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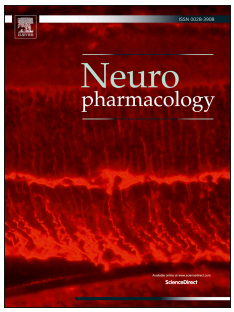

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Oxaliplatin evokes P2X7-dependent glutamate release in the cerebral cortex: a pain mechanism mediated by Pannexin 1

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Abstract

Anticancer therapy based on the repeated administration of oxaliplatin is limited by the development of a neuropathic syndrome difficult to treat. Oxaliplatin neurotoxicity is based on complex nervous mechanisms, the comprehension of the role of single neurotransmitters and the knowledge of the signal flow among cells is matter of importance to improve therapeutic chances.

In a rat model of oxaliplatin-induced neuropathy, we report increased P2X7-evoked glutamate release from cerebrocortical synaptosomes. The release was abolished by the P2X7 receptor (P2X7R) antagonists Brilliant-Blue-G (BBG) and A-438079, and significantly reduced by Carbenoxolone and the Pannexin 1 (Panx1) selective inhibitors Erioglaucine and ^{10}Panx suggesting the recruitment of Panx1. Aimed to evaluate the significance of P2X7R-Panx1 system activation in pain generated by oxaliplatin, pharmacological modulators were spinally infused by intrathecal catheteter in oxaliplatin-treated animals. BBG, Erioglaucine and ^{10}Panx reverted oxaliplatin-dependent pain. Finally, the influence of the P2X7R-Panx1 system blockade on oxaliplatin anticancer activity was evaluated on the human colon cancer cell line HT-29. Prevention of HT-29 apoptosis and mortality was dependent by kind and concentration of P2X7R antagonists. On the contrary, the inhibition of Panx1 did not alter oxaliplatin lethality in tumor cells.

It is concluded that glutamate release dependent on P2X7R is increased in cerebrocortical nerve terminals from oxaliplatin-treated rats; the increase is mediated by functional recruitment of Panx1; P2X7R antagonists and Panx1 inhibitors revert oxaliplatin-induced neuropathic pain; Panx1 inhibitors do not alter the oxaliplatin-induced mortality of cancer cells HT-29. The inhibition of Panx1 channel is suggested as a new and safe pharmacological target.

Keywords: chemotherapy-induced neuropathy; oxaliplatin; P2X7; Pannexin 1; synaptosome

1. Introduction

Neuropathic pain evolves from the physiological role of nociceptive pain to pathological aspects depending on the complex response of the nervous system to a lesion or disease of its somatosensory component. Electrical, molecular and cellular activity participate in sensitizing nervous circuits of periphery, dorsal horn as well as anterior cingulate gyrus, prefrontal cortex, amygdala, and periaqueductal gray leading to pain-mediating signals [52]. The maladaptive plasticity of the central nervous system (CNS) assumes increasing evidence in the pathophysiology of chemotherapy-induced neuropathies [19,49], a peculiar iatrogenic damage of the nervous tissue that results in therapy dose reduction or discontinuation and negatively influences quality of life on cancer survivors [29].

ATP is recognized as one of the keys for the relay of sensory information from the periphery to the CNS [10]. Both sensory neurons and glial cells inside and outside of the CNS release ATP to affect surrounding cells [45]. Accumulated evidence indicates that ATP and its purine receptors are involved in the regulation of neuropathic pain [12,51]. Among the ATP-gated ionotropic P2X receptors the P2X7 subtype (P2X7R) plays a central role in glial/neuron crosstalk [8,47]. Functional P2X7Rs are found on different nerve terminals [39,47]. In CNS glutamatergic neurons [5,47], P2X7R triggers the release of glutamate, a neurotransmitter crucially involved in the central sensitization and in the related changes in the properties of central neurons [36].

P2X7R exhibits peculiar physiological and pharmacological characteristics [45]. Differently from the micromolar affinity for ATP shown by the other family members, P2X7R is activated by high concentrations of ATP (>100 μM), further its prolonged exposure to ATP causes the formation of a reversible plasma membrane pore permeable to hydrophilic solutes up to 900 Da [45]. The large pore is either formed by P2X7 itself in a process termed pore dilation [10] or as a result of recruitment and activation of Pannexin 1 (Panx1) [22,42].

Aimed to study the role of P2X7R-Panx1 system in chemotherapy-induced neuropathic pain, the pharmacological analysis of glutamate release from nerve terminals was performed in the cerebral

cortex of oxaliplatin-treated rats. P2X7R-Panx1 modulation was evaluated as pain reliever focusing on molecules that not interfere with oxaliplatin antitumor mechanisms.

2. Materials and Methods

2.1 Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200-250 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (N°54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guideline [32]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Intrathecal catheterization

Rats were anesthetized with 2% isoflurane and intrathecal catheter was surgically implanted according to [52]. Rats were shaved on the back of the neck and placed in the stereotaxic frame with the head securely held between ear bars. The skin over the nap of the neck was cleaned with

ethyl alcohol and incised for 1 cm. The muscle on either side of the external occipital crest was detached and retracted to expose about 3-4 mm^2 of the atlanto-occipital membrane. The membrane was incised by a needle, which led to the escape of cerebrospinal fluid. The caudal edge of the cut was lifted and about 7.0 cm of 28G polyurethane catheter (0.36 mm outer diameter; 0.18 mm inner diameter; Alzet, USA) was gently inserted into the intrathecal space in the midline, dorsal to the spinal cord until the lumbar enlargement. The exit end of the catheter was connected to 4.0 cm polyurethane (0.84 mm outer diameter; 0.36 mm inner diameter) and was taken out through the skin, flushed with saline solution, sealed and securely fixed on the back of the head with a silk wire. All animals used during behavioral tests did not shown motor impairment induced by surgical operation for the catheter implantation. The evaluation of potential motor dysfunctions were investigated using Rota rod test. The animals who represented any kind of motor disability were excluded from the behavioral measurements.

2.3 Oxaliplatin model

Oxaliplatin treatment started 4 days after the surgical procedure for the spinal catheter implantation. Rats were treated with 2.4 mg kg^{-1} oxaliplatin (Sequoia Research Products, Pangbourne, UK), administered intraperitoneally (i.p.) for 5 consecutive days every week for 2 weeks (10 i.p. injections) [14,19]. Oxaliplatin was dissolved in a 5% glucose-water solution. The model used for the present research is consistent with the clinical practice [14,59].

2.4 Preparation of purified nerve terminals

Purified nerve terminals (synaptosomes) were prepared from the cerebral cortex of oxaliplatin-treated (and vehicle-treated) rats on day 15 of treatment, as previously reported [39]. Briefly, the cerebral cortex was rapidly removed and placed in ice-cold medium, then homogenized in 0.32 mM sucrose with Tris–HCl, pH 7.4, using a glass-Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 4°C, 1000 g) to remove nuclei and debris; the supernatant was

stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33500 g (5 min). The layer containing synaptosomes (between 10% and 20% Percoll) was collected and washed by centrifugation; synaptosomes were then suspended in HEPES medium (mM: NaCl 128, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, and HEPES 10 with glucose 10, pH 7.4).

2.5 Glutamate release from superfused synaptosomes

Glutamate release was studied by measuring tritium efflux from synaptosomes pre-labeled with [^{3}H]D-aspartate (Amersham Radiochemical Centre, Buckinghamshire, UK (see [2,15,39]). Briefly, synaptosomes were incubated (15 min, 37°C) with [^{3}H]D-aspartate (0.03 µM), transferred to parallel superfusion chambers at 37°C and superfused (0.5 ml/min) with standard medium. After 33 min superfusion, superfusate fractions were collected (3-min samples) till the end of the experiment; after 38 min superfusion, synaptosomes were exposed (120 s) to 2'-3'-O-(benzoylbenzoyl) ATP (BzATP; Sigma-Aldrich, Milan, Italy). The effect of Brilliant Blue G (BBG; Sigma-Aldrich, Milan, Italy), A-438079 (Tocris Bioscience, Bristol, UK), Carbenoxolone (Sigma-Aldrich, Milan, Italy), Brilliant Blue FCF (Erioglaucine; Sigma-Aldrich, Milan, Italy) or ^{10}Panx (Trp-Arg-Gln-Ala-Ala-Phe-Val-Asp-Ser-Tyr; WRQAAFVDSY; Proteogenix, Schiltigheim, France) was evaluated by adding the drug 8 min before BzATP. The radioactivity in synaptosomes and superfusate fractions was determined by liquid scintillation counting at the end of superfusion. The radioactivity released in each fraction was calculated as a percentage of the synaptosomal tritium content at the start of the respective collection period (fractional efflux). The BzATP-evoked release (overflow) in the presence or absence of antagonists was calculated by subtracting the basal efflux from the total tritium released in the fractions collected during and after stimulation. In each experiment at least one chamber was used as a control for each condition and was superfused with standard medium or with medium appropriately modified.

When possible, drugs were dissolved in distilled water or in physiological medium. ^{10}Panx was

dissolved in 1% DMSO to obtain 1 mM solution and then diluted in physiological medium at final concentration.

2.6 In vivo pharmacological treatments

BBG and Erioglaucine were dissolved in sterile saline solution. ^{10}Panx was dissolved in 1% DMSO to obtain 1 mM solution and then diluted in sterile saline solution to obtain the final concentrations. Behavioral measurements were performed on day 15 of oxaliplatin treatment after the intrathecal (i.t.) infusion of 0.01 – 1 nmol BBG, 0.01 – 1 nmol Erioglaucine and 0.01 – 10 ^{10}Panx. All compounds were infused in a final volume of 10 µl. Control animals received equivalent volumes of vehicles. The i.t. route of administration was used for all compounds in order to compare the effects induced by BBG and Erioglaucine with those of 10Panx (unable to cross the blood brain barrier) and to specifically evaluate their central properties.

2.7 Paw-pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to a previously published method [37]. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g.

