

## Novel Nicotinic Receptor Agonist Reduces Hyperalgesia and Allodynia of Neuropathic Pain in Diabetic Rats

Roberto C Debom<sup>1,2</sup>, Margarete M Trachez<sup>3,4</sup>, Gisele Zapata Sudo<sup>4\*</sup>, Jaqueline S da Silva<sup>4</sup>, Kesley M Oliveira<sup>2</sup>, Ariel L Garcia<sup>2</sup>, Lidiane M Godoy<sup>2</sup>, Ogari C Pacheco<sup>2</sup> and Roberto T Sudo<sup>1,4</sup>

<sup>1</sup>Programa de Pós-graduação em Medicina (Cirurgia Geral), Universidade Federal de Rio de Janeiro, Rio de Janeiro, Brazil

<sup>2</sup>Cristália Produtos Químicos e Farmacêuticos Ltda, Itapira, São Paulo, Brazil

<sup>3</sup>Disciplina de Anestesiologia, Universidade Federal Fluminense, Rio de Janeiro, Brazil

<sup>4</sup>Programa de Desenvolvimento de Fármacos, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

\*Corresponding author: Gisele Zapata-Sudo, M.D., Ph.D., Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Instituto de Ciências Biomédicas, Bloco J, Sala 14, Rio de Janeiro, RJ, Brazil, 21941-590 Tel: 55-21-25626478; Email: gsudo@icb.ufrj.br

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### Abstract

Diabetic Peripheral Neuropathic Pain (DPNP) has been attributed to abnormal glucose metabolism and oxidative stress. It is among the most common consequence of chronic diabetes and is treated with antiepileptic compounds, antidepressants, and opioids. However, many patients with DPNP experience lack of treatment efficacy and/or adverse secondary effects that impair quality of life. Here we report the identification of a novel agonist targeting neuronal nicotinic Acetylcholine Receptors (nAChRs) that may alleviate neuropathic pain. A new pyrazole analogue, Cris-104, has been observed to produce antinociceptive activity in acute and chronic pain models. In the present study, Cris-104 effects on thermal hyperalgesia and mechanical allodynia were evaluated in a rat DPNP model. Oral administration of Cris-104 (35 mg.kg<sup>-1</sup>.day<sup>-1</sup> for 7 days) induced antinociception as evidenced by reductions in thermal hyperalgesia and mechanical allodynia in DPNP rats. The mechanism of antinociception cannot be attributed to a diabetes-alleviating effect because blood glucose levels were not reduced significantly by the treatment. The analgesia could be mediated by activation of nAChRs since Cris-104 is a specific ligand for the nAChR. In conclusion, Cris-104 is a compound with potential to be used as a pain reliever in patients with DPNP.

**Keywords:** Diabetic rats; Nicotinic receptor agonist; Neuropathic pain; Hyperalgesia; Allodynia

### Introduction

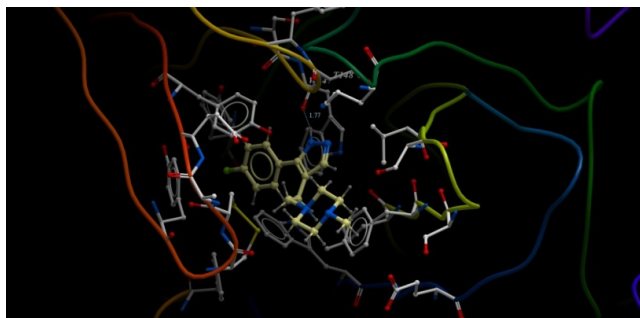
Diabetes is a metabolic disease that can induce long-term complications, such as nephropathy, retinopathy, and neuropathy. Diabetes affects approximately 383 million people worldwide currently and its prevalence is expected to increase in the coming decades [1]. Diabetic Peripheral Neuropathic Pain (DPNP) is a highly debilitating sequela of chronic diabetes that affects some 60% of diabetic patients. It is a consequence of nervous system lesions induced by abnormal glucose metabolism and oxidative stress [2,3]. DPNP is treated with a variety of classes of drugs including anticonvulsants, antidepressants, narcotic analgesics, and local anesthetics. However, many patients are unable to manage their neuropathic pain effectively and the currently prescribed drugs can produce adverse secondary effects that impair quality of life. The pharmaceutical industry is challenged with identifying new pharmacological treatments that can produce greater clinical benefits for patients with DPNP, while reducing side effects and frequency of dosing [4].

