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ORIGINAL ARTICLE

A rat model of FOLFOX-induced neuropathy: effects of oral dimiracetam in comparison with duloxetine and pregabalin

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Abstract

Background and aim The FOLFOX family of chemotherapy regimens are hampered by the development of a painful neuropathy. Current clinical treatments are inadequate, and furthermore, the research of innovative drugs is strongly disadvantaged by the absence of a preclinical model based on the complete mixture of FOLFOX components. The aim of this study was to set up a rat model of FOLFOX-induced neuropathy in rats, validate its predictability by reference drugs, and evaluate the effectiveness of the new anti-neuropathic compound dimiracetam.

Methods Male Sprague–Dawley rats were treated intraperitoneally with the FOLFOX components (6 mg kg^{-1} oxaliplatin, 50 mg kg^{-1} 5-FU, 90 mg kg^{-1} leucovorin calcium salt) or oxaliplatin alone (6 mg kg^{-1}) on days 0, 7, 14, and 21, whereas a separate group received one more injection of FOLFOX on day 28. Pain behavioural measurements (paw pressure, cold plate, and electronic Von Frey tests) and motor coordination (Rota-rod test) were assessed before and after treatments. Behavioural, motor, neurological, and autonomic parameters (open field and Irwin tests) were evaluated.

Results FOLFOX reduced the pain threshold in response to mechanical noxious and thermal (cold) non-noxious stimuli beginning from day 14 up to day 42 comparably to oxaliplatin alone. A fifth FOLFOX injection enhanced the severity but not the duration of painful alterations. Spontaneous activity, behavioural, autonomic, and neurological functions were also affected, whereas the motor coordination was not altered. On day 22, duloxetine (15 mg kg^{-1}, per os), morphine (10 mg kg^{-1}, subcutaneously), or pregabalin (20 mg kg^{-1}, per os), acutely administered, reduced the FOLFOX-dependent hypersensitivity. Repeated treatments with dimiracetam (150 mg kg^{-1}, per os, twice daily, from day 22) significantly protected rats from FOLFOX-induced alterations of pain threshold as well as from autonomic and neurological impairments taking effect after 7 days treatment. Pregabalin repeatedly administered (20 mg kg^{-1}, per os, twice daily, from day 22) was less effective in reducing mechanical hypersensitivity.

Conclusion A clinically consistent model of FOLFOX-induced neurotoxicity has been developed in rats. Dimiracetam fully reduced hypersensitivity and neurological alterations showing a relevant profile as anti-neuropathic resource.

Keywords FOLFOX · Oxaliplatin · Pain model · Neuropathy · Dimiracetam · Pregabalin · HT-29

Introduction

The addition of oxaliplatin to 5-fluorouracil (5-FU) and leucovorin (LV) proved to be a significant improvement in the treatment of metastatic colon cancer [1]. This combination, called the FOLFOX regimen, has become part of the world-wide standard of care for the adjuvant and the

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palliative treatment of the disease [2]. Among others, the prominent side effect of FOLFOX therapy is a debilitating, dose-limiting neurotoxicity, which leads to a painful sensory neuropathy. Symptoms evolve in two distinct phases. The acute phase, experienced by 85–95% of treated patients, is characterized by an increased sensitivity to touching cold items or swallowing cold liquids, throat discomfort, and muscle cramping. It is most pronounced 3 days after a given oxaliplatin infusion [3]. A chronic phase comprises sensory impairment of the distal peripheral nerves of the extremities. Chronic symptoms affect roughly 60, 40, 20, and 10% of treated subjects at 1, 6 months, 1, and 2 years after completion of the FOLFOX regimen, respectively [4–6].

The symptoms of neurotoxicity observed during and after FOLFOX therapy are largely attributable to oxaliplatin [5]. Monotherapy with 5-FU rarely induced neurotoxicity, but fluoropyrimidine metabolites could trigger further nervous alterations [7]. On the other hand, clinical reports are mainly based on the evaluation of the FOLFOX combination effects without distinction among single components and knowledge about possible additive effects. The molecular bases of the neurotoxicity evoked by FOLFOX therapy in its entirety are unknown, even because the lack of a preclinical model of FOLFOX neuropathy. The currently available data about the most neurotoxic component oxaliplatin reveal a complex panel of damages (peripheral nerves, dorsal root ganglia, and central nervous system areas) and altered responses due to maladaptive plasticity (of both neurons and glia; [8–11]) that may be complicated by the presence of 5-FU and LV.