2.8 Von Frey test

The animals were placed in 20 cm × 20 cm Plexiglas boxes equipped with a metallic mesh floor, 20 cm above the bench. Animals were allowed to habituate themselves to their enviroment for 15 min before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the

withdrawal threshold was evaluated by applying forces ranging from 0 to 50 g with a 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the mesh floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum force required to elicit a robust and immediate withdrawal reflex of the paw. The anterior paw was chosen as the more valuable to unmask an altered sensitivity to this test [20]. Voluntary movements associated with locomotion were not considered as a withdrawal response. Stimuli were applied to each anterior paw at 5 s intervals. Measurements were repeated 5 times and the final value was obtained by averaging the 5 measurements [44].

2.9 Cold plate test

The animals were placed in a stainless box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4°C ± 1°C. Pain-related behaviors (i.e. lifting and licking of the hind paw) were observed and the time (s) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s.

2.10 EON test

The neurological examination consisted of all 6 tests as developed and described by [27]. The score assigned to each rat at the completion of the evaluation equals the sum of all 6 test scores: (1) spontaneous activity; (2) symmetry in the movement of 4 limbs; (3) forepaw outstretching; (4) climbing; (5) body proprioception; and (6) response to vibrissae touch. Of the 6 tests, 3 have a minimum score of 1 and 3 have a minimum score of 0, and all 6 tests have a maximum score of 3. Thus, the final minimum score is 3 and the maximum is 18. The test was performed 30 and 60 min after administration.

2.11 Irwin test

This test consists of evaluation of behavioral, autonomic, and neurological manifestations produced by compound administration in rats: motor displacement, motor reflexes, stereotypies, grooming, reaction to painful or environmental stimuli (analgesia, irritability), startle response, secretions, excretions, respiratory movements, skin color and temperature, piloerection, exophthalmos, eyelid and corneal reflexes, muscle tone, ataxia, tremors, head twitches, jumps, convulsions, Straub tail, and other signs or symptoms. For postural reflexes (righting reflex) and other signs such as piloerection, exophthalmia (exaggerated protrusion of the eyeball), ataxia, tremors, and Straub tail, only presence or absence was recorded. Skin color was evaluated qualitatively (pale, red or purple); other signs were evaluated semiquantitatively, according to the observer's personal scale (0 to +4, −4 to 0, or −4 to +4). The terms sedation and excitation express the final interpretation of a group of signs: reduced motor activity, reduced startle response, eyelid ptosis, and reduced response to manual manipulation, for the former; and increased motor activity, increased startle response, increased response to manual manipulation, and exophthalmia, for the latter. Hyperactivity includes running, jumps, and attempts to escape from the container. The animals were placed in transparent cages (26 x 41 cm). The total observation period was 2 hour, beginning 15 minutes after administrations (saline or compound). Trained observers not informed about the specific treatment of each animal group carried out this test.

2.12 Rota-rod test

The Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 r.p.m. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the

rotating rod up to a maximum of 10 min (600 s). The number of falls from the rod was also measured. After a maximum of 6 falls, the test was suspended and the time was recorded.

2.13 Cell culture and treatments

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (Rockville, MD). HT-29 were cultured in DMEM high glucose with 20% FBS in 5% CO_2 atmosphere at 37° C. Media contained 2 mM L-glutamine, 1% essential aminoacid mix, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Sigma, Milan, Italy). HT-29 cells were plated in 96-wells cell culture ($1 \cdot 10^4$/well) for MTT assay or in 6-wells cell culture ($5 \cdot 10^5$/well) for caspase 3 activity measurements and, 48 h after, treated with oxaliplatin (0-100 µM) for 8 or 48h. Carbenoxolone, BBG, Erioglaucine, A-438079 and ^{10}Panx were used in the presence of oxaliplatin for 8 or 48h.The chosen concentrations are in accord with previous published data [21,42,48,53] and, as regards oxaliplatin, with plasmatic concentration of treated rats [59].

2.14 Cell viability assay

HT-29 cell viability was evaluated by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Cells were plated into 96-well cell culture plates, and treated after 48 h. Oxaliplatin, at various concentrations, was incubated in DMEM in the presence of described compounds for 48. After extensive washing, 1 mg/ml MTT was added into each well and incubated for 30 minutes at 37 °C. After washing, the formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance was measured at 550 nm. Experiments were performed in quadruplicate on at least three different cell batches.

2.15 Caspase 3 activity

HT-29 cells were incubated with 100 µM oxaliplatin in the presence or in the absence of described compounds for 8 hours. After treatment cells were scraped in 100 µM lysis buffer (200 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl, 20 mM EDTA, and 0.2% Triton X-100). Fifty microliters of the supernatants were incubated with 25 µM fluorogenic peptide caspase substrate rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-Laspartic acid amide) (Z-DEVD-R110; Molecular Probes) at 25°C for 30 minutes. The amount of cleaved substrate in each sample was measured in a 96-well plate fluorescent spectrometer (Perkin-Elmer; excitation at 496 nm and emission at 520 nm).

2.16 Statistic analysis

Behavioral measurements were performed on 8 rats for each treatment carried out in 2 different experimental sets. Results were expressed as means ± s.e.m. and the analysis of variance was performed by one way ANOVA. A Bonferroni's significant difference procedure was used as post-hoc comparison. P values of less than 0.05 or 0.01 were considered significant. Data were analyzed using the "Origin 9" software (OriginLab, Northampton, USA). The area under the curve (AUC) of the pain reliever effects was calculated using the "Origin 9" software, evaluating the data from 0 – 60 min. For release experiments mean ± s.e.m. of the numbers of experiments (n) are indicated throughout; significance of the difference was analyzed by the *t* test, with statistical significance being taken at $P < 0.05$.

3. Results

3.1 Evaluation of P2X7 receptor and Pannexin 1 on glutamate efflux from cerebrocortical nerve terminal

The basal fractional tritium outflow in the first two fractions collected from superfused purified rat

cerebrocortical synaptosomes amounted to 0.282 ± 0.023%/min (n = 9) in oxaliplatin-treated rats, and to 0.246 ± 0.026%/min (n = 6) in vehicle-treated rats.

BzATP (100 μM)-evoked tritium overflow was higher from synaptosomes obtained from the oxaliplatin-treated with respect to vehicle-treated rats; the P2X7R antagonists BBG (0.1 μM) or A-438079 (10 μM) counteracted the BzATP (100 μM)-evoked tritium overflow (Figure 1). Carbenoxolone (10 μM), a non-selective inhibitor of Panx1 currents (at 1–20 μM; [42,48]) inhibited the BzATP-evoked [^{3}H]D-aspartate overflow in oxaliplatin-treated rats, while not affecting the [^{3}H]D-aspartate overflow in vehicle rats (Figure 1). Analogously, the Panx1 selective inhibitors Erioglaucine (10 μM) and ^{10}Panx (10 μM) inhibited the BzATP-evoked [^{3}H]D-aspartate overflow in oxaliplatin-treated rats, while were ineffective in vehicle rats (Figure 1). Addition of BBG, A-438079, Carbenoxolone, Erioglaucine or ^{10}Panx at the concentrations used did not affect the basal efflux of tritium (data not shown).

3.2 Pain threshold measurements

In the rat, oxaliplatin daily treatments (2.4 mg kg^{-1}) at a clinically relevant dose [59] induce an increasing painful condition [19]. On day 15, oxaliplatin induced mechanical hypersensitivity revealed by the response to a noxious mechanical stimulus. The weight tolerated on the posterior paw, measured by the Paw-pressure test, significantly decreased from the control value of 63.4 ± 2.2 g (Figure 2) to 43.7 ± 2.9 g for oxaliplatin-treated animals. Increasing doses of the P2X7 antagonist BBG (0.01 - 1 nmol), administered i.t. at the lumbar level of the spinal cord, reduced oxaliplatin-induced hypersensitivity in a dose-dependent manner (Figure 2a). The peak effect was measured between 15 and 30 min from infusion for all doses. The lower dose (0.01 nmol) reduced oxaliplatin-induced hypersensitivity by about 74%. The maximal effect corresponding to 65.8 ± 0.8 g was obtained in response to 1 nmol BBG and the pain reliever effect was significant until 60 min and vanished 90 min after administration. In Figure 2b the effect the Panx1 inhibitor Erioglaucine is shown. After i.t. infusion of 0.01 nmol Erioglaucine decreased mechanical hypersensitivity by about

62%. The higher dose (1 nmol) induced a maximal effect corresponding to 65.8 ± 1.1 g (Figure 2b). The Panx1 blocking peptide ^{10}Panx was tested in 0.01 – 10 nmol range dose. Fifteen min after the injection of the lower dose (0.01 nmol) oxaliplatin-dependent hypersensitivity was decreased by 72% (Figure 3a). The weight tolerated on the posterior paw increased to 60.4 ± 2.1 g in animals treated with 1 nmol ^{10}Panx. Furthermore, 10 nmol ^{10}Panx increased the pain threshold over the control value (67.5 ± 2.5 g) showing analgesic effects. The efficacy of 10 nmol ^{10}Panx vanished 90 min after administration (Figure 3a). The dose response curve to i.t ^{10}Panx expressed as area under the curve is shown in the panel b of Figure 3. ^{10}Panx (1 and 10 nmol i.t.) did not alter pain threshold of naïve animals as measured by Paw pressure test (Supplementary Table S1). Acute infusion of ^{10}Panx (1 and 10 nmol i.t.) did not induce behavioral, autonomic and neurological manifestations as evaluated by EON and Irwin test (Supplementary Table S2 and Table S3).

As shown in Figure 4, oxaliplatin caused a lowering of the threshold to mechanical stimuli which do not normally provoke pain measurable by the electronic Von Frey apparatus. The withdrawal threshold to non-noxious mechanical stimuli was decreased in oxaliplatin-treated animals (day 15) from 23.9 ± 0.5 g, vehicle + vehicle, to 16.1 ± 0.1 g, oxaliplatin + vehicle (Figure 4). The acute i.t. infusion of 1 nmol BBG and Erioglaucine and 0.1 nmol ^{10}Panx fully reverted pain threshold alteration (Figure 4a, b and c; 15 min after administration).

