Small Synthetic Hyaluronan Disaccharide BIS014 Mitigates Neuropathic Pain in Mice

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Abstract: Neuropathic pain (NP) is a challenging condition to treat, as the need for new drugs to treat NP is an unmet goal. We investigated the analgesic potential of a new sulfated disaccharide compound, named BIS014. Oral administration (p.o.) of this compound induced ameliorative effects in formalin-induced nociception and capsaicin-induced secondary mechanical hypersensitivity in mice, but also after partial sciatic nerve transection (spared nerve injury), chemotherapy (paclitaxel)-induced NP, and diabetic neuropathy induced by streptozotocin. Importantly, BIS014, at doses active on neuropathic hypersensitivity (60 mg/kg/p.o.), did not alter exploratory activity or motor coordination (in the rotarod test), unlike a standard dose of gabapentin (40 mg/kg/p.o.) which although inducing antiallodynic effects on the NP models, it also markedly decreased exploration and motor coordination. In docking and molecular dynamic simulation studies, BIS014 interacted with TRPV1, a receptor involved in pain transmission where it behaved as a partial agonist. Additionally, similar to capsaicin, BIS014 increased cytosolic Ca2+ concentration ([Ca2+]i) in neuroblastoma cells expressing TRPV1 receptors; these elevations were blocked by ruthenium red. BIS014 did not block capsaicin-elicited [Ca2+]i transients, but inhibited the increase in the firing rate of action potentials in bradykinin-sensitized dorsal root ganglion neurons stimulated with capsaicin.

Perspective: We report that the oral administration of a new sulfated disaccharide compound, named BIS014, decreases neuropathic pain from diverse etiology in mice. Unlike the comparator gabapentin,
BIS014 does not induce sedation. Thus, BIS014 has the potential to become a new efficacious non-sedative oral medication for the treatment of neuropathic pain.

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Key words: Compound BIS014, neuropathic pain, TRPV1, hyaluronan disaccharide, hyaluronic, analgesic drugs, antiallodynic drugs.

The International Association for the Study of Pain (IASP) defines neuropathic pain (NP) as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system.”27 NP can have multiple etiologies, and some examples include mechanical lesions of peripheral nerves, diabetic neuropathy or chemotherapy-induced NP (among many others).2,18 It affects 7 to 8% of the population,3,54 and importantly, less than 50% of patients under treatment with first-line medications (such as tricyclic antidepressants, dual reuptake inhibitors of serotonin and norepinephrine, or gabapentinoids) experience satisfactory pain relief. Furthermore, several cross-sectional studies performed in clinical practice have found that patients with NP continue to have pain of moderate severity despite taking the prescribed medications.23,24,34,39 Thus, the efficient pharmacological control of NP is still a therapeutic challenge and new medications are strongly needed.

Disaccharidic sulfated compounds were initially conceived as treatments for osteoarthritis or diseases and injuries of tendons, ligaments, and bones as proposed in patents EP1300411B156 and WO2008/151898A1.21 The disaccharide with the highest potential to repair damaged tendons was BIS014. This compound has a molecular structure similar to the disaccharidic units composing the large molecular weight (MW) of hyaluronan or hyaluronic acid (HA), an unsulfated straight chain glycosaminoglycan polymer, composed of repeating units of the disaccharide (-D-glucuronic acid-β1-3-N-acetyl-D-glucosamine-β1-4). From a physiological point of view, it is known that large hyaluronan polymers are space-filling, impede cell differentiation and have anti-angiogenic and immunosuppressive properties. Also, high molecular weight (MW) HA chains are involved in ovulation, embryogenesis, protection of epithelia, wound repair, and regeneration. Low molecular weight fragments appear to function as “danger signals.”52 To be used as a drug, the large MW of HA precludes its oral administration. This was the idea behind BIS014, which was sulfated to increase its activity upon oral administration to patients with osteoarthritis, or several other joint diseases of bone and tendons. In the process of studying the pharmacological properties of BIS014, we found that it exhibited antinociceptive effects. We then decided to expand these findings exploring the possible effects of BIS014 on several models of NP of different etiologies. Finally, we performed initial studies on its mechanism of action. Since it has been recently reported that hyaluronan has antinociceptive properties through the modulation of TRPV1,7 we tested whether BIS014 is also able to modulate TRPV1-mediated responses.

Methods

Details of our methods and providers are shown in Supplementary Materials and Methods and are outlined here.

Experimental Animals

Adult Swiss mice (Hsd: ICR-CD-1, Harlan, Barcelona, Spain) 3 to 4 months old were used. Animal protocols were approved by regional (Junta de Andalucía) and Institutional (Research Ethics Committee of the University of Granada) authorities.

Drugs and Drug Administration

BIS014 is a disaccharide with 3 bonds (1 → 3) between glucuronic acid and a derivative of glucosamine, having sulfate groups in the C-4 and C-6 position of the glucosamine ring (Fig 1). Gabapentin and indomethacin were
used as control drugs with known antinociceptive effects. Drugs were administered orally (p.o.) in a final volume of 10 mL/Kg animal weight. The experimenters who evaluated drug effects were blinded to the treatment group of each experimental animal.