The symptoms arisen after FOLFOX therapy significantly affect patients' daily activities and quality of life, and they are difficult to treat: randomized, controlled clinical trials have to-date demonstrated only for duloxetine a modest treatment effect [12, 13]. Thus, the discovery of novel effective strategies remains a crucial objective. Recently, dimiracetam, a racetam derivative with nootropic properties, has been proposed as a pain reliever active against chemotherapy-induced neuropathy (CIN) distinguished by an optimal safety profile. It has been shown to be a potent inhibitor of glutamate-induced glutamate release in the rat spinal cord: dimiracetam inhibits NMDA + glycine-stimulated [3H]-D-aspartate release from rat spinal synaptosomes with an IC50 between 10 and 20 nM [14]. Glutamate-induced glutamate release is believed to be directly involved in sensitization of spinal and central pain pathways [15].

The present research undertook the development of an animal model of chemotherapy-induced neuropathic pain evoked by the combined administration of all FOLFOX components. The hypersensitivity to mechanical and thermal noxious and non-noxious stimuli, as well as behavioural, neurological, autonomic parameters, and motor functions were analyzed to offer a clinical relevant tool to study the pathophysiological mechanisms of FOLFOX neurotoxicity and screen new treatments. The properties of dimiracetam were characterized in comparison with currently applied drugs.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats (Envigo, 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 1 week after their arrival. Four rats were housed per cage (size 26 × 41 cm) kept at 23 ± 1 °C with a 12 h light/dark cycle, with lights on at 7 a.m; they were fed a standard laboratory diet and tap water ad libitum. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. 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 Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [16]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Treatments

Oxaliplatin (6 mg kg^{-1}) or a mixture of FOLFOX components (6 mg kg^{-1} oxaliplatin, 50 mg kg^{-1} 5-FU, 90 mg kg^{-1} LV calcium salt) were administered intraperitoneally (i.p.) on days 0, 7, 14, and 21. Oxaliplatin was dissolved in 5% glucose; the other compounds were dissolved in saline. Regarding to the FOLFOX regimen, 5-FU and LV were administered 2 h after the oxaliplatin administration. Doses were established accordingly with Robinson et al. [17]. On day 28, FOLFOX-treated animals were divided into two groups, one of them, namely, FOLFOX (5), was treated once again on the same day. The other group received four total administrations—FOLFOX (4).

The effect of the pain relieving drugs was evaluated after acute or repeated treatment. As regards the acute treatment, duloxetine (15 mg kg^{-1}, per os—p.o.), morphine (10 mg kg^{-1}, subcutaneously—s.c.), pregabalin (20 mg kg^{-1}, p.o.), and dimiracetam (150 mg kg^{-1}, p.o.) were administered on day 22 or on day 28. For the chronic treatment, dimiracetam (150 mg kg^{-1}, p.o.), pregabalin

(20 mg kg^{-1}, p.o.), and duloxetine (15 mg kg^{-1}, p.o.) were administered daily bis in die (b.i.d.) for three consecutive weeks (starting from day 22). Morphine was dissolved in saline, while dimiracetam, pregabalin, and duloxetine were suspended in 1% carboxymethylcellulose sodium salt. Body weight was constantly measured during the experiment, and results obtained during treatments are reported in the Supplementary Figure S1.

Paw pressure test

Paw mechanical sensitivity was determined using a Randall and Selitto apparatus exerting a force that increased at constant rate (32 g s^{-1}). The threshold stimulus at which rats withdrew the paw was evaluated before and at different timepoints after treatment. Results represent the group mean of mechanical thresholds expressed as grams. To avoid any possible damage to the rats' paw, the maximum applied force was fixed at 150 g [18].

Cold plate test

The rats were placed in a stainless box (12 cm × 20 cm × 10 cm) with a temperature-controlled cold steel plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behaviours (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 [10].

Von Frey test

The rats were placed in 20 × 20 cm plexiglas boxes equipped with a metallic screen-mesh floor, 20 cm above the bench. A habituation of 15 min was allowed before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying a force ranging from 0 to 50 g with a 0.2 g accuracy. The punctuate stimulus was delivered to the mid-plantar area of each posterior paw from below the meshy floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum pressure required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each posterior paw with an interval of 5 s. The measure was repeated five times and the final value was obtained by averaging the five measures [10].

Open field test

Locomotive activity was assessed by the open-field test. The observation apparatus consisted of a 60 × 60 cm wooden box with the field bordered by 45 cm high sidewalls. Time spent in the corners and in the center; number of rearing; number of crossings to the center and the periphery; and spontaneous activity and inactivity of each rat were monitored for 20 min. The data were analyzed using the X-Plo-rat software system version 3.3.

Irwin test

Each rat was individually placed in a transparent cage (26 × 41 cm), and 26 neurobehavioural or physiological parameters were systematically assessed according to Irwin (1968) [19] 4 and 7 weeks after the beginning of the experiment.