The demonstration that the nicotinic cholinergic receptor (nAChR) agonist epibatidine is an effective analgesic agent shifted the attention of pain researchers to the cholinergic system, and to nicotinic subtype receptors in particular [5]. Moreover, there are a variety of neuronal nAChR subtypes in the central and peripheral nervous systems that could thus be targeted with good potential specificity [6]. Neuronal nAChRs are pentamers (i.e. five subunits) of various combinations of

$\alpha(2-9)$  and  $\beta(2-4)$  subunits, with the  $(\alpha4)2(\beta2)3$  configuration being the most prevalent subtype present in the central nervous system. Receptors composed of  $\alpha4$  and  $\beta2$  subunits, collectively referred to as the  $\alpha4\beta2$  subtype, are bound by epibatidine, an alkaloid isolated from the skin of a frog (*Epipedobates tricolor*), which exhibits analgesic activity that is 100 times more effective than that of morphine [7,8]. Although epibatidine produces analgesia through activation of  $\alpha4\beta2$  subtype nAChRs [7], it also binds muscarinic cholinergic receptors, resulting in side effects such as bradycardia and hypotension [8].

Based on the importance of activation of nicotinic receptors as target for drugs acting to relieve pain, we conducted structure-based design of new ligands for nAChR using the Bionet Screening Compounds Program (Key Organics). One hundred compounds with molecular similarity with epibatidine which could act in the central nervous system were selected to the docking study. Thus, molecular docking was carried out to predict binding mode and orientation of compounds at the active sites of nAChR. Glide XP program successfully docked epibatidine and its derivatives into the active sites of  $\alpha4\beta2$  receptor subtype structure [9,10]. Docking scores showed that some analogues had high binding affinity to the nicotinic receptor. The most promising derivative which was designated as Cris-160 was found to dock into the active site of nAChR with fitness XP G-score of -18.08 Kcal/mol. The docking complex was analyzed and it formed one hydrogen bond between Cris-160 and the residue Trp147 of nAChR with bonding distance of 1.77Å (Figure 1).

The antinociceptive activity was investigated observing the hind paw withdrawal in a hot plate.



**Figure 1:** The structural of nAChR ligand binding domain in complex with Cris-160 obtained by flexible docking

The latency to respond was measured in mice after treatment with either morphine or Cris-160 (12 mg. kg<sup>-1</sup>). Morphine and Cris-160 produced antinociceptive activity because 5 min after intraperitoneal administration, the analgesic effect was 68.5 ± 7.9% and 47.4 ± 5.2%, respectively. Thus, Cris-160 induced antinociceptive effect in an acute pain model comparable to morphine and its action could be mediated by the activation of nAChR.

With the aim of developing new selective nAChR radioligands, structural modification to the prototype molecule Cris-160 were proposed and 14 new analogues were synthesized and subjected to a variety receptor binding assays. The ability of the new analogues to inhibit the binding of agonists to the adenosine, adrenergic (α1, α2 and β), cannabinoid (CB1 and CB2), dopamine (D1 and D2), gamma-aminobutyric acid, gabapentin, glutamate (AMPA and NMDA), histamine (H1-H4), muscarinic (M1-M5), opiate (δ, κ, μ), prostanoid (EP2 and EP4), purinergic (P2X and P2Y) and serotonin (5-HT1-5-HT5) receptors was tested (performed by MDS Pharma Services, Taiwan). The binding assay experiments implicated only one of the pyrazole analogues as a specific ligand for the nAChR, namely the analogue Cris-104 (Figure 2), which exhibited inhibition of >60%.

Furthermore, the new pyrazole, Cris-104 (12 mg.kg<sup>-1</sup>) showed evidence of antinociceptive activity investigated using the hot plate test. The latency to respond with paw withdrawal was increased after intraperitoneal administration of Cris-104 when compared to vehicle-treated mice (data not shown).

Based on the evidence that Cris-104 induced antinociceptive effect in acute pain model and had high affinity for the nAChR, this compound was selected for further assessment as a potential analgesic in chronic pain model. The effects of Cris-104 on thermal hyperalgesia and mechanical allodynia, common symptoms of DPNP, were examined in diabetic model rats.

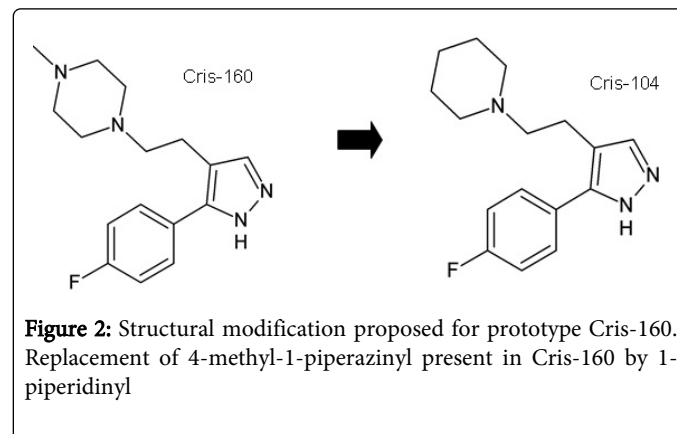
## Methods

The experiments were approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro, Brazil.

### Diabetes model

Male Wistar rats (*Rattus norvegicus*) weighing 180–220 g received an intravenous (i.v.) injection of streptozotocin (STZ, 60 mg.kg<sup>-1</sup>) dissolved in citrate buffer (pH 4.5) to induce diabetes. Four weeks after induction, a significant increase in blood glucose concentration was

observed in STZ-injected animals compared to the non-injected controls. STZ-treated rats with glucose levels >350 mg/dL were randomly divided into two groups (n=6 each) and given vehicle (saline) or Cris-104 (35 mg. kg<sup>-1</sup>) orally by gavage once a day for 7 days. Glucose levels were examined with an Accu-Check® Performa glucometer (Roche, Mannheim, Germany) before and weekly after the STZ injection for 4 weeks and at the end of treatment period.



**Figure 2:** Structural modification proposed for prototype Cris-160. Replacement of 4-methyl-1-piperazinyl present in Cris-160 by 1-piperidinyl

## Assessment of thermal hyperalgesia and mechanical allodynia

### Pain responsivity

Thermal hyperalgesia and mechanical allodynia were measured before and 4 weeks after diabetes induction. Animals with a paw withdrawal response significantly lower than that of non-diabetic animals were considered to have neuropathic pain. Paw withdrawal latency and threshold were measured at 1, 3 and 7 days of Cris-104 administration.

### Thermal hyperalgesia

Heat withdrawal latency was assessed by applying a focused radiant heat source (halogen lamp) to the plantar surface of each hind paw through a glass plate [9,10]. The light beam was deactivated when the animal lifted its hind paw, allowing measurement of the length of time the paw remained in contact with the heat source. Paw withdrawal latency was determined using a plantar analgesia meter (Model 33; ITC, Woodland Hills, CA, USA). A maximal cutoff of 30 s was used to prevent tissue damage. Control latency was determined using the average response of three measurements separated by 5 min intervals prior to the STZ injection. Both hind paws were tested. Nociceptive threshold, as determined by heat withdrawal latency was measured prior to and after 4 weeks of diabetic.

### Mechanical allodynia

Withdrawal threshold to pressure applied to the hind paw (in g) was measured with a digital analgesimeter (Model EFF301; Insight, Ribeirão Preto, Brazil) [10,11]. The pressure meter consisted of a hand-held force transducer fitted with a 0.7 mm<sup>2</sup> polypropylene tip. Upon paw withdrawal, the pressure was released immediately and the nociceptive threshold was read on a scale. Stimulation of the paw was repeated five times and the readings were accepted if the difference between the highest and lowest measurements was <10 g. An upper

limit of 150 g was used to avoid potential tissue injury in the absence of a response.

## Statistical Analysis

Data are expressed as means  $\pm$  standard errors of the mean (SEM). One-way Analysis of Variance (ANOVA) or two-way repeated measures ANOVA was used to analyze the overall effects of treatments. When significant, Dunnett's or Bonferroni's post hoc tests were applied, respectively. Differences were considered statistically significant at  $P < 0.05$ . All tests were performed using GraphPad Prism (Version 4.0; Graph-Pad Software).

## Results

### Blood glucose levels in streptozotocin-induced diabetes in rats

Blood glucose levels in the Cris-104 group did not differ significantly from levels observed in non-treated diabetic controls. After oral administration of Cris-104, serum glucose level was  $557.2 \pm 27.3$  mg/dL compared to  $511.5 \pm 38.0$  mg/dL measured in the non-treated diabetic rats.

### Cris-104 reduced thermal hyperalgesia and mechanical allodynia

Four weeks after STZ injection, mean paw withdrawal latency (PWL) and paw withdrawal threshold (PWT) of STZ-treated rats were reduced significantly from  $12.2 \pm 0.4$  s to  $8.9 \pm 0.2$  s and from  $38.9 \pm 0.6$  g to  $27.7 \pm 1.9$  g, respectively (Figure 3A and 3B), demonstrating the installation of neuropathic pain. As shown in Figure 3A, subsequent daily oral administration of Cris-104 ( $35 \text{ mg.kg}^{-1}$ ) increased PWL significantly after 3 ( $12.4 \pm 0.3$  s) and 7 days ( $12.1 \pm 0.7$  s) of treatment. Additionally, as shown in Figure 3B, Cris-104 increased PWT significantly after 3 days ( $27.8 \pm 0.5$  g) and 7 days ( $32.6 \pm 1.4$  g) of treatment. Both parameters returned completely to pre-treatment level 7 days after suspension of treatment.

### Cris-104 in locomotor activity

Sedative property of Cris-104 was investigated by recording locomotor activity of diabetic rats in an open field. The mobility of Cris-104-treated rats ( $113.0 \pm 4.6$  movements/min) over 60 min did not differ significantly from that of diabetic rats given only saline ( $147.2 \pm 23.5$ ) in an open field after 7 days of treatment (Figure 4). Meanwhile, diazepam ( $10 \text{ mg.kg}^{-1}$ ), the sedation positive reference treatment, reduced locomotor activity significantly from  $147.2 \pm 23.6$  movements/min before injection to  $50.7 \pm 6.4$  movements/min after injection.

## Discussion

In the current study, we investigated the antinociceptive activity of a new non-opioid compound in a STZ-induced rat model of diabetes. Symptom of thermal hyperalgesia is commonly observed in diabetic rats submitted to intense lesion of pancreatic  $\beta$ -cells induced by streptozotocin ( $60 \text{ mg.kg}^{-1}$  i.v.) [11,12]. Thermal hyperalgesia and mechanical allodynia are detected after 4 weeks after streptozotocin administration which suggest the development of diabetic neuropathy. Those animal signs have been useful to test efficiency of drugs for the

treatment of neuropathic pain. Daily oral administration of Cris-104 for 7 days induced antinociception in diabetic rats evidenced by reductions in thermal hyperalgesia and mechanical allodynia. Our results suggest that Cris-104 may be a valuable adjuvant compound for treatment of DPNP. The 7 of days Cris-104 treatment did not alter blood glucose levels significantly, suggesting that Cris-104's mechanism is probably not related to an antidiabetic activity per se. additionally, locomotor activity was not affected significantly by Cris-104, indicating that it should not have a sedative effect.

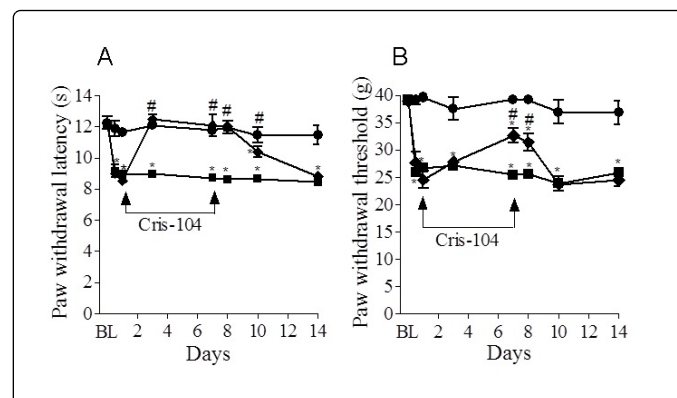


Figure 3: Thermal hyperalgesia (A) and mechanical allodynia (B) of non-diabetic rats ( $\bullet$ ,  $n=4$ ) and diabetic rats treated with either saline ( $\blacksquare$ ,  $n=6$ ) or Cris-104 ( $\blacklozenge$ ,  $n=6$ ) at dose of  $35 \text{ mg.kg}^{-1}$ . Treatment was initiated 4 weeks after STZ injection to induce diabetes. Data are mean  $\pm$  SEM. \*  $P < 0.05$  compared to control (BL) #  $P < 0.05$  compared to diabetic+saline

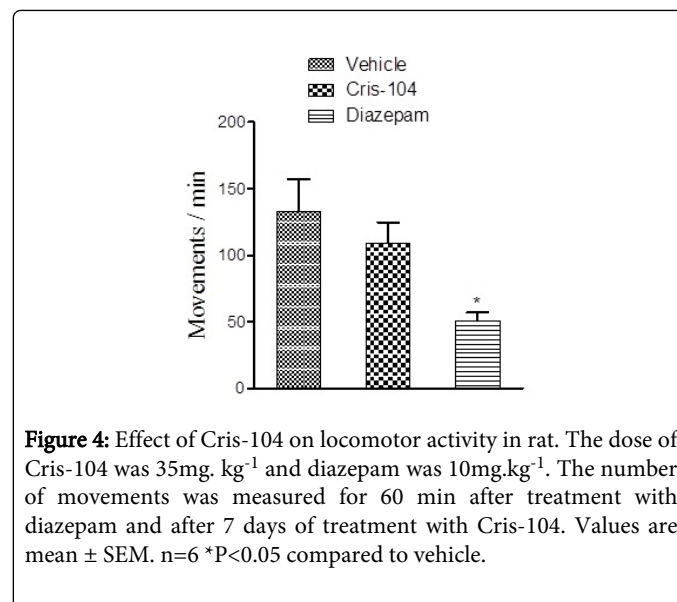


Figure 4: Effect of Cris-104 on locomotor activity in rat. The dose of Cris-104 was  $35 \text{ mg.kg}^{-1}$  and diazepam was  $10 \text{ mg.kg}^{-1}$ . The number of movements was measured for 60 min after treatment with diazepam and after 7 days of treatment with Cris-104. Values are mean  $\pm$  SEM.  $n=6$  \* $P < 0.05$  compared to vehicle.

Nicotine-induced antinociception appears to be a complex phenomenon that involves multiple nAChR subtypes depending on the pain type and sites of action. The discovery of epibatidine, a potent nAChR agonist, was of great importance for treatment of chronic pain. Unfortunately, early tests showed that it had several toxic effects when used clinically [4]. With the aim of introducing new analgesics chemically related to epibatidine, a new compound named ABT-594 was synthesized with the advantages of causing few effects on the

cardiovascular system [12] and being more potent than morphine [13]. However, intolerable adverse secondary effects (i.e. nausea, vomiting, and dizziness) were observed, terminating development of ABT-594 during phase II clinical trials [14]. With the identification of the structure of epibatidine and using [125I]-epibatidine [15], it was possible to identify the distribution of nAChRs in the brain and the role of  $\alpha 4\beta 2$  subunits, generally considered to be the major neuronal nAChR in the brain [16].

The  $\alpha 4\beta 2$  nAChR subtype exists in two stoichiometric configurations,  $(\alpha 4)_2(\beta 2)_3$  and  $(\alpha 4)_3(\beta 2)_2$ , which are found in different brain areas. These two stoichiometries (also called “subtypes” by some authors) have been distinguished by their sensitivity to cytosine in competitive ligand binding assays, with receptors of the  $(\alpha 4)_2(\beta 2)_3$  configuration having greater affinity for cytosine and  $(\alpha 4)_3(\beta 2)_2$  having lower affinity for cytosine [17]. Recently, binding assays showed that Cris-104 has a high affinity for  $(\alpha 4)_3(\beta 2)_2$  receptors (61% inhibition) and a low affinity for  $(\alpha 4)_2(\beta 2)_3$  receptors.

The pharmacological differences between receptor subtypes are therapeutically explored through the development and use of drugs that are selective for each receptor. Increasing the selectivity of a drug for a particular tissue or receptor subtype can enable it to produce the desired therapeutic response without additional unwanted effects. Gao et al. demonstrated that the nAChR agonist varenicline (prescribed for smoking cessation) was effective in a neuropathic pain model in the dose range of 3-10 mg/kg [18] and that at higher doses (two orders of magnitude) may have a non-selective activating effect on multiple receptor subtypes including receptors with  $\alpha 4$ ,  $\beta 2$ ,  $\alpha 7$ , and  $\alpha 3$  subunits, and that these other binding activities may contribute to an analgesic effect, but with a low therapeutic index [19].

The mechanisms mediating analgesia via involvement with nAChRs are poorly understood. Nicotinic receptors are found in the spinal cord dorsal horns, along the ascending and descending nociceptive pathways, and in supraspinal sites. Antinociceptive effects mediated through activation of  $\alpha 4\beta 2$  nAChRs have been attributed to enhancement of descending monoaminergic inhibition in the nucleus raphe magnus and locus ceruleus [10] and stimulation of GABAergic and glycinergic inhibitory transmission in the spinal cord [20,21].

The measurement of motor activity in rodents is a fundamental behavioral test in drug safety testing. Several compounds with high analgesic activity, such as morphine, tramadol, and benzodiazepine analgesic-adjuvant compounds can impair motor activity. Daily administration of Cris-104 for 7 days did not have a significant effect on locomotor activity. This result suggests that its analgesic activity is unlikely to impair motor activation through central or peripheral action.

In conclusion, data from the present study suggest that the recently discovered new nAChR agonist Cris-104 is a compound with the potential to be developed into a treatment for the symptoms of DPNP. It appears to work primarily through activation of the  $(\alpha 4)_2(\beta 2)_3$  nAChR, which may enable it to produce pain relief without untenable adverse secondary effects. Currently used medications, such as antiepileptic compounds (gabapentin and pregabalin), antidepressants (amitriptyline and duloxetine), and opioids, have considerable adverse secondary effects that have limited their use [22]. The present results are promising in suggesting that Cris-104 has the potential to be developed into an efficacious treatment for DPNP symptoms without excessive side effects [23-26].

## Acknowledgements

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