Formalin-Induced Nociception
The formalin test was performed as previously described. A volume of 20 μL 2.5 % formalin solution (0.92 % formaldehyde) dissolved in saline solution (NaCl) was injected intraplantarly (i.pl.) in the right hind paw. The first phase of formalin-induced pain was recorded from time 0 to 5 minutes after the injection and then, the second phase of pain was recorded from 15 to 30 minutes after injection. BIS014 (3, 10, and 30 mg/Kg), indomethacin (5 mg/Kg) or their solvent were administered p.o. 30 minutes before formalin injection.

Capsaicin-Induced Secondary Mechanical Hypersensitivity
Capsaicin was dissolved in 1 % DMSO in physiological sterile saline to a concentration of 0.05 μg/μL and injected intraplantarly (i.pl.) into the right hind paw proximate to the heel, in a volume of 20 μL (i.e., 1 μg per mouse). Fifteen minutes after the administration of capsaicin or its vehicle, punctate mechanical stimulation was applied with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy), which uses a nonflexible filament (0.5 mm diameter) electronically driven into the ventral side of the hind paw at least 5 mm away from the site of capsaicin injection (or its solvent) toward the fingers. The intensity of the stimulation was fixed at 0.5 g force. When a paw withdrawal response occurred, the stimulus was automatically terminated, and the response latency was automatically recorded. BIS014 (3–30 mg/Kg/p.o.), gabapentin (20–40 mg/Kg/p.o.) or their solvent were administered 30 minutes before capsaicin injection.

Spared Nerve Injury: Surgical Procedure and Assessment of Neuropathic Hypersensitivity
The spared nerve injury (SNI) was performed as previously described. The tibial and common peroneal branches were ligated with a silk suture and transected distally, and the sural nerve was left intact. Mechanical threshold was assessed with von Frey filaments with the up–down paradigm in the sural nerve territory. Cold sensitivity was tested by gently touching the plantar skin of the hind paw with an acetone drop and recording the duration of biting or licking of the hind paw, as previously described. In all cases, each mouse was evaluated in only one nociceptive test. Baseline sensory sensitivity was measured before SNI. The effect of BIS014 (30 and 60 mg/Kg/p.o.), gabapentin (40 mg/Kg/p.o.), or their solvent on neuropathic hypersensitivity was assessed 7 days after SNI, because that is when mechanical and cold allodynia are fully developed.

Assessment of Diabetic Neuropathy
The administration of streptozotocin (STZ) was used to induce diabetes. STZ (60 mg/Kg) was dissolved in physiological saline and administered intraperitoneally (i.p.) in a volume of 10 mL/Kg once per day for 5 consecutive days. Mechanical threshold was determined using the procedure described above. The effect of BIS014 (3–30 mg/Kg/p.o.), gabapentin (40 mg/Kg/p.o.), or their solvent, was assessed 45 minutes after their administration at day 25 after STZ administration, when neuropathic mechanical alldynia was fully developed.

Assessment of Chemotherapy (Paclitaxel)-Induced Neuropathic Pain
Paclitaxel (2 mg/Kg) was administered i.p. in a volume of 10 mL/Kg once per day for 5 consecutive days. Two protocols were used to explore the effects of BIS014 on the mechanical allodynia associated to chemotherapy. We first administered BIS014 (7.5–30 mg/Kg/p.o.) when mechanical allodynia was fully developed, 10 days after the first administration of paclitaxel, and mechanical threshold was determined 45 minutes after drug administration. We also tested whether BIS014 (30 mg/Kg/p.o.) was able to exert a preventive effect on paclitaxel-induced NP, administering BIS014 30 minutes before each paclitaxel administration; the antiallodynic effect of BIS014 was evaluated also at day 10.

To assess the extent of mechanical alldynia, threshold force for hind paw withdrawal was measured with a Dynamic Plantar Aesthesiometer as previously described. Briefly, an electronically driven monofilament was applied with an increasing force (from 0 to 10 g) against the plantar surface of the hind paw over a 20-second period. The nociceptive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evoked the response is recorded.

Evaluation of Locomotor Activity: Open Field and Rotarod Tests
Animals were introduced for 6 minutes in a 50×50×50 cm opaque plastic box with the floor divided into 16 squares of equal size. Mice were treated orally with saline, BIS014 (60 mg/Kg) or gabapentine (40 mg/Kg), 30 minutes before introducing them in the box. The parameters evaluated were the number of square crossings and the time that mice spent performing rearings, during the 6 minutes duration of the test. For the rotarod test, animals were placed on the roller with an initial speed of 10 rpm at a constant acceleration of 1 rpm for a maximum time of 2 minutes. The time that the animal stayed on the rotating roller before falling was assessed. This test was carried out for 2 consecutive days so that the animals were habituated and learned to walk on the roller (first and second training day) and on the third day (test day), the animals were treated with saline, BIS014 (60 mg/Kg/p.o.) or gabapentine (40 mg/Kg/p.o.), 30 minutes before putting them on the rotarod.
Cytosolic Calcium Transients in SH-SY5Y Neuroblastoma Cells Expressing TRPV1 Receptors

SH-SY5Y human neuroblastoma cells stably transfected with a TRPV1-expressing plasmid (IBMC–UMH), were cultured in a monolayer in Earle's minimum essential medium with Earle's salts supplemented with 10% FBS, 1% nonessential amino acids, 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.4 μg/mL puromycin (37°C, 5% CO2, ThermoScientific Incubator). Calcium fluorometry was monitored when cells were at 80% confluence (3–4 days); medium was replaced by 100 μL/well of Fluo-4 solution in HBSS containing probenecid following manufacturer's instructions. Data acquisition (“cycle” of acquisition) was performed every 10 seconds by means of a fluorescence plate reader (Polastar BMG).

Recording of the Excitability of Sensory Neurons After Sensitization With Bradykinin

Neonatal dorsal root ganglia (DRG) from Wistar rats (3–5 days-old) were isolated and placed into a Petri dish containing DMEM-glutamax with 1% P/S (5000 U/mL). Extracellular recordings were made using multiple electrode planar arrays of 60-electrode thin MEA chips. The electrical activity of sensory neurons was recorded with the MEA1060 System (Multi Channel Systems GmbH) and MC_Rack software version 4.3.0 at a sample rate of 25 kHz. Data were analysed using MC-RACK spike sorter and Neuroexplorer Software (Nex Technologies). A spike was defined when the amplitude of the neuronal electrical activity overcame a threshold set at -18 μV. The recorded signals were then processed to extract mean spike frequency.

Statistics

Data are expressed throughout as means ± S.E.M. The statistical analysis was done by one–way ANOVA followed by Dunnett (formalin-induced nociception, capsaicin-induced mechanical hypersensitivity, open field and rotarod experiments), or Tukey (neuropathic pain models: SNI, STZ and paclitaxel) post hoc tests. When the time-course of drug effects on the SNI was analysed, we used a 2-way repeated measures ANOVA followed by Tukey post-hoc test. In the case that only single group of values was compared, a paired two-tailed t-test was performed.

In the case of calcium fluorometry experiments, statistical analysis was performed by a 1-way ANOVA followed by Bonferroni’s test by comparing fluorescence signals of cells treated with active compounds with vehicle-treated cells for inhibition and to ruthenium-treated cells for activation. Data for the electrophysiological experiments were statistically analysed using paired t-test or 1e-way ANOVA for repeated measures.

The limit of statistical significance between groups was established at P < .05.

Results

Effects of BIS014 in the Formalin Test

The effects of BIS014 on the formalin test were studied in the frame of its preclinical pharmacological profile. Oral treatments were given 30 minutes before formalin injection into the paw. The formalin test has 2 phases of paw licking, the first is neurogenic and the second has inflammatory and central sensitization components.26 In the first phase, the saline group exhibited a licking time of 90 ± 4.2 s (n = 8). BIS014 significantly reduced this time by 26%, 45%, and 32% at the doses of 3, 10, and 30 mg/kg/p.o., respectively, however, the comparator indomethacin (at 5 mg/kg/p.o.) did not affect the duration of the pain-like responses during this phase (Fig 2A). During the second phase, the saline group exhibited a licking time of 197 ± 25 s duration. Again, BIS014 significantly reduced this time by 39%, 60% and 41% at doses of 3, 10, and 30 mg/kg/p.o., respectively. Here, indomethacin also reduced significantly the second phase by 38% (Fig 2B). This led us to predict that BIS014 could exhibit efficacy in reducing other pain models which also involve central sensitization.

Effects of BIS014 on Capsaicin-Induced Mechanical Allodynia

Both the second phase of the formalin test and capsaicin-induced secondary tactile allodynia have a component of central sensitization.47 Thus, and taking into account the ameliorative effects of BIS014 on formalin-induced nociception commented above, we next decided to test the effects of BIS014 on capsaicin-induced allodynia. First, animals received orally either saline, increasing doses of BIS014 (3, 10, 30 or 60 mg/kg/p.o.), or gabapentin (20 or 40 mg/kg/p.o.), and then, mechanical allodynia was induced by capsaicin injection in the right hind paw of mice. The 3 highest doses tested of BIS014 inhibited significantly allodynia, reaching a full reversal of mechanical hypersensitivity at 60 mg/kg (Fig 3). The comparator gabapentin also induced a dose-dependent antiallodynic effect, and at 40 mg/kg/p.o. it blocked completely capsaicin-induced allodynia, a reference control that validated the positive effects of BIS014 in this surrogate model of NP.
Figure 2. Effects of BIS014 on formalin-induced nociception. Data represent the time that mice spent licking the formalin injected hind paw during the first (A) and second (B) phases of the test. Indomethacin (5 mg/kg) and increasing doses of BIS014 3, 10, and 30 mg/kg were administered orally (p.o.) 30 minutes prior to formalin injection. Data are means ± S.E.M. *P < .05, **P < .01, and ***P < .001 with respect to animals treated with saline (1–way ANOVA followed by Dunnett post-hoc test). The number of animals (n) used is indicated above each bar.

Figure 3. Effects of BIS014 on capsaicin-induced allodynia. Mechanical allodynia was elicited by intraplantar injection of capsaicin in mice. The latency to paw withdrawal in response to an electronically driven monofilament at 0.5 g, was measured 45 min after oral (p.o.) administration of saline, increasing doses of BIS014 (3–60 mg/Kg) or gabapentin (20–40 mg/Kg). Data are means ± S.E.M. of the number of mice (n) shown above each bar. ###P < .001 with respect to capsaicin alone, in the absence of drug (one–way ANOVA followed by Dunnett post-hoc test).
Effects of BIS014 on Allodynia Induced by Spare nerve Injury

After SNI the injured animals developed a pronounced hypersensitivity to otherwise innocuous mechanical stimulation in control mice; such hypersensitivity (allodynia) was located at the sural nerve skin area, the lateral surface of the hind paw. Monitored with von Frey filaments, mechanical 50 % threshold decreased from 1.63 ± 0.09 g to 0.39 ± 0.08 g after SNI (Fig 4A). We explored the effects of gabapentin and BIS014 at several time-points postadministration, using saline as a control. As expected, the p.o. administration of saline did not significantly alter the reduced mechanical threshold of neuropathic animals (Fig 4A). At 40 mg/kg/p.o., gabapentin exerted a maximal antiallodynic effect at 45 to 60 minutes after its oral administration, which was maintained up to the rest of the evaluation period (180 minutes) (Fig 4A). Interestingly, this dose of gabapentin also markedly increased the 50% threshold of non-injured animals from 60 to 120 minutes postadministration (Fig 4B). At the dose of 60 mg/kg/p.o., BIS014 produced a much more sustained effect on the mechanical threshold of SNI mice, with a full recovery of tactile allodynia at 45 to 60 minutes, which was still significant at 180 minutes. This high dose of BIS014 increased the mechanical threshold of SNI mice up to values above their baseline levels, inducing therefore robust antinociception beyond the antiallodynic effect, in particular at the peak of its effect at 90 minute postadministration (Fig 4A). We also tested the effects of BIS014 on the 50% mechanical threshold of non-injured animals, and found that 30 mg/kg of this compound did not significantly altered the responses to
mechanical stimulation. However, BIS014 60 mg/kg increased mechanical threshold of non-injured mice with a peak effect at 90 minute postadministration (Fig 4B). We also tested the effects of the administration of saline (p.o.) on the mechanical threshold of non-injured animals, as a control. Saline-treated non-injured animals did not significantly alter their sensory threshold at any time-point evaluated (Fig 4B).

In addition to the effects of BIS014 on mechanical hypersensitivity, we tested the effects of this compound on cold allodynia. Acetone application to the paw of uninjured mice was almost unable to induce a measurable effect (see “Basal” measure in Fig 4D). However, 7 days after SNI, the duration of paw licking/biting produced by acetone was markedly increased, reaching values above 8 seconds. While treatment with saline (p.o.) did not significantly modified the response to acetone, treatment with BIS014 (30–60 mg/kg/p.o.) dose dependently reduced the duration of acetone-induced paw licking/biting (Fig 4D). The time-course of the antiallodynic effect to cold stimulus induced by BIS014 30 mg/Kg/p.o. was similar to the effect on mechanical hypersensitivity described above, peaking at 45 minute after drug administration. BIS014 60 mg/Kg/p.o. induced a much more robust effect, almost abolishing cold hypersensitivity from 30 to 60 minutes after drug administration (Fig 4D).

**Effects of BIS014 on Mechanical Allodynia in a Model of Diabetic Neuropathy**

Mice developed diabetes 3 weeks after the injection of 5 single daily doses of 60 mg/kg/i.p. of streptozotocin (STZ). Blood glucose changed from a baseline value of 132.5 ± 2.3 to 328.2 ± 15.3 mg/dL after 21 days from the first dose of STZ (Fig 5A). 17 % of the animals treated with STZ from a cohort of 60 animals were discarded because they did not present a minimum baseline blood glucose value higher than 250 mg/dL. Therafter, animals exhibited a significant decrease of the mechanical threshold, which was maintained during (at least) the days 21 to 30 after STZ administration (data not shown).

BIS014 was orally administered at 3 different doses (3, 10, and 30 mg/Kg) 45 minutes before the assessment of the 50% mechanical threshold. The test was carried out 25 days after STZ injection. The oral administration of a saline solution (used as a control) did not induce any significant effect in neuropathic animals (from 0.70 ± 0.04 to 0.76 ± 0.06 g), but BIS014 induced a dose-dependent antiallodynic effect reaching a full reversion of the sensory hypersensitivity at 10 to 30 mg/kg/p.o. The comparator gabapentin at 40 mg/kg/p.o. also increased mechanical threshold, and to values much higher than the pre-STZ level of hind paw sensitivity (2.32 ± 0.29 g) (see Fig 5B).

**Effects of BIS014 on Paclitaxel-Induced Alloodynia**

Chemotherapy-induced peripheral neuropathy is a clinically-relevant side effect of neurotoxic chemotherapeutic agents, such as paclitaxel, and in mice it is manifested by the development of alldynia.44,51 To test for the effects of BIS014 on paclitaxel-induced allodynia, we used 2 different protocols: the first explored the...
The effects of this compound once allodynia was established, while the second explored the prophylactic effect of BIS014 on the development of allodynia.

Fig 6A shows that paclitaxel treatment induced mechanical allodynia at day 10 after the first administration of the antineoplastic, and mechanical threshold was measured 45 min after drug administration. BIS014 (30 mg/Kg/p.o.) was given 30 min before each taxol administration (at days 1–5 of the protocol), and the mechanical threshold was tested at day 10. Data are the mean ± S.E.M. of the number of mice (n) shown above each bar. *P < .05, ***P < .001 with respect to the basal values, and ###P < .001 with respect to the pre-treatment measures (1-way ANOVA followed by Tukey posthoc test).

**Effect of BIS014 on Locomotor Activity**

To determine whether BIS014 could alter locomotion, the open field (exploratory locomotion) and the rotarod test (motor coordination) were employed. For comparative purposes, gabapentin was also included. A single oral dose of 60 mg/kg/p.o. of BIS014, administered 30 minutes before testing, did not produce significant differences in the number of crossings or in the time spent rearing in the open field when compared to vehicle treated animals. However, gabapentin at a dose of 40 mg/Kg/p.o., significantly reduced the number of crossings by 57% and the time spent rearing by 87%, compared to vehicle treated animals (Fig 7A and B). Therefore, gabapentin, but not BIS014, decreased the exploratory activity of mice.

The rotarod was used to determine the impact of drug effects on motor coordination. Animals showed an improvement in the time spent in the rotarod with the repeated training (from day 1 to 3). On the test day, animals were distributed into 3 groups (vehicle, BIS014, and gabapentin). Vehicle-treated animals showed a latency to fall of 85.33 ± 7.39 seconds, and those treated with BIS014 (60 mg/Kg/p.o.) showed a similar latency value (85.33 ± 7.39 seconds); no significant differences were observed between both groups. However, gabapentin (40 mg/Kg/p.o.) induced a robust and significant reduction in the latency to fall (21.89 ± 1.02 seconds) when compared with vehicle or BIS014 treated animals (Fig 7C).

**Interactions of BIS014 With the TRPV1 Receptor Protein: Docking and Molecular Dynamic Simulations**

On the basis of its pharmacological profile in the mouse models of NP above mentioned, we felt plausible
that the TRPV1 receptor, could be involved in the antiallodynic effects of BIS014. To find out whether this hypothesis was sound, we investigated the potential interactions of BIS014 with TRPV1, using computational approaches.

A first docking study suggested that an area of TRPV1 differs from the rest of the protein as far as the intensity of protein-ligand was concerned; in other words, BIS014 has a pronounced preference to interact with this protein fragment. This is illustrated in Fig 8A and B, where BIS014 is modulating the channel opening. This is essentially due to the hydrophobic interactions that the compound finds during its penetration into the channel and the hydrogen bonds established by the different functional groups with the close residues, as schematically shown in Fig 8C. These interactions suggest that BIS014 could modulate the opening of the TRPV1 channel. However, this conclusion has been reached on results obtained with static interactions. So, to predict more solidly such interaction, we performed computer simulations of molecular dynamics, taking the docking results as a reference.

Molecular dynamic (MD) simulations were done with TRPV1 bound to BIS014 (TRPV1_complex) and the resulting unbound form obtained upon removal of the ligand (TRPV1_free). The effective radius of the TRPV1 channel was calculated with the program HOLE. Before MD simulation, pore radius was very small, .004 nm in the narrowest point (Fig 8D). At the end of the MD trajectories (after 20 ns), the effective radius was increased to .02 nm in the free protein and to .08 nm in the complex (Fig 8E, F). When the space occupied by the ligand was

Figure 7. BIS014 does not modify locomotion in the open field and rotarod tests. Animals were evaluated in the open field for 6 min after oral (p.o.) administration of saline, BIS014 (60 mg/Kg) or gabapentine (40 mg/Kg). Drugs were administered 30 minutes before testing. Data represent the (A) number of crossings and (B) time spent rearing (in seconds) in the open field. (C) Effect of BIS014 and gabapentin in the rotarod test. All animals were trained during 2 days in the rotarod (day 1 and day 2); on the third day (test day), animals were divided into 3 groups and were treated p.o., 30 minutes prior to testing, with saline, BIS014 (60 mg/Kg) or gabapentin (40 mg/Kg). The latency to fall was measured. Data are the mean ± S.E.M. of the number of mice (n) shown above each bar. **P < .01 and ***P < .001 compared to saline (1−way ANOVA followed by Dunnett posthoc tests).
considered in the calculation, an effective radius of .02 nm, similar to that resulting for the free protein, was obtained (Fig 8F).

**Figure 8.** Docking of BIS014 to protein receptor TRPV1. (A) Structure of the protein TRPV1 in a vertical transversal cross section. (B) Top view of the result of the docking of compound BIS014 with the protein TRPV1. (C) Diagram 2D of the main interactions among BIS014 and the closer residues of the binding site, defined with BINDSURF for the TRPV1 protein. (D-F) Molecular dynamics of BIS014 on the TRPV1 receptor. (D) Effective radius of TRPV1 along the principal axis before simulations of molecular dynamics (MD) and (E) in the last structure (after 20 ns) of the trajectories corresponding to the free protein and (F) the complex BIS014-TRPV1, in which it can be observed the variation of the pore radius with and without BIS014 in the calculation of the radius. (G and H) Snapshots corresponding to the last conformation (after 20 ns of MD simulation) for TRPV1 free, and in the panels (I and J) for the TRPV1 complex. Lateral and top views are shown. Notice that the conformational changes experienced by the unbound structure are more relevant than those undergone by the complexed protein.

**Table 1.** Simulations of TRPV1 Bound to BIS014 (TRPV1-Complex) and Unbound (TRPV1-Free).

<table>
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<th>TRPV1-COMPLEX</th>
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<td>H-bond TRPV1-BIS014</td>
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<td>Intramolecular H-bonds</td>
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<td>H-bond TRPV1-water</td>
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<td>H-bonds BIS014-water</td>
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<td>Intramolecular BIS014 H-bonds</td>
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<td>Residues of TRPV1 around of the binding pocket of BIS014</td>
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<td>Positively charged aminoacids</td>
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<td>Interaction energy TRPV1-BIS014 (KJ/mol)</td>
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</table>

A representation of the last protein conformation (after 20 ns of MD simulation) is shown in Fig 8G-J. It can be clearly noticed that the conformational changes experienced by the unbound structure are more relevant than those undergone by the complexed protein.
experienced by the unbound structure are more evident than those undergone by the complexed protein. However, a single snapshot is not usually representative for what has happened throughout a MD simulation, being necessary a battery of analysis to understand the global trajectory. Table 1 summarizes the results of such analyses.

**Effects of BIS014 on Functional Parameters Mediated by TRPV1 Receptors**

As docking and MD studies suggested that BIS014 interacts with TRPV1 receptors, it was of interest to test the potential of the compound on functional parameters mediated by TRPV1 receptors in cell cultures. First, we tested the effects of BIS014 on the cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{c}\)) in neuroblastoma cells stably expressing TRPV1 receptors, loaded with Fluo-4. The initial basal [Ca\(^{2+}\)]\(_{c}\) was elevated by increasing concentrations of BIS014 (left arrow of Fig 9A). Added on top of BIS014, 100 μM capsaicin evoked further increments of [Ca\(^{2+}\)]\(_{c}\) (Fig 9A, right arrow). Both [Ca\(^{2+}\)]\(_{c}\) signals were drastically blocked by the TRP receptor inhibitor ruthenium red at 10 μM (Fig 9B). This compound blocked the BIS014 elicited [Ca\(^{2+}\)]\(_{c}\) responses by 75 to 85% at the concentrations of 10 to 100 μM, and by 95% the responses elicited by 100 μM capsaicin. This indicates that the changes in [Ca\(^{2+}\)]\(_{c}\) elicited by BIS014 and capsaicin involve the activation of Ca\(^{2+}\) entry through TRPV1 channels, as reflected by increased [Ca\(^{2+}\)]\(_{c}\).

Further experiments were done on the more physiological preparation of primary cultures of sensory neurons. TRPV1-mediated neuronal firing activity was evoked by short (15 seconds) applications of 500 nM capsaicin given in the external superfusing solution. The protocol consisted in three sequential applications of capsaicin (P1, P2, and P3) to evoke neuronal firing. Bradykinin at 1 μM was applied during 5 minutes before P3, in order to sensitize the TRPV1 receptor and increase the neuronal electrical activity elicited by capsaicin. Fig 10A shows an original recording of neuronal firing elicited by capsaicin in P1 and P2; this response was enhanced upon BK application (P3). At the end of the recording, a pulse of KCl (40 mM) was applied to ensure cell viability and excitability. In the experiments of Fig 10B, DRG neurons were preincubated for 1 h with 10 μM BIS014. This caused a decrease of neuronal firing during P3. Averaged data are plotted in Fig 10C-E. Note the reduction of spike frequency in P2 versus P1, and its augmentation in P3 elicited by BK (Panel C). This augmentation was blocked by cell preincubation with BIS014 (Panel D). This is better observed when the ratios P3/P2 were represented: in cells preincubated with vehicle, BK potentiated by about 3-fold the rate of spike firing; however, in cells preincubated with BIS014, the rate of spike firing increase was reduced to 1.4-fold (Panel E).

**Discussion**

Here, we found that the oral administration of BIS014, a novel sulfated disaccharidic compound, exerted a consistent antinociceptive effect in formalin-induced nociception, and an antiallodynic effect not only in capsaicin-induced mechanical hypersensitivity, but also in three mouse models of neuropathic pain, which include SNI, paclitaxel-induced NP and diabetic neuropathy.

In an attempt to clarify the mechanism of action of BIS014, we focused on transient receptor potential (TRP) receptor channels that, from a medicinal chemistry perspective, are being intensely explored as targets to treat pain (analgesia) and/or NP (antiallodynia). BIS014 was highly effective in reverting formalin-induced nociceptive responses. It is relevant to note that the effects of BIS014 were more prominent in the second than in the first phase of nociceptive responses induced by this chemical algogen. Whereas the first phase is due to
direct activation of nociceptors, the second phase is markedly contributed by central sensitization. We also show that BIS014 was able to reduce capsaicin-induced secondary hypersensitivity, which occurs in the area beyond the site of capsaicin injection, where C-nociceptors are activated. C-fiber barrage due to capsaicin application augments the responses of dorsal horn neurons in the spinal cord, leading to central sensitization. The involvement of TRPV1 vanilloid receptors in the capsaicin-induced responses is proven by the observation that mice deprived of those receptors lose their sensitivity to capsaicin. This central sensitization also occurs in the formalin test or by administration of irritants that cause pain intraplantarly, such as phorbol esters. Here, they excite sensory neurons by directly activating TRP channels.

Another model in which BIS014 exhibited antiallodynic effects was the chemotherapy-elicited NP. At 7.5-30 mg/kg/p.o. the compound acutely reversed paclitaxel-induced tactile allodynia. TRPV1 receptors have also been implicated in paclitaxel-induced peripheral neuropathy, and it has been shown that the selective inhibition of those receptors with capsapegpine reduced NP in this model. Therefore, actions on TRPs might (at least partially) account for the acute effects of BIS014 on this particular type of neuropathic pain. Even more interesting was the finding that when co-administered with paclitaxel, BIS014 prevented the development of allodynia. These latter findings might have a clear therapeutic implication, since chemotherapy-induced NP is one of the few exceptions where the moment of onset of the nerve insult is known and preventive treatment can be given. Neuroprotective approaches have been proposed as a preventive therapy for the neurotoxicity associated to chemotherapy-induced neuropathic pain. In this regard, we previously found that BIS014 exhibited neuroprotective effects in rat hippocampal slices subjected to hypoxia followed by re-oxygenation, and in a photothrombotic mouse model of stroke. Thus, the neuroprotective effects of BIS014 might account for the preventive effects on paclitaxel-induced NP that we described here.

Figure 10. Effects of BIS014 on TRPV1 mediated spike frequency in cultures of dorsal root ganglion neurons. Three consecutive pulses (P1, P2, P3) of 500 nM capsaicin were applied for 15s (Cap, top horizontal lines). Bradykinin (BK) at 1 μM was perfused during 5 minutes between P2 and P3. Finally, a pulse of 40 mM KCl was applied to ensure cell viability and excitability. (A and B) Show original traces and the augmentation of the frequency of spikefiring. (C) Normalized mean spike frequency upon capsaicin stimulation in vehicle treated cells; data were normalized to P1. (D) Normalized mean spike frequency upon capsaicin stimulation in BIS014 treated cells (10 μM for 1 h). (E) Ratios P3/P2 showing the blockade by BIS014 of capsaicin potentiation elicited by bradykinin (BK). Data are the mean ± S.E.M. of 3 independent experiments. The total number of registered electrodes were 92 for vehicle and 147 for BIS014. ***P < .001, 1-way ANOVA for repeated measures followed by Bonferroni’s posthoc test (C and D) and paired t-test (E).
with a specific area of the crystallized TRPV1 receptor protein. This interaction was corroborated by molecular dynamic simulations showing lesser conformational changes of the complex BIS014-TRPV1, compared with the unbound structure. Those computational interactions were in line with some functional data i.e., the direct augmentation by BIS014 of the $[Ca^{2+}]$, signal and its blockade by the TRP receptor blocker ruthenium red; this suggests that the compound is interacting functionally with TRPV1 receptors expressed by human neuroblastoma cells. However, BIS014 did not block the $[Ca^{2+}]$, transients elicited by capsaicin in these cells. Nevertheless, the compound effectively inhibited the action potential firing in DRG neurons sensitized by BK. These data suggest that BIS014 may not simply behave as a blocker of the TRPV1 ion pore; a more complex modulatory allosteric effect of TRPV1 receptors could explain (at least partially) the ability of compound BIS014 to block the transmission of the NP signal generated at peripheral nociceptors, thus preventing the state of CNS sensitization occurring in NP.

BIS014 has a similar structure to the disaccharidic units that compose HA (patents EP1300411B1 and WO2008/151898A1). On the other hand, intra-articular HA alleviates pain in osteoarthritic patients through inhibition of TRPV1 channel activity and reduction of action potential firing in nociceptive neurons. In fact, HA has been reported to prevent TRPV1 sensitization by bradykinin, similar to what we reported here with BIS014. Although we cannot rule out a direct effect of BIS014 (or HA) on bradykinin receptors, it could be suggested that BIS014 has a mechanism similar to HA to reduce peripheral nociceptive activity via TRPV1 receptors, with the advantage over HA of its small molecular weight and its oral activity to reduce NP in the variety of mouse models here studied.

Our results show that BIS014 can inhibit not only SN1-induced mechanical allodynia, but also cold hypersensitivity. It is well known that cold allodynia is not related to TRPV1-expressing neurons, but that it is driven by TRPM8+ nociceptors. Therefore, not all effects of BIS014 on pain can be attributed to TRPV1 modulation. In fact, BIS014 was initially designed to combat inflammatory processes (see Introduction), and it is widely known that neuroinflammation plays a key role on the pathogenesis of NP. For instance, peripheral nerve damage such as that occurring in the SNI mouse model causes central sensitization and microglia activation in the spinal cord; this leads to the release of pro-apoptotic cytokines of the TNF type and reactive oxygen species (ROS), provoking chronic neuroinflammation and neuronal death. Also, TRPV1 receptors are required for the nociceptive and inflammatory effects of vanilloid compounds. Furthermore, paclitaxel enhances the activity of voltage-dependent sodium and calcium channels as well as of TRP channels, with production of ROS which disrupts the mitochondrial electron transport chain, altering ATP production in sensory neurons. Neuropathy induced by paclitaxel also involves a strong inflammatory component, due to activation of microglia, astrocytes and satellite glial cells in the dorsal horn of the spinal cord. Thus, inflammation might be contributing to the persistent NP elicited by paclitaxel. Additionally, in diabetic neuropathy microglia is transformed into a pro-inflammatory phenotype; this contributes to mechanical pain-related hypersensitivity in animal models of painful diabetic neuropathy. It is reported that inflammation, oxidative stress, degeneration, and death of nerve fibers and neurons frequently occurs in different NP states. In this context, the inflammatory component of NP could explain the "wide spectrum" antiallodynic effects of compound BIS014 in the variety of NP models here studied. Target deconvolution strategies will be needed to fully unveil the mechanisms of the antinociceptive actions of BIS014.

Gabapentin, a first-line medication for NP, affected the mechanical threshold in both injured and non-injured mice. We also show that this drug markedly impaired exploratory activity and motor coordination, as reported previously. Substances producing motor impairment might attenuate pain-like responses (since flinching, licking/biting or withdrawal of the stimulated paw are motor responses), and thereby induce false analgesic-like effects. Therefore, it is likely that in our experimental conditions, motor impairment partially influence the increase in sensory thresholds induced by gabapentin, and suggests a narrow therapeutic window for this compound. In fact, dizziness and somnolence are usual complaints of patients treated with gabapentin. Doses of BIS014 that induce a prominent effect on NP hypersensitivity or that even increase mechanical threshold in non-injured animals, did not induce measurable alterations of exploratory locomotion or motor coordination. Therefore, and in contrast to the effects induced by pregabalin, the behavioral effects induced by BIS014 in the pain models tested are not likely to be attributable to motor interference.

From a translational point of view, the antiallodynic effects of BIS014 in the mouse models of NP here observed, constitute a solid proof-of-concept for the potential therapeutic effects of this compound in different NP syndromes in patients. For example, pain on light stimuli generates allodynia in the skin beyond the area where C-nociceptors have been directly activated by capsaicin; this is similar to the characteristic allodynia present in NP states such as postherpetic neuralgia in humans. Although osteoarthritis has traditionally been associated with nociceptive pain of inflammatory origin, neuropathic-like pain is frequently present in this condition, as well as during reumatoid arthritis. On the other hand, the SNI model reminds the NP of some clinical conditions of nerve transaction such as after traumatic accidents or after some surgical procedures. Also, the diabetic STZ mouse model of peripheral neuropathy is a good model of the very frequent painful peripheral neuropathy in diabetic patients, and the paclitaxel mouse model neuropathy resemble the peripheral NP associated to chemotherapy in human patients. Of particular translational interest is the experiment here presented showing not only a therapeutic effect, but also a prophylactic effect of BIS014 to prevent the development of paclitaxel-induced neuropathy in mice.

In conclusion, the new disaccharidic compound BIS014 exhibits antiallodynic effects in different mouse models.
of NP. In addition to blocking TRPV1 receptors, BIS014 exhibits antinflammatory, antioxidative, and neuroprotective effects that may contribute to nerve repair and neuronal protection against the damage occurring in different NP clinical syndromes. If ongoing safety and pharmacokinetic preclinical studies generate positive outcomes, compound BIS014 may result a good candidate to test its efficacy and safety in clinical trials in patients suffering different types of NP.

References


9. Cotter M, Brown NJ, Myrick E, Drummond C, Taylor A, Allen L, petals K, Spence JD: Determination of pain thresholds in patients suffering different types of NP. In particular, antinflammatory effects may contribute to nerve repair and neuronal protection against the damage occurring in different NP clinical syndromes. If ongoing safety and pharmacokinetic preclinical studies generate positive outcomes, compound BIS014 may result a good candidate to test its efficacy and safety in clinical trials in patients suffering different types of NP.

BIS014 decreases neuropathic pain

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Supplementary data

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