Behavioural, autonomic, and neurological manifestations produced by compound administration in rats were evaluated: motor displacement, motor reflexes, stereotypies, grooming, reaction to painful or environmental stimuli (analgesia, irritability), startle response, secretions, excretions, respiratory movements, skin colour 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 colour was evaluated qualitatively (pale, red, or purple); other signs were evaluated semi-quantitatively, 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. Trained observers not informed about the specific treatment of each animal group carried out this test.

Rota-rod test

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 four equal sections by five disks. Thus, up to four rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed

based on the number of falls from the rod for a maximum of 600 s. After a maximum of six falls from the rod, the test was suspended and the time was recorded. Each rat was assessed once, and the group mean average score was calculated.

Cell culture and treatments

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (Rockville, MD, USA). 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 (1×10^4/well) plates, and after 48 h, they were treated with increasing concentrations of FOLFOX components for 24 or 48 h. The molar ratio among oxaliplatin/5-FU/LV used in vivo was maintained (6/50/90 mg kg^{-1} are 15/384/176 µg kg^{-1}). Experiments were repeated in the absence and in the presence of dimiracetam (100 µM).

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 after 48 h, they were treated as previously described. After extensive washing, 1 mg ml^{-1} MTT was added into each well and incubated for 30 min at 37 °C. After washing, the formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance was measured at 550 nm [8].

Statistical analysis

Behavioural measurements were performed on ten rats for each treatment carried out in two different experimental sets. Cell culture measurements were performed in sextuplicate on at least three different cell batches. All experimental results (excepted for the Irwin test) were expressed as means ± SEM and the analysis of variance was performed by two-way ANOVA. A Bonferroni's significant difference procedure was used as post-hoc comparison. P values ≤ 0.05 were considered statistically significant.

Results

Pain threshold measurements: effect of FOLFOX treatment

On days 0, 7, 14, and 21, FOLFOX (4) (6 mg kg^{-1} oxaliplatin, 50 mg kg^{-1} 5-FU, and 90 mg kg^{-1} leucovorin calcium salt) or oxaliplatin alone (6 mg kg^{-1}) was administered i.p. once a week for four times; furthermore, a separate group received one more injection of FOLFOX (5) on day 28. Pain measurements were assessed before treatment and 24 h after, up to day 28, and once a week up to day 63. As shown in the Supplementary Figure S1, all treatments reduced the body weight increase in comparison with vehicle-treated animals.

As measured by the paw pressure test, FOLFOX (4) lowered the paw withdrawal threshold in response to a noxious mechanical stimulus starting from day 14 and picking on day 29 [43.3 ± 0.7 g, FOLFOX (4) vs. 66.6 ± 2.3 g, vehicle]. Oxaliplatin treatment also reduced the paw withdrawal threshold starting from day 14 and picking on day 29 (46.7 ± 1.7 g, oxaliplatin vs. 66.6 ± 2.3 g, vehicle). The effect of FOLFOX was numerically greater than oxaliplatin alone, but did not reach the threshold of statistical significance. The paw withdrawal threshold induced by a fifth injection of FOLFOX picked in severity on day 42 [41.5 ± 1.6 g, FOLFOX (5) vs. 69.4 ± 1.5 g, control]; this peak was lower than that achieved in the FOLFOX (4) group. By day 64, the paw-withdrawal threshold of the FOLFOX groups had not quite returned to the level of the injection vehicle-treated group, whereas the oxaliplatin group had more completely recovered (Fig. 1a).

The cold plate test measured the response to a thermal non-noxious stimulus as the time latency of licking or retracting paw after a cold stimulus. As shown in Fig. 1b, both FOLFOX and oxaliplatin alone significantly enhanced the sensitivity to cold from day 14 until, day 35 and day 42, respectively, and picking on day 28 [15.0 ± 0.7 s, FOLFOX (4)] and day 35 (12.5 ± 0.5 s, oxaliplatin), respectively. A fifth injection of FOLFOX reduced the pain threshold in response to a thermal non-noxious stimulus up to day 42, reaching a nadir on that day [10.9 ± 0.5 s, FOLFOX (5) vs. 23.6 ± 0.8 s, control] (Fig. 1b).

Neither FOLFOX nor oxaliplatin alone altered at any timepoint the pain sensitivity in response to a mechanical non-noxious stimulus in the Von Frey apparatus (Fig. 1c).