Oxaliplatin administration also altered the sensitivity to thermal stimuli (Figure 5) inducing lowered threshold to cold stimuli which do not normally provoke pain (Cold plate test). The licking latency decreased from 21.08 ± 0.4 s (Figure 5; vehicle + vehicle) to 12.8 ± 0.6 s (oxaliplatin + vehicle). Acute i.t. administration of 0.01 nmol BBG and Erioglaucine as well as ^{10}Panx significantly relieved pain 15 min after treatment. The higher dose tested (1 nmol) for all compounds was able to fully normalize pain threshold (Figure 5).

3.3 Evaluation of P2X7 receptor and Pannexin 1 modulation on oxaliplatin antitumor effect (HT-29 cells)

Aimed at evaluating the potential interaction between P2X7R antagonists or Panx1 blocker and the therapeutic property of oxaliplatin, we measured the viability of the human colon cancer cell line HT-29. Table 1 shows the lack of influence of Erioglaucine and ^{10}Panx (concentration range 100 nM - 10 µM) on the concentration-dependent (0.3–100 µM) oxaliplatin lethal effect after 48h incubation. BBG did not alter oxaliplatin-induced toxicity till 1 µM. Fifty µM Carbenoxolone significantly impaired 300 µM oxaliplatin effect. Compound A-438079 significantly prevented oxaliplatin-dependent mortality of HT-29 cells. All tested compounds did not modify oxaliplatin-induced apoptosis evaluated as caspase 3 activity (Table 2).

4. Discussion

Oxaliplatin is effective in various solid tumours [25,40]. Colorectal cancer is the main target, introduced for the management of the advanced stages oxaliplatin is currently the regimen of choice for the adjuvant treatment of patients with curative resection of node-positive colon cancer [3,24]. The dose-limiting toxicity of this compound is the development of peripheral neuropathy [55] related to the total cumulative dose and to the treatment dose-intensity [4]. Although acute symptoms typically resolve within a week, cumulative oxaliplatin causes chronic neuropathy in approximately 50% of patients receiving doses higher than 1000 mg/m^2. Signs and symptoms of chronic neuropathy include pain, paresthesia, hypoesthesia, dysesthesia, and changes in proprioception that persist between cycles [36].

The pathophysiology of oxaliplatin neurotoxicity involves multiple aspects. Molecular damages to peripheral nerves and dorsal root ganglia [19] result in decreased cellular metabolism and axoplasmatic transport [50]. Despite the oxaliplatin limited ability to cross the blood brain barrier (discussed in [21,59]), the CNS appears strongly involved since oxidative damage [21], peroxisome

derangement and intracellular signalling alteration have been shown at spinal and supraspinal level [19,59]. In the spinal dorsal horn of oxaliplatin-treated mice, Renn and co-workers [49] observed an increased activity of wide dynamic range neurons. On the other hand, oxaliplatin induces a significant glial cell density increase differently according to cell type, anatomical region and treatment time-points revealing a prominent role of astrocytes [19].

The present research highlights the role of the P2X7R-Panx1 system in neuropathic pain induced by oxaliplatin. In the cerebral cortex of oxaliplatin-treated rats, a P2X7R-dependent increase of glutamate release was shown in synaptosomes (i.e. in nerve terminals prepared from cerebrocortical neurons of the rats exposed to oxaliplatin). P2X7R was described in CNS glutamatergic neurons [5,47]; glutamate release may be due to both a Ca^{2+}-dependent exocytotic [2,38,39] and a non-exocytotic [39] mechanisms modulate by P2X7R. On the other hand, astrocytes are a major source of ATP released in response to glutamate acting on their AMPA receptors [54]. Circularly, ATP might act on presynaptic P2X7R facilitating glutamate release from the terminals. The widespread P2X7R expression [45] allows to hypothesize on a central role of the receptor in the synergy between the neural network and the surrounding glial cells to ensure a high level of synaptic transmission reinforcing pain signals [8]. The present data agree with the oxaliplatin-dependent P2X7R activation observed in cell cultures [41].

Interestingly, the present results show that the oxaliplatin-induced glutamate release is prevented by the Panx1 inhibitors Carbenoxolone (10 µM; [42,48], erioglaucine [53] and ^{10}Panx, a mimetic peptide of the first extracellular loop domain of Panx1 [42]. Panx1 is a vertebrate homolog of the invertebrate innexin gap junction proteins [30]. However, Panx1 rarely forms functional gap junctions, but acts as a channel that carries ions and signaling molecules between the cytoplasm and extracellular space [30]. Panx1 plays a role in releasing ATP and glutamate in nervous cells and can be opened at the resting membrane potential by extracellular ATP via P2X7R, especially in pathological conditions [34]; long-lasting gating of Panx1 causes aberrant ionic currents and dysregulated neuronal firing patterns by increasing Ca^{2+} entry [34]. Our findings in synaptosomes

indicate that *in vivo* treatment with oxaliplatin induced plastic changes of glutamatergic transmission in the cerebral cortex, likely indicating that P2X7 and P2X7R-linked Panx1 mechanism plasticity might also occur at different levels. In any case, the changes we found in the cerebral cortex glutamatergic neurons might indeed contribute to central sensitization of pain. In fact, neuropathic pain was reported associated with presynaptic and postsynaptic changes resulting in ongoing potentiated excitatory activity at cerebrocortical synapses and descending facilitation contributing to enhanced sensory transmission in the spinal cord [6,57,60]. Interestingly, the persistent molecular changes that potentiate cerebral cortex glutamatergic transmission in peripheral neuropathic injury, have been proposed as potential target mechanisms to guide the treatment of neuropathic pain [56].

Against this background, we can suggest that the neuronal P2X7R-Panx1 activation cooperates to neuropathic pain induced by oxaliplatin. The intrathecal infusion, bypassing the blood-brain barrier, of the P2X7R antagonist and the blockers of Panx1 channel dose-dependently relieve oxaliplatin-dependent alteration of pain threshold (induced by non-noxious stimuli; allodynia-related measures) and hypersensitivity (induced by noxious stimuli; hyperalgesia-related measures). The higher doses are able to fully control pain. Accordingly, Chessell et al. [17] have shown that P2X7R gene-ablated mice do not develop either mechanical allodynia or thermal hyperalgesia after a neuropathic injury. On the contrary, normal nociceptive thresholds were unaltered by P2X7 gene deletion suggesting a specific contribution of P2X7Rs to states of pathological nociception [17]. Moreover, the administration of selective P2X7R antagonists reduces both allodynia and hyperalgesia in several animal models of neuropathic and inflammatory pain [7,12]. In neuropathic pain models induced by trauma the efficacy of P2X7R antagonists has been prevalently related to a reduction of cytokine release and to the inhibition of microglia [28]. In the oxaliplatin model a different mechanism is suggested since the task of spinal microglia cells is limited to the early phase of treatment [19]. The role of Panx1 in pain signaling is lesser enquired. Panx1 is considered an important target for treatment of neurological disorders, such as stroke and epilepsy [34]; despite the Panx1 involvement

in the responses to ATP and glutamate, neurotransmitters strongly related to pain [8,36], few recent research only [9,46] establish a relationship between Panx1 and pain. After sural nerve transection Panx1 expression levels in spinal cord were not modified in comparison to sham animals. The selective Panx1 inhibitor ^{10}Panx and the non specific Carbenoxolone depressed the spinal C-reflex wind-up activity induced by peripheral nerve lesion and decreased pain hypersensitivity [9].

Interestingly, in mice in which P2X7R with defective C-terminal tail have impaired pore formation and coupling to Panx1, less allodynia was shown than in mice with the pore-forming P2X7R; in humans genetic association between lower pain intensity and hypofunctional P2X7R with low pore-forming capability was found [46]. It is to note that the P2X7R C-terminal tail was required for the receptor function as a pore permeable to glutamate [16] and is involved in the receptor interaction with Panx1 [31]. To the best of our knowledge, the present results are the first evidence of the involvement of Panx1 in chemotherapy-induced neuropathic pain. Oxaliplatin treatment induces Panx1 channel recruitment by P2X7R on the nerve terminals increasing the glutamate release from cortical glutamatergic neurons; P2X7R-Panx1 inhibitors i.t. infused at spinal level are able to reduce oxaliplatin-dependent pain suggesting that P2X7R-linked Panx1 plasticity may also occur at different levels, or the possibility that molecules administered i.t. may exert therapeutic effects on supraspinal areas (as recently suggested by [13]). We hypothesize that increased P2X7R/Panx1-dependent release of glutamate (and consequent glutamate receptor activation) might contribute to the increased neuron excitability involved in central sensitization and generation of pain hypersensitivity (see [36]). ATP release through Panx1 channel might also be hypothesized [18]. Although further investigation at other anatomical levels and cell types (e.g., glial cells) is required to a full comprehension of the P2X7R-linked Panx1 mechanisms contributing to oxaliplatin neuropathic pain, this response suggests a role of the P2X7R-Panx1 complex in neuron pain-mediating signals.

The Panx1 blockade does not alter the normal pain threshold since ^{10}Panx has not analgesic effect. The intrathecal administration of this peptide (0.01 – 10 nmol) does not induce behavioral, autonomic or neurological manifestations.

Finally, the theoretical translation of a new pharmacological target in a therapeutic opportunity for chemotherapy-induced neuropathic pain needs the exclusion of interaction with the anticancer effect. The P2X7R profile in tumor cell proliferation is debated since there are evidence that P2X7R-mediated signal promotes tumor growth in vivo suggesting antagonists as antiproliferative compounds [1,23]; on the contrary P2X7-dependent apoptosis seems to be an important mechanism to control the development and progression of neoplasia in the mouse [26]. The scarce information regarding Panx1 reveal a similar unclear condition, in glioma cells Panx1 showed tumor-suppressive effects [35] whereas the loss of Panx1 attenuated melanoma progression [43]. In the present research, the P2X7R-Panx1 modulators were tested in the oxaliplatin-sensitive human colon cancer line HT-29. Panx1 inhibition by the specific blocker ^{10}Panx [42] and by the more recent Erioglaucine [53] do not interfere neither with oxaliplatin-induced lethality nor with apoptosis activation. Carbenoxolone is safe when the low concentration, able to block Panx1 instead of connexin 43 gap junctions [42,48], is used. As regards P2X7R antagonists, A-438079 prevented cell mortality induced by oxaliplatin, BBG causes statistically significant inhibition only at the higher concentration suggesting a dependence from molecule. None of tested compounds interfere with oxaliplatin-induced caspase 3 activation. Nonetheless, *in vivo* experiments are needed to further enquire the safety of P2X7R-Panx1 modulators in cancer conditions.

The 2014 clinical practical guideline from the American Society of Clinical Oncology states that there are no agents recommended for the prevention of chemotherapy-induced neuropathic pain [29]. The insufficient information on the molecular basis of the neuropathy is an important limit to the development of new treatments. The present results highlight the relevance of P2X7R-Panx1 complex in the maladaptative response of spinal cord to oxaliplatin neurotoxicity. P2X7R-Panx1 participates in alteration of neuronal functions leading to central sensitization and pain

chronicization. The selective inhibition of Panx1 channel is suggested as new pharmacological target for oxaliplatin-induced neuropathic pain relief.

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References

[1] Adinolfi E, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, and Di Virgilio F. Expression of P2X7 receptor increases in vivo tumor growth. Cancer Res 2012;72:2957-2969.

[2] Alloisio S, Cervetto C, Passalacqua M, Barbieri F, Maura G, Nobile M, Marcoli M. Functional evidence for presynaptic P2X7 receptors in adult rat cerebrocortical nerve terminals. FEBS Lett 2008;582:3948–3953.

[3] André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A. Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343-2351.

[4] Argyriou AA, Bruna J, Marmiroli P, Cavaletti G. Chemotherapy-induced peripheral neurotoxicity (CIPN): an update. Crit Rev Oncol Hematol 2012;82:51-77.

[5] Atkinson L, Batten TF, Moores TS, Varoqui H, Erickson JD, Deuchars J. Differential co-localisation of the P2X7 receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. Neuroscience 2004;123:761-8.

[6] Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell 2009;139:267-84.

[7] Battilocchio C, Guetzoyan L, Cervetto C, Di Cesare Mannelli L, Frattaroli D, Baxendale IR, Maura G, Rossi A, Sautebin L, Biava M, Ghelardini C, Marcoli M, Ley SV. Flow synthesis and biological studies of an analgesic adamantane derivative that inhibits P2X(7)-evoked glutamate Release. ACS Medicinal Chemistry Letters 2013;4:704-709.

[8] Bennett MR, Farnell L, Gibson WG. P2X7 regenerative-loop potentiation of glutamate synaptic transmission by microglia and astrocytes. J Theor Biol 2009;261:1–16.

[9] Bravo D, Ibarra P, Retamal J, Pelissier T, Laurido C, Hernandez A, Constandil L. Pannexin 1: A novel participant in neuropathic pain signaling in the rat spinal cord. Pain 2014;155:2108-15.

[10] Browne LE, Compan V, Bragg L, North RA. P2X7 receptor channels allow direct permeation of nanometer-sized dyes. J Neurosci 2013;33:3557-3566.

[11] Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 2007;87:659–797.

[12] Burnstock G. Physiopathological Roles of p2x Receptors in the Central Nervous System. Curr Med Chem 2015;22:819-844.

[13] Calias P, Banks WA, Begley D, Scarpa M, Dickson P. Intrathecal delivery of protein therapeutics to the brain: a critical reassessment. Pharmacol Ther 2014;144:114-122.

[14] Cavaletti G, Tredici G, Petruccioli MG, Dondè E, Tredici P, Marmiroli P, Minoia C, Ronchi A, Bayssas M, Etienne GG. Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. Eur J Cancer 2001;37:2457-2463.

[15] Cervetto C, Mazzotta V, Frattaroli D, Alloisio S, Nobile M, Maura G, Marcoli M. Calmidazolium selectively inhibits exocytotic glutamate release evoked by P2X7 receptor activation. Neurochem Int 2012;60:768-72.

[16] Cervetto C, Alloisio S, Frattaroli D, Mazzotta MC, Milanese M, Gavazzo P, Passalacqua M, Nobile M, Maura G, Marcoli M. The P2X7 receptor as a route for non-exocytotic glutamate

release: dependence on the carboxyl tail. J Neurochem 2013;124:821-31.

[17] Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 2005;114:386–396.

[18] Dahl G, Locovei S. Pannexin: to gap or not to gap, is that a question? IUBMB Life 2006; 58:409–419.

[19] Di Cesare Mannelli L, Bonaccini L, Mello T, Zanardelli M, Pacini A, Ghelardini C. Morphologic features and glial activation in rat oxaliplatin-dependent neuropathic pain. J Pain 2013;14:1585-600.

[20] Di Cesare Mannelli L, Zanardelli M, Failli P, Ghelardini C. Oxaliplatin-induced neuropathy: oxidative stress as pathological mechanism. Protective effect of silibinin. J Pain 2012;13:276-284.

[21] Di Cesare Mannelli L, Zanardelli M, Failli P, Ghelardini C. Oxaliplatin-induced oxidative stress in nervous system-derived cellular models: Could it correlate with in vivo neuropathy? Free Radical Biology and Medicine 2013;61:143-150.

[22] Di Virgilio F. Liaisons dangereuses: P2X(7) and the inflammasome. Trends Pharmacol. Sci. 2007;28:465–472.

[23] Di Virgilio F. Purines, purinergic receptors, and cancer. Cancer Res 2012;72:5441-5447.

[24] Ducreux M, Bennouna J, Hebbar M, Ychou M, Lledo G, Conroy T, Adenis A, Faroux R, Rebischung C, Bergougnoux L, Kockler L, Douillard JY. Capecitabine plus oxaliplatin (XELOX) versus 5-fluorouracil/leucovorin plus oxaliplatin (FOLFOX-6) as first-line treatment for metastatic colorectal cancer. Int J Cancer 2011;128:682–690.

[25] Fu S, Kavanagh JJ, Hu W, Bast RC Jr. Clinical application of oxaliplatin in epithelial ovarian cancer. Int J Gynecol Cancer 2006;16:1717-1732.

[26] Fu W, McCormick T, Qi X, Luo L, Zhou L, Li X, Wang BC, Gibbons HE, Abdul-Karim FW, Gorodeski GI. Activation of P2X(7)-mediated apoptosis Inhibits DMBA/TPA-induced formation of skin papillomas and cancer in mice. BMC Cancer 2009;9:114.doi: 10.1186/1471-2407-9-114.

[27] Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke 1995;26:627-634.

[28] He WJ, Cui J, Du L, Zhao YD, Burnstock G, Zhou HD, Ruan HZ. Spinal P2X7 receptor mediates microglia activation-induced neuropathic pain in the sciatic nerve injury rat model. Behaviour Brain Res 2012;226:163–170.

[29] Hershman DL, Lacchetti C, Dworkin RH, Lavoie Smith EM, Bleeker J, Cavaletti G, Chauhan C, Gavin P, Lavino A, Lustberg MB, Paice J, Schneider B, Smith ML, Smith T, Terstriep S, Wagner-Johnston N, Bak K, Loprinzi CL. American Society of Clinical Oncology. Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: American Society of Clinical Oncology clinical practice guideline. J Clin Oncol 2014;32:1941-67.

[30] Huang Y, Grinspan JB, Abrams CK, Scherer SS. Pannexin1 is expressed by neurons and glia but does not form functional gap junctions. Glia 2007;55:46–56.

[31] Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, Scemes E. P2X7 receptor-Pannexin1 complex: pharmacology and signaling. Am J Physiol Cell Physiol 2008;295:C752-C760.

[32] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother 2010;1:94-99.

[33] Kiernan MC. The pain with platinum: oxaliplatin and neuropathy. Eur J Cancer 2007; 43,2631-2633.

[34] Kim JE, Kang TC. The P2X7 receptor-pannexin-1 complex decreases muscarinic acetylcholine receptor-mediated seizure susceptibility in mice. J Clin Invest 2011;121:2037-47.

[35] Lai CP, Bechberger JF, Thompson RJ, MacVicar BA, Bruzzone R, Naus CC. Tumor-suppressive effects of pannexin 1 in C6 glioma cells. Cancer Res 2007;67:1545-54.

[36] Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 2009;10:895–926.

[37] Leighton GE, Rodriguez RE, Hill RG, Hughes J. k-opioid agonist produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. Br J Pharmacol 1988;93:553-560.

[38] Leon D, Sanchez-Nogueiro J, Marõn-Garcõa P, Miras-Portugal MT. Glutamate release and synapsin-I phosphorylation induced by P2X7 receptors activation in cerebellar granule neurons. Neurochem Int 2008;52:1148–1159.

[39] Marcoli M, Cervetto C, Paluzzi P, Guarnieri S, Alloisio S, Thellung S, Nobile M, Maura G. P2X7 presynaptic receptors in adult rat cerebrocortical nerve terminals: a role in ATP-induced glutamate release. J Neurochem 2008;105:2330–2342.

[40] Martín M. Platinum compounds in the treatment of advanced breast cancer. Clin Breast Cancer 2001;2:190-208.

[41] Massicot F, Hache G, David L, Chen D, Leuxe C, Garnier-Legrand L, Rat P, Laprévote O, Coudoré F. P2X7 Cell Death Receptor Activation and Mitochondrial Impairment in Oxaliplatin-Induced Apoptosis and Neuronal Injury: Cellular Mechanisms and In Vivo Approach. PLoS One 2013;8(6):e66830.

[42] Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 2006;25:5071-5082.

[43] Penuela S, Gyenis L, Ablack A, Churko JM, Berger AC, Litchfield DW, Lewis JD, Laird DW. Loss of pannexin 1 attenuates melanoma progression by reversion to a melanocytic phenotype. J Biol Chem 2012;287:29184-93.

[44] Sakurai M, Egashira N, Kawashiri T, Yano T, Ikesue H, Oishi R. Oxaliplatin-induced neuropathy in the rat: involvement of oxalate in cold hyperalgesia but not mechanical allodynia. Pain 2009;147:165-174.

[45] Skaper SD, Debetto P, Giusti P. The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J 2010;24:337-45.

[46] Sorge RE, Trang T, Dorfman R, Smith SB, Beggs S, Ritchie J, Austin JS, Zaykin DV, Meulen HV, Costigan M, Herbert TA, Yarkoni-Abitbul M, Tichauer D, Livneh J, Gershon E, Zheng M, Tan K, John SL, Slade GD, Jordan J, Woolf CJ, Peltz G, Maixner W, Diatchenko L, Seltzer Z, Salter MW, Mogil JS. Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. Nat Med 2012;18:595-600.

[47] Sperlagh B, Vizi ES, Wirkner K, Illes P. P2X7 receptors in the nervous system. Prog Neurobiol 2006;78:327–346.

[48] Spray DC, Ye ZC, Ransom BR. Functional connexin ‘‘hemichannels’’: a critical appraisal. Glia 2006;54:758–773.

[49] Renn CL, Carozzi VA, Rhee P, Gallop D, Dorsey SG, Cavaletti G. Multimodal assessment of painful peripheral neuropathy induced by chronic oxaliplatin-based chemotherapy in mice. Mol Pain 2011;7:29.

[50] Ta LE, Espeset L, Podratz J, Windebank AJ. Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum-DNA binding. Neurotoxicology 2006;27:992-1002.

[51] Tsuda M, Tozaki-Saitoh H, Inoue K. Pain and purinergic signaling. Brain Res Rev 2010; 63:222–32.

[52] von Hehn CA, Baron R, Woolf CJ. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. Neuron 2012;73:638-652.

[53] Wang J, Jackson DG, Dahl G. The food dye FD&C Blue No. 1 is a selective inhibitor of the ATP release channel Panx1. J Gen Physiol 2013;141:649–656.

[54] Werry EL, Liu GJ, Bennett MR. Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P. J Neurochem 2006;99:924–936.

[55] Wolf S, Barton D, Kottschade L, Grothey A, Loprinzi C. Chemotherapy induced peripheral neuropathy: prevention and treatment strategies. Eur J Cancer 2008;44:1507-1515.

[56] Xu B, Descalzi G, Ye H-R, Zhuo M, Wang Y-W. Translational investigation and treatment of neuropathic pain. Molecular Pain 2012;8:15.

[57] Xu H, Wu LJ, Wang H, Zhang X, Vadakkan KI, Kim SS, Steenland HW, Zhuo M: Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. J Neurosci 2008;28:7445-7453.

[58] Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976;17:1031-1036.

[59] Zanardelli M, Micheli L, Cinci L, Failli P, Ghelardini C, Di Cesare Mannelli L. Oxaliplatin neurotoxicity involves peroxisome alterations. PPARγ agonism as preventive pharmacological approach. PLoS One 2014;9:e102758. doi: 10.1371/journal.pone.0102758.

[60]Zhao MG, Ko SW, Wu LJ, Toyoda H, Xu H, Quan J, Li J, Jia Y, Ren M, Xu ZC, Zhuo M. Enhanced presynaptic neurotransmitter release in the anterior cingulate cortex of mice with chronic pain. J Neurosci 2006;26:8923-8930.

Figure legends

Figure 1. Nerve terminals: glutamate release. *Glutamate release evoked by P2X7 receptor activation and involvement of pannexin 1*. Effect of the P2X7R antagonists A-438079 or BBG and of the panx1 inhibitors Carbenoxolone, Erioglaucine or ^{10}Panx on the BzATP-evoked [^{3}H]D-aspartate overflow from cerebrocortical nerve terminals in vehicle-treated and oxaliplatin-treated

rats. Bars represent tritium overflow in the presence of the drugs. BzATP was added for 120 s during superfusion; A-438079, BBG, Carbenoxolone, Erioglaucine or ^{10}Panx was added 8 min before the agonist. Data are mean ± s.e.m. of 3–9 independent experiments in triplicate. *****P<0.05 vs BzATP alone; $^{\#}$P<0.05 vs vehicle-treated.

Figure 2. Pain: mechanical noxious stimuli. *Effect of BBG and Erioglaucine on oxaliplatin-induced hypersensitivity.* Oxaliplatin (2.4 mg kg^{-1}) was administered i.p. daily for 2 weeks. On day 15 a) BBG (0.01 – 1 nmol) and b) Erioglaucine (0.01 – 1 nmol) were infused in a final volume of 10 µl at the lumbar level of the spinal cord by intrathecal catheter. The Paw-pressure test was used to measure over time the sensitivity to a mechanical noxious stimulus. Control animals were treated with vehicles. The values represent the mean of 8 rats performed in 2 different experimental sets. **P<0.01 in comparison to vehicle + vehicle treated rats; ^P<0.05 and ^^P<0.01 in comparison to oxaliplatin + vehicle treated rats.

Figure 3. Pain: mechanical noxious stimuli. *Effect of ^{10}Panx on oxaliplatin-induced hypersensitivity.* Oxaliplatin (2.4 mg kg^{-1}) was administered i.p. daily for 2 weeks. a) On day 15 ^{10}Panx (0.01 – 10 nmol) was infused in a final volume of 10 µl at the lumbar level of the spinal cord by intrathecal catheter. The Paw-pressure test was used to measure over time the sensitivity to a mechanical noxious stimulus. Control animals were treated with vehicles. b) The Area Under Curve (AUC) represents the pain reliever effect induced by intrathecal administration of ^{10}Panx between 0 and 60 minutes. The values represent the mean of 8 rats performed in 2 different experimental sets. **P<0.01 in comparison to vehicle + vehicle treated rats; ^P<0.05 and ^^P<0.01 in comparison to oxaliplatin + vehicle treated rats.

Figure 4. Pain: mechanical non-noxious stimuli. *Effect of BBG, Erioglaucine and ^{10}Panx on oxaliplatin-induced pain threshold alteration.* The Von Frey test was used to measure the pain

threshold as a response evoked by a non-noxious mechanical stimulus. Oxaliplatin (2.4 mg kg^{-1}) was administered i.p. daily for 2 weeks. On day 15, BBG, Erioglaucine and ^{10}Panx were intrathecally infused in a final volume of 10 µl at the lumbar level of the spinal cord. Behavioral measurements were performed 15 min after the administration of different doses of tested compounds. Control animals were treated with vehicles. The values represent the mean of 8 rats performed in 2 different experimental sets. **P<0.01 in comparison to vehicle + vehicle treated rats; ^P<0.05 and ^^P<0.01 in comparison to oxaliplatin + vehicle treated rats.

Figure 5. Pain: thermal non-noxious stimuli. *Effect of BBG, Erioglaucine and ^{10}Panx on oxaliplatin-induced pain threshold alteration.* The Cold plate test was used to evaluate the pain threshold measuring the latency to pain-related behavior (lifting or licking of the paw). Oxaliplatin (2.4 mg kg^{-1}) was administered i.p. daily for 2 weeks. On day 15, BBG, Erioglaucine and ^{10}Panx were intrathecally infused in a final volume of 10 µl at the lumbar level of the spinal cord. Behavioral measurements were performed 15 min after the administration of different doses of tested compounds. Control animals were treated with vehicles. The values represent the mean of 8 rats performed in 2 different experimental sets. **P<0.01 in comparison to vehicle + vehicle treated rats; ^P<0.05 and ^^P<0.01 in comparison to oxaliplatin + vehicle treated rats

Table 1. HT-29 cell viability %, 48h

	Oxaliplatin (μM)							
	0	**0.3**	**1**	**3**	**10**	**30**	**100**	**300**
control	100.0 ± 2.4	100.9 ± 2.5	99.5 ± 1.1	94.9 ± 3.7	88.8 ± 1.3*	79.4 ± 1.6*	37.9 ± 3.5*	17.1 ± 1.2*
Carbenoxolone 10 μM	104.4 ± 6.1	100.3 ± 7.1	107.9 ± 3.0	99.8 ± 6.1	83.5 ± 4.1	79.2 ± 4.7	40.5 ± 3.8	20.7 ± 7.1
Carbenoxolone 50 μM	105.9 ± 2.4	101.6 ± 2.9	104.7 ± 3.3	99.5 ± 2.9	90.7 ± 6.2	82.3 ± 3.9	33.7 ± 2.3	32.6 ± 1.1^
A-438079 1 μM	108.5 ± 5.4	106.8 ± 3.9	108.7 ± 1.7	112.3 ± 1.5^	114.6 ± 1.6^	85.9 ± 5.8	41.6 ± 5.7	18.3 ± 1.5
A-438079 10 μM	116.4 ± 3.3^	118.7 ± 1.4^	122.4 ± 12.1^	120.3 ± 8.7^	115.9 ± 8.9^	94.6 ± 0.6^	43.0 ± 6.5	20.2 ± 0.3
BBG 100 nM	100.5 ± 6.7	99.7 ± 2.3	101.5 ± 4.1	90.8 ± 8.6	83.2 ± 8.7	78.5 ± 8.3	41.2 ± 5.9	16.7 ± 1.9
BBG 1 μM	91.9 ± 2.5	96.6 ± 1.8	96.0 ± 3.3	88.9 ± 2.7	81.3 ± 5.7	81.4 ± 7.1	39.5 ± 5.7	14.9 ± 1.9
BBG 10 μM	93.4 ± 6.3	121.6 ± 6.6^	125.9 ± 4.1^	123.6 ± 6.2^	110.3 ± 9.7^	110.6 ± 6.4^	76.8 ± 3.9^	24.9 ± 5.2
Erioglaucine 100 nM	97.4 ± 3.4	98.6 ± 4.8	95.3 ± 4.5	91.2 ± 3.9	85.1 ± 7.7	82.1 ± 3.7	45.9 ± 5.3	17.6 ± 0.8
Erioglaucine 1 μM	101.4 ± 6.8	103.2 ± 2.7	98.5 ± 5.7	95.5 ± 6.2	90.3 ± 4.0	84.4 ± 5.3	30.4 ± 4.6	18.8 ± 1.9
Erioglaucine 10 μM	103.6 ± 3.6	99.0 ± 5.4	104.8 ± 7.1	97.3 ± 4.8	88.5 ± 3.3	73.2 ± 8.1	28.7 ± 3.3	16.0 ± 1.1
^{10}Panx 100 μM	98.5 ± 4.6	102.3 ± 2.0	94.9 ± 0.9	92.1 ± 1.8	89.6 ± 2.3	81.0 ± 2.5	44.2 ± 6.7	22.2 ± 2.4
^{10}Panx 200 μM	95.2 ± 1.8	95.1 ± 3.2	92.5 ± 0.3	93.8 ± 0.9	88.7 ± 1.2	79.1 ± 2.4	43.4 ± 6.3	13.9 ± 4.9

HT-29 cells were treated with increasing concentrations of oxaliplatin (1 - 300 μM) in the presence or in the absence of the described compounds. Incubation was allowed for 48h. Cell viability was measured by MTT assay. Control condition was arbitrarily set as 100% and values are expressed as the mean ± s.e.m. of three experiments. *P<0.05 in comparison to control (oxaliplatin 0 μM); ^P<0.05 in comparison to the same concentration of oxaliplatin in the absence of tested compounds.

Table 2. HT-29 cells, Caspase 3 activity %, 8h

		Oxaliplatin 100 µM
control	100.0 ± 2.4	132.2 ± 1.8*
Carbenoxolone 10 µM		131.6 ± 1.8
Carbenoxolone 50 µM		121.6 ± 5.1
A-438079 1 µM		125.9 ± 4.7
A-438079 10 µM		137.1 ± 7.5
BBG 100 nM		133.1 ± 1.0
BBG 1 µM		136.9 ± 2.3
Erioglaucine 100 nM		137.8 ± 3.1
Erioglaucine 1 µM		140.9 ± 4.3
^{10}Panx 100 µM		125.2 ± 5.0
^{10}Panx 200 µM		145.6 ± 6.8

HT-29 cells were treated with 100 µM oxaliplatin in the presence or in the absence of the described compounds. Incubation was allowed for 8h. Apoptosis induction was evaluated by measuring Caspase 3 activity. Control condition was arbitrarily set as 100% and values are expressed as the mean ± s.e.m. of three experiments. *P<0.05 in comparison to control (oxaliplatin 0 µM).

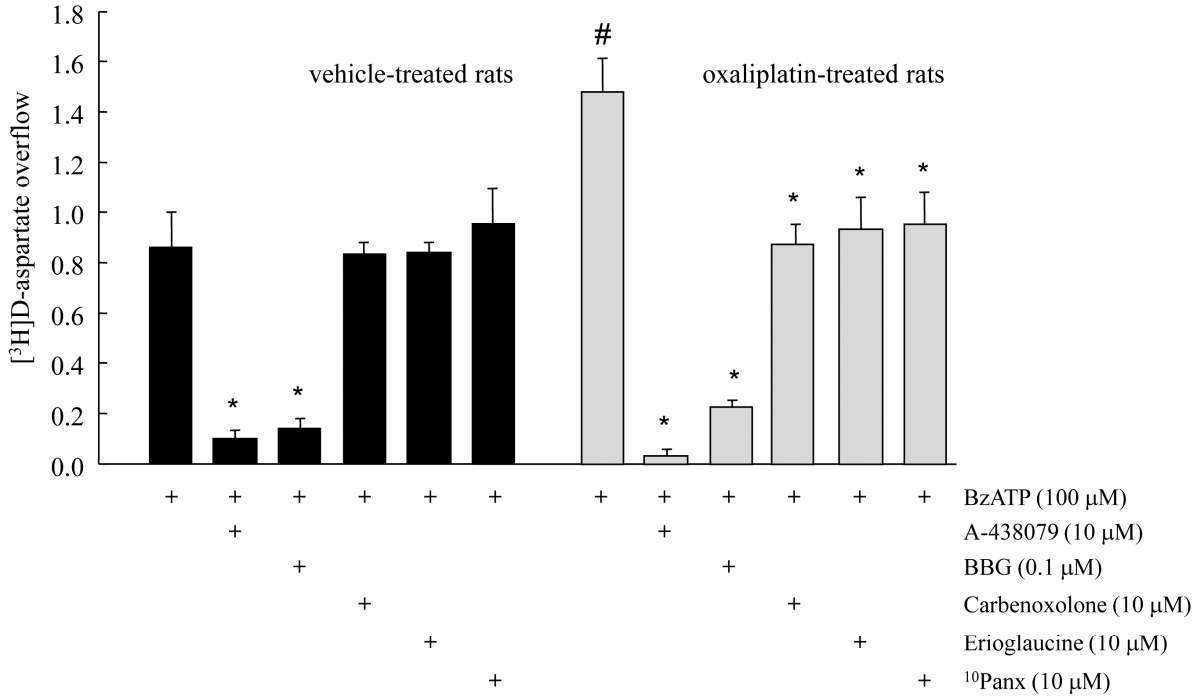
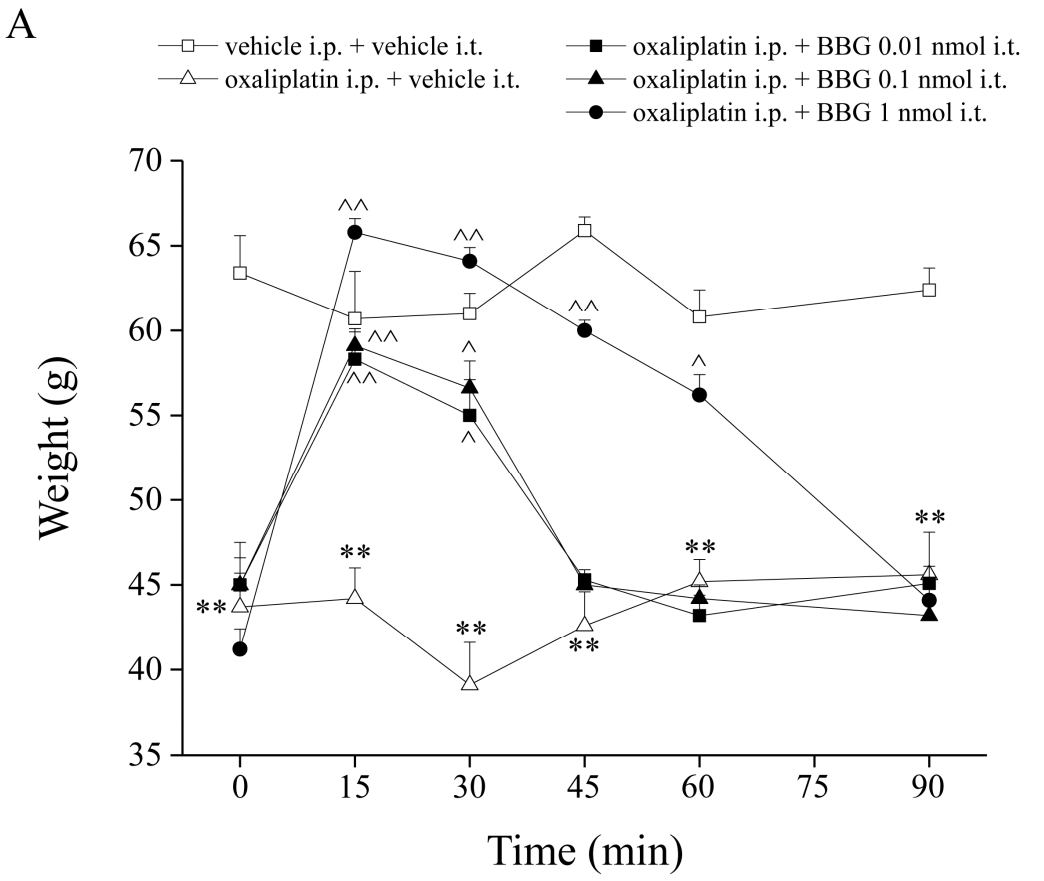


Figure 1


A
vehicle i.p. + vehicle i.t.
oxaliplatin i.p. + vehicle i.t.
oxaliplatin i.p. + BBG 0.01 nmol i.t.
oxaliplatin i.p. + BBG 0.1 nmol i.t.
oxaliplatin i.p. + BBG 1 nmol i.t.
Weight (g)
Time (min)

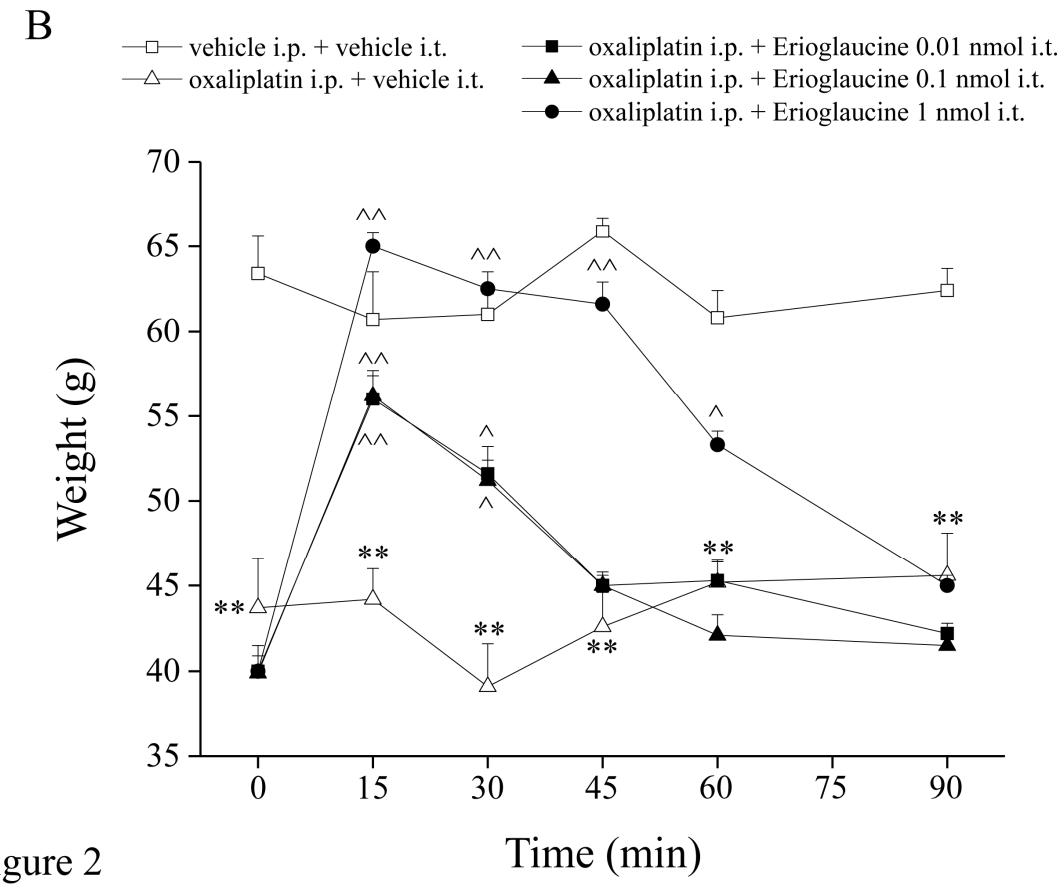

B
vehicle i.p. + vehicle i.t.
oxaliplatin i.p. + vehicle i.t.
oxaliplatin i.p. + Erioglaucine 0.01 nmol i.t.
oxaliplatin i.p. + Erioglaucine 0.1 nmol i.t.
oxaliplatin i.p. + Erioglaucine 1 nmol i.t.
Weight (g)
Time (min)

Figure 2

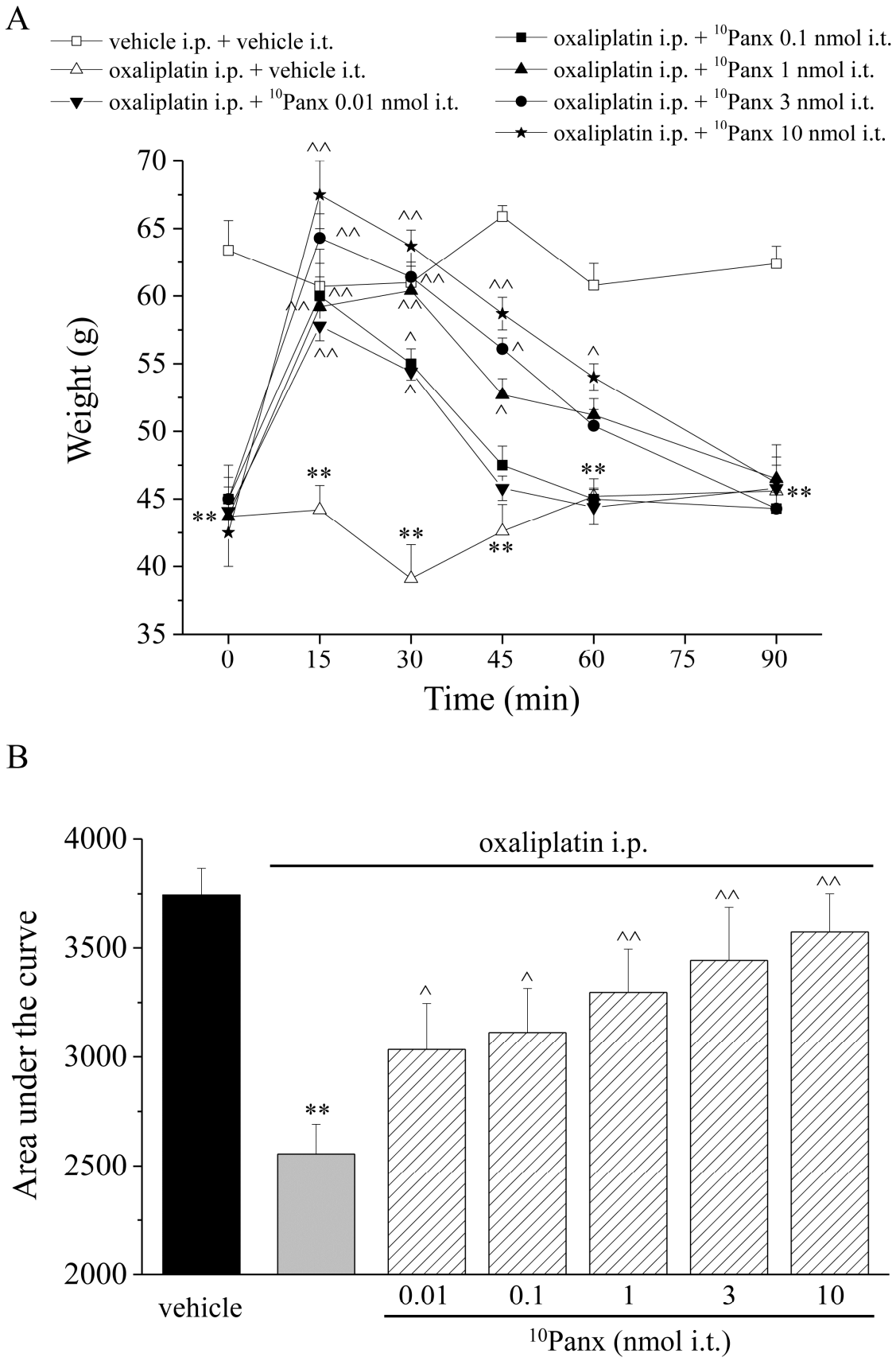
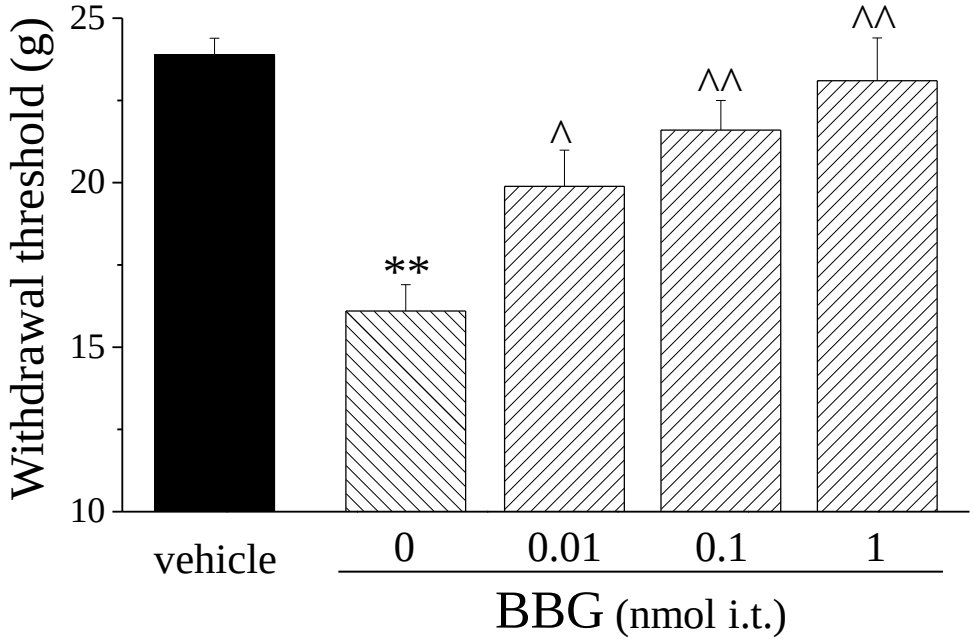
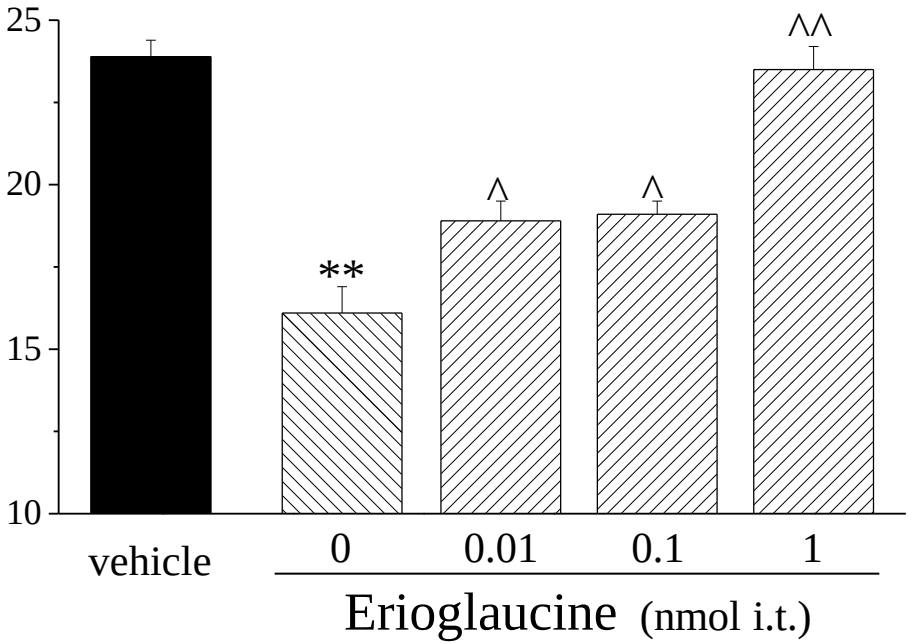
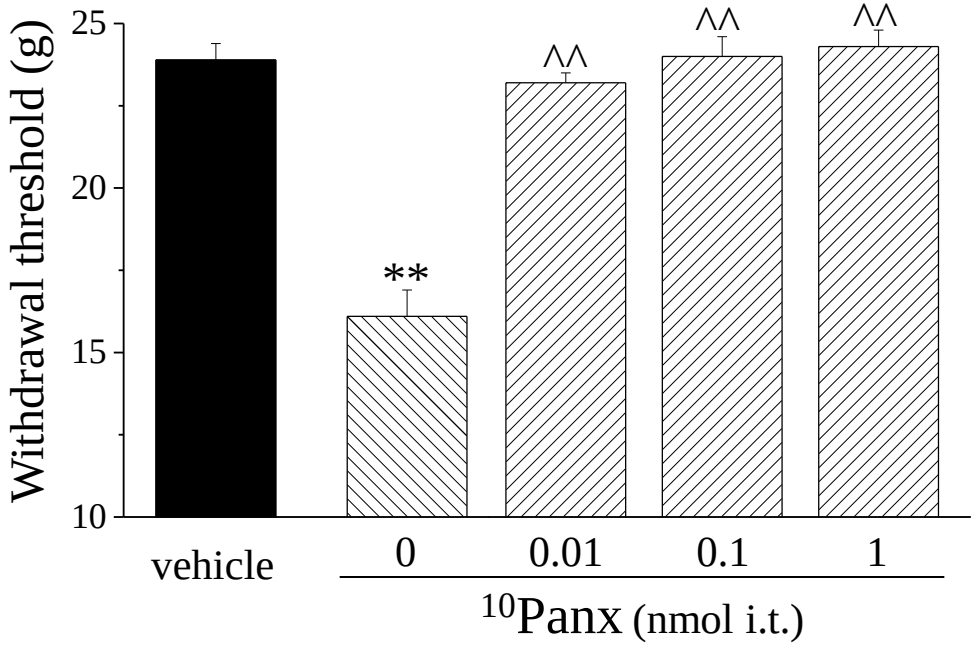

A
vehicle i.p. + vehicle i.t.
oxaliplatin i.p. + vehicle i.t.
oxaliplatin i.p. + 10Panx 0.01 nmol i.t.
oxaliplatin i.p. + 10Panx 0.1 nmol i.t.
oxaliplatin i.p. + 10Panx 1 nmol i.t.
oxaliplatin i.p. + 10Panx 3 nmol i.t.
oxaliplatin i.p. + 10Panx 10 nmol i.t.
Weight (g)
Time (min)
B
oxaliplatin i.p.
Area under the curve
vehicle
0.01
0.1
1
3
10
10Panx (nmol i.t.)

Figure 3


Withdrawal threshold (g)
25
20
15
10
**
^
^^
^^
vehicle
0
0.01
0.1
1
BBG (nmol i.t.)


25
20
15
10
**
^
^
^^
vehicle
0
0.01
0.1
1
Erioglaucine (nmol i.t.)


Withdrawal threshold (g)
25
20
15
10
**
^^
^^
^^
vehicle
0
0.01
0.1
1
10Panx (nmol i.t.)

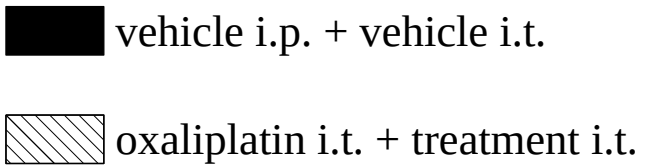

vehicle i.p. + vehicle i.t.
oxaliplatin i.t. + treatment i.t.

Figure 4

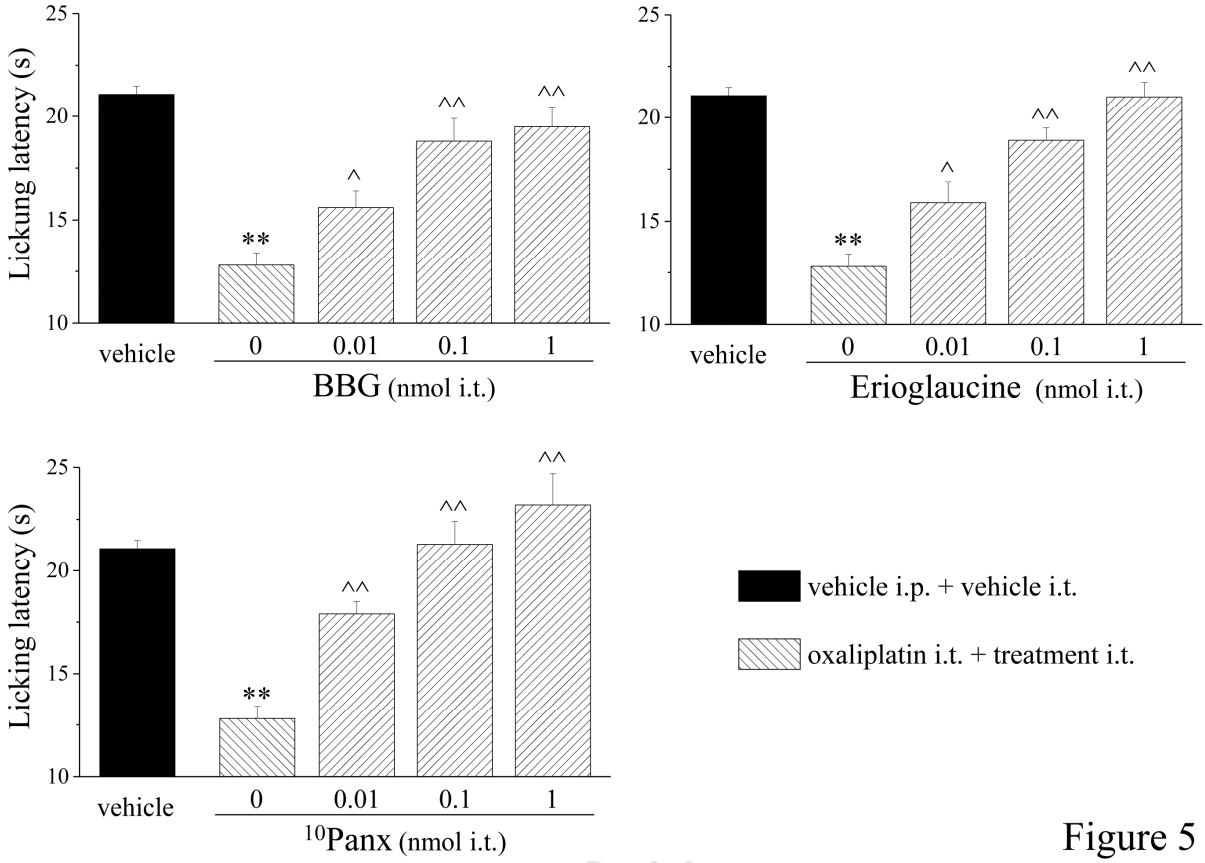


Figure 5

Highlights

- Glutamate release dependent on P2X7 receptor is increased in cerebrocortical nerve terminals from oxaliplatin-treated rats; the increase is mediated by functional recruitment of Pannexin 1
- P2X7 receptor antagonist and Pannexin 1 inhibitors revert oxaliplatin-induced neuropathic pain
- Pannexin 1 inhibitors do not alter the oxaliplatin-induced mortality of cancer cells HT-29

Table S1. Effect of ^{10}Panx on normal pain threshold, Paw pressure test

	Weight (g)					
		min after i.t. treatment				
	pretest	**15**	**30**	**45**	**60**	**90**
vehicle	62.8 ± 1.9	65.1 ± 1.7	63.7 ± 3.0	63.2 ± 2.4	60.3 ± 1.9	63.5 ± 2.1
^{10}Panx 1 nmol	65.5 ± 3.2	60.0 ± 2.8	62.5 ± 1.6	65.4 ± 4.1	64.7 ± 3.8	62.7 ± 2.5
^{10}Panx 10 nmol	61.9 ± 3.3	65.5 ± 2.6	66.0 ± 3.8	62.7 ± 2.2	64.3 ± 1.7	60.8 ± 2.8

Pain threshold was evaluated by Paw pressure test. ^{10}Panx (1 and 10 nmol) was infused in a final volume of 10 µl at the lumbar level of the spinal cord by intrathecal catheter. Measurements were repeated over time in comparison to vehicle-treated animals. The values represent the mean of 8 rats performed in 2 different experimental set.

Table S2. Neurological measurements after ^{10}Panx administration, EON test

	vehicle i.t.		^{10}Panx 1 nmol i.t.		^{10}Panx 10 nmol i.t.	
min after treatment	**30**	**60**	**30**	**60**	**30**	**60**
Spontaneous activity	3	3	3	3	3	3
Symmetry in the movement of 4 limbs	3	3	3	3	3	3
Forepaw outstretching	3	3	3	3	3	3
Climbing	3	3	3	3	3	3
Body proprioception	3	3	3	3	3	3
Response to vibrissae touch	3	3	3	3	3	3
Total	**18**	**18**	**18**	**18**	**18**	**18**

Neurological examinations were performed by the rat EON test. ^{10}Panx (1 and 10 nmol) was infused in a final volume of 10 µl at the lumbar level of the spinal cord by intrathecal catheter. Control group was treated with vehicle. Evaluations were performed 30 and 60 min after administration. Each value represents the mean of 8 rats performed in 2 different experimental set.

Table S3. Behavioral, autonomic, and neurological manifestations after ^{10}Panx administration, Irwin test

	vehicle i.t.		^{10}Panx 1 nmol i.t.		^{10}Panx 10 nmol i.t.	
min after treatment	**30**	**60**	**30**	**60**	**30**	**60**
Motor displacement (0-4)	4	4	4	4	4	4
Motor reflexes (0-4)	4	4	4	4	4	4
Stereotypies	absent	absent	absent	absent	absent	absent
Grooming (0-4)	4	4	4	4	4	4
Reaction to painful or environmental stimuli (analgesia, irritability) (-4 / +4)	0	0	0	0	0	0
Startle response (-4 / +4)	0	0	0	0	0	0
Secretions (-4 / +4)	0	0	0	0	0	0
Excretions (-4 / +4)	0	0	0	0	0	0
Respiratory movements (-4 / +4)	0	0	0	0	0	0
Skin color	pale	pale	pale	pale	pale	pale
Skin temperature	normal	normal	normal	normal	normal	normal
Piloerection	absent	absent	absent	absent	absent	absent
Exophthalmos	absent	absent	absent	absent	absent	absent
Eyelid and corneal reflexes (-4 / 0)	0	0	0	0	0	0
Muscle tone (-4 / +4)	0	0	0	0	0	0
Ataxia	absent	absent	absent	absent	absent	absent

Tremors	absent	absent	absent	absent	absent	absent
Head twitches (0-4)	0	0	0	0	0	0
Jumps (0-4)	0	0	0	0	0	0
Convulsions (0-4)	0	0	0	0	0	0
Straub tail	absent	absent	absent	absent	absent	absent
Other signs or symptoms	absent	absent	absent	absent	absent	absent

Irwin test was performed in the rat to evaluate behavioral, autonomic and neurological manifestations after ^{10}Panx administration. ^{10}Panx (1 and 10 nmol) was infused in a final volume of 10 µl at the lumbar level of the spinal cord by intrathecal catheter. Control group was treated with vehicle. The total observation period was 2 hour, beginning 15 min after administration. The measurements recorded after 60 and 120 min are reported. Each value represents the mean of 8 rats performed in 2 different experimental set.