continuous morphine infusions. Again, tolerance to

morphine developed in a time-dependent manner. Co-administration of oral amitriptyline (10 mg/kg/d) prevented tolerance after 12 to 16 days of administration (Figure 2B). However, morphine-induced tolerance was completely prevented by 16 days of LASSBio-981 (10 mg/kg/d) administration (Figure 2B). This effect was spontaneously reversed by discontinuation of LASSBio-981 treatment.

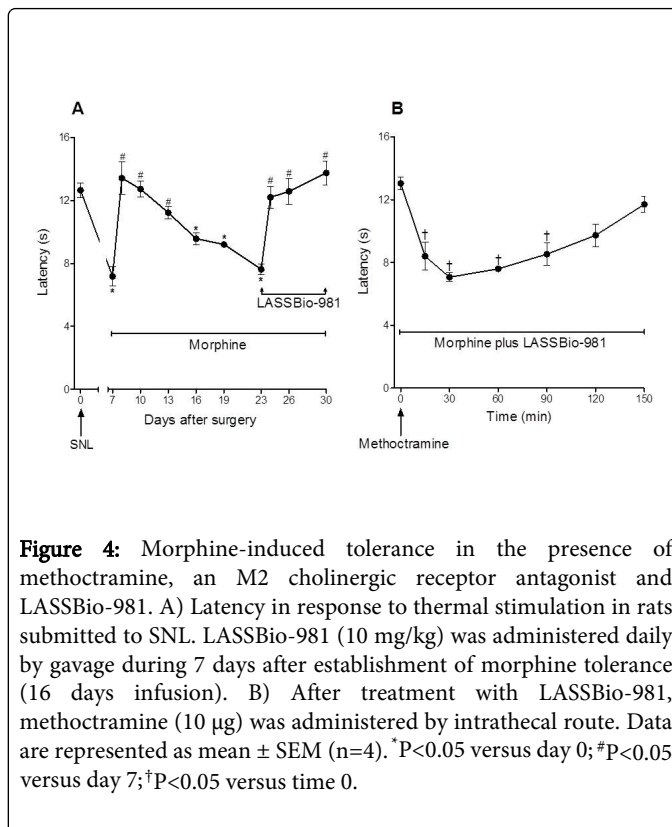
### Effect of LASSBio-981 on reversal of morphine-induced tolerance

The thermal and mechanical stimulation-induced paw withdrawal responses were reduced 7 days after SNL treatment ( $P < 0.05$ ). However, these response increased back to control levels after 1 day of continuous morphine infusion (Figures 3A and 3B). Morphine gradually developed tolerance over 16 days at which time pain responses returned to pre-morphine levels. After 14 days of oral administration, both amitriptyline (10 mg/kg/d;  $8.0 \pm 0.3$  s to  $10.2 \pm 0.5$  s;  $P < 0.05$ ) and LASSBio-981 (10 mg/kg/d;  $7.7 \pm 0.2$  to  $13.3 \pm 1.2$  s;  $P < 0.05$ ) increased the thermal stimulation-induced withdrawal latency. LASSBio-981 was more effective than amitriptyline reversing the morphine-induced tolerance (Figure 3A).

Amitriptyline and LASSBio-981 both increased the mechanical stimulation-induced withdrawal threshold ( $19.9 \pm 1.0$  to  $29.0 \pm 1.1$  g and  $21.0 \pm 0.6$  to  $37.0 \pm 0.1$  g, respectively;  $P < 0.05$ ). LASSBio-981 was more effective than amitriptyline in reversing tolerance to antinociceptive effect of morphine. No effects of vehicle



**Figure 3:** Reversal of the morphine-induced tolerance by LASSBio-981 or amitriptyline. Morphine was infused (2.5 mg/kg/d, i.p.) through an osmotic mini-pump during 30 days. LASSBio-981 (10 mg/kg), amitriptyline (10 mg/kg) or vehicle was administered daily by gavage during 14 days after development of morphine tolerance. (●) LASSBio-981, (▲) amitriptyline, (○) vehicle. A) Latency in response to thermal stimulation in rats submitted to SNL. B) Withdrawal threshold in response to mechanical stimulation applied to the paw of rats submitted to SNL. Data represent the mean  $\pm$  SEM ( $n = 4$ ). \* $P < 0.05$  versus day 0; # $P < 0.05$  versus day 7.



**Figure 4:** Morphine-induced tolerance in the presence of methoctramine, an M2 cholinergic receptor antagonist and LASSBio-981. A) Latency in response to thermal stimulation in rats submitted to SNL. LASSBio-981 (10 mg/kg) was administered daily by gavage during 7 days after establishment of morphine tolerance (16 days infusion). B) After treatment with LASSBio-981, methoctramine (10  $\mu$ g) was administered by intrathecal route. Data are represented as mean  $\pm$  SEM ( $n=4$ ). \* $P < 0.05$  versus day 0; # $P < 0.05$  versus day 7; † $P < 0.05$  versus time 0.

administration (DMSO) were found on reversal of morphine tolerance (Figure 3B).

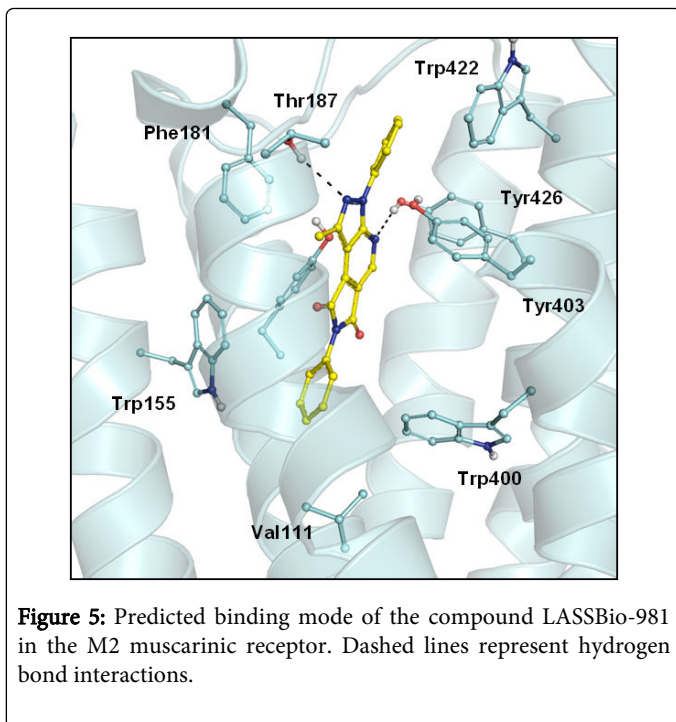
### Analgesic mechanism of LASSBio-981 on morphine tolerance

Daily oral administration of LASSBio-981 (10 mg/kg/d) completely reversed morphine-induced tolerance (Figure 4A). Intrathecal methoctramine (10  $\mu$ g) completely inhibited this effect within 30 min of administration followed by spontaneous recovery of the LASSBio-981-induced reversal of morphine tolerance (Figure 4B).

### Molecular docking analysis

The RMSD of redocked QBN and co-crystallized QBN structures was 0.43 Å for all atoms validating the chosen fitness function. Due to the proximity of the Thr187 side chain and a hydrogen bond acceptor group in LASSBio-981, we performed a semi-rigid docking conferring flexibility to the Thr187 side chain. The semi-rigid methodology provides a higher score than the rigid methodology and the best associated pose was chosen for further analyses.

Molecular docking analysis revealed that LASSBio-981 interacted primarily in the orthosteric binding site of the human M2 muscarinic receptor. This interaction was with two hydrogen bonds between the pyrazolo-pyridine moiety of LASSBio-981 and the hydroxyl groups of residues Tyr426 and Thr187 of the M2 muscarinic receptor. In addition, LASSBio-981 exhibited hydrophobic interactions with a pocket of amino acids formed by the side chains of residues Tyr104, Trp155, Trp400 and Tyr403 (Figure 5).



**Figure 5:** Predicted binding mode of the compound LASSBio-981 in the M2 muscarinic receptor. Dashed lines represent hydrogen bond interactions.

## Discussion

Morphine is very effective treatment for pain, but prolonged use can result in several serious side effects, including tolerance, cross-tolerance to others  $\mu$ -opioids agonists and physical dependence. The current study found that 16 days of continuous morphine exposure (via osmotic mini-pumps) in SNL-treated rats produced a complete tolerance in reducing thermal hyperalgesia and mechanical allodynia. Osmotic mini-pumps are a reliable methods for continuous drug infusion that overcome the plasma drug concentration fluctuation associated with intermittent administration [29,30]. Here, we used this method to investigate the effect of a zolpidem derivative, LASSBio-981, on the prevention or reversal of the morphine-induced tolerance in an animal model of neuropathic pain.

Chronic morphine use has been suggested to produce tolerance through a number of different mechanisms, including desensitization and endocytosis of opioid receptors [12], activation of glia cells [31], sustained morphine-mediated augmentation of spinal calcitonin gene-related peptide immunoreactivity [29], increased spinal NK1 receptor and NMDA receptor expression [8,30].

Previously, we demonstrated that a new zolpidem analogue, LASSBio-873, was effective at reducing thermal hyperalgesia and mechanical allodynia in rats submitted to SNL-treatment [20]. The structural difference between LASSBio-981 (used in the current study) and LASSBio873 is the presence of a nitro group in the para position of one of the phenyl rings connecting the pyrazolo[3,4-b]pyrrolo[3,4-d]pyridine moiety of LASSBio-873. Oral administration of LASSBio-981 has been found to produce greater pain reduction than LASSBio-873 in an animal model of neuropathic pain (data not shown). Thermal hyperalgesia and mechanical allodynia induced by SNL rats were partially (ca. 50%) reversed by treatment with LASSBio-981 [31]. The antinociceptive effect of LASSBio-981 was sustained with no observation of tolerance; however animals were short-term treated for 7 days. Notably, LASSBio-981, but not

amitriptyline, completely prevented and reversed morphine-induced tolerance even after 14 days of treatment. Combination treatment with morphine and LASSBio-981 was more effective than with LASSBio-873 alone [20]. The effects of LASSBio-981 were inhibited by intrathecal administration of methoctramine, suggesting that the M2 muscarinic receptor-signaling pathway aids in mediating these effects. Those effects could occur through direct activation of muscarinic receptors or by inhibition of the enzyme acetylcholinesterase (AChE) which in turn could increase ACh level. However, LASSBio-981 did not inhibit the activity of AChE (< 10%, data not shown), indicating that the action of LASSBio-981 occurs by direct interaction with muscarinic receptors.

Morphine tolerance can be reduced in rats by several other compounds, including lamotrigine (an anticonvulsant drug) [33], vigabatrin (an antiepileptic drug) [34] and gabapentin (an anti-epileptic drug) [35]. Tricyclic antidepressants (TCAs), commonly used for the treatment of major depressive disorders, are also used in the treatment of neuropathic pain. In agreement with others [36], here we found that the TCA amitriptyline attenuated tolerance only to the antinociceptive effects of morphine.

Several reports have shown that the cholinergic system plays an important role in pain modulation. The intrathecal administration of acetylcholine increased L-enkephalin,  $\beta$ -endorphin and dynorphin A1-13 concentrations in the rat spinal cord. This effect was reduced by either the non-selective muscarinic receptor antagonist atropine or the nicotinic receptor antagonist gallamine [17]. The activation of M2 muscarinic receptors regulated glutamate release in spinal cord primary afferent neurons was demonstrated using electrophysiological technique [37]. In addition, the M3 and M2/M4 muscarinic receptor subtypes are involved in regulation of glutamate release from a subpopulation of interneurons in the rat spinal cord [37].

Following tight ligation of the L5 and L6 spinal nerves in rats, there was an increase in M2 muscarinic receptor immunoreactivity in both ipsilateral and contralateral DRG neurons [15]. In a streptozotocin-induced neuropathic pain model in rats, intrathecal administration of the non-selective muscarinic receptor agonist, muscarine, had antinociceptive effects [16]. Antinociceptive effect produced by muscarinic receptor activation has also been demonstrated with direct cholinergic agonists [38] and acetylcholinesterase inhibitors [39]. Intrathecal administration of a nicotinic agonist has also been shown to produce antinociceptive effects [40].

The intrathecal administration of the muscarinic receptor antagonist atropine and the M1/M4 antagonist pirenzepine produced a dose-dependent inhibition of morphine-induced antinociception. Interestingly, neither the M2 muscarinic receptor antagonist methoctramine nor the M3 muscarinic receptor antagonist 4-DAMP was found to inhibit morphine-induced antinociception [41].

The elucidation of the crystal structure of the human M2 muscarinic receptor combined with mutagenesis data has led to important advancements in molecular recognition of agonists and antagonists binding sites. LASSBio-981 was found to interact with amino acids residues involved primarily in agonist binding sites, such as Trp155 and Trp400 [42]. Importantly, using semi-rigid methodology, we were able to establish a hydrogen bond with LASSBio-981. We determined that the binding site was near the Thr187 residue of M2 muscarinic receptor. In support of our findings that LASSBio-981 is a potential M2 muscarinic receptor agonist,

others have shown that the Thr187 residue is involved in the binding of agonists but not antagonists [42].

## Conclusion

Our findings suggest that LASSBio-981 can prevent and reverse the development of morphine tolerance. This study also indicates that M2 muscarinic receptor activation can enhance morphine-induced antinociception.

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