Pain threshold measurements: effect of single doses of duloxetine, morphine, pregabalin, and dimiracetam against FOLFOX-induced hypersensitivity

Following single doses of duloxetine (15 mg kg^{-1}, p.o.), morphine (10 mg kg^{-1}, s.c.), pregabalin (20 mg kg^{-1}, p.o.), or dimiracetam (150 mg kg^{-1}, p.o.) on FOLFOX (4)-treated or oxaliplatin-treated rats on day 22, or on FOLFOX (5)-treated animals on day 28, the response to a mechanical noxious stimulus was measured over time up to 45 min after administration. As depicted in Fig. 2a, duloxetine significantly decreased the hypersensitivity induced by FOLFOX (4) up to 30 min exerting the maximum effect 15 min after treatment [57.9 ± 0.5 g, FOLFOX (4) + duloxetine] and

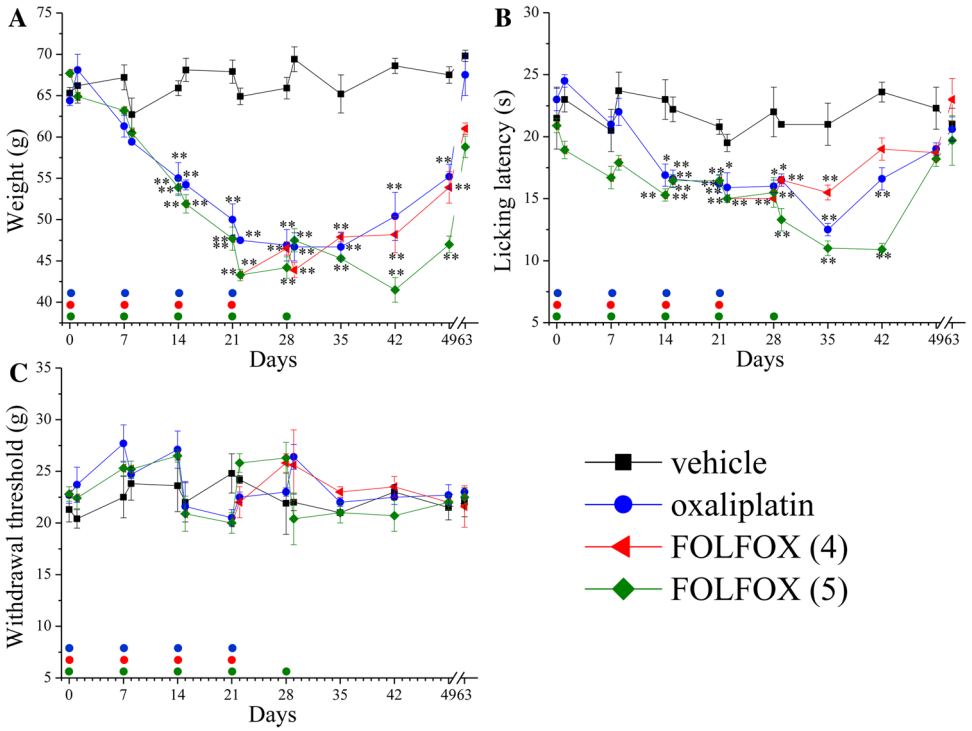

Fig. 1 Pain-related measurements. Oxaliplatin (6.0 mg kg^{-1} i.p.) (blue circle) or FOLFOX (oxaliplatin 6.0 mg kg^{-1} i.p/5-FU 50 mg kg^{-1} i.p./leucovorin 90 mg kg^{-1} i.p.) [FOLFOX (4) red circle] were injected on days 0, 7, 14, and 21 after the behavioural measurements. On day 28, animals were divided into two groups, one was treated once again on the same day with FOLFOX [FOLFOX(5) green circle]. On days 0, 1, 7, 8, 14, 15, 21, 22, 28, 29, 35, 42, 49, and 63, the sensibility to a noxious mechanical stimulus was measured by paw pressure test (**a**), the response to a thermal non-noxious stimulus was evaluated by cold plate test measuring the latency (s) to pain-related behaviours (lifting or licking of the paw) (**b**), and the withdrawal threshold in response to a non-noxious mechanical stimulus was evaluated by the Von Frey apparatus (**c**). Control rats were treated with vehicle. Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle + vehicle-treated animals

morphine and pregabalin reduced FOLFOX (4)-induced hypersensitivity 15 min after injection [56.3 ± 0.3 g, FOLFOX (4) + morphine; 52.3 ± 0.3 g, FOLFOX (4) + pregabalin]. Otherwise, dimiracetam had no effect (Fig. 2a). Similar single-dose profiles were also evident in FOLFOX (5)- and in oxaliplatin-treated rats (Fig. 2b, c).

Pain threshold measurements: effect of repeated doses of duloxetine, morphine, pregabalin, and dimiracetam against FOLFOX-induced hypersensitivity

To evaluate the efficacy of compounds after repeated administrations, FOLFOX (4)-treated animals were daily p.o. treated b.i.d. with pregabalin (20 mg kg^{-1}), duloxetine (15 mg kg^{-1}), or dimiracetam (150 mg kg^{-1}) starting from day 22 for four consecutive weeks. The response to a mechanical noxious stimulus was measured every week over time until 24 h after administration. As shown in Fig. 3, dimiracetam significantly increased the pain threshold of FOLFOX-injected rats, starting from day 28. Pregabalin was effective from day 35. The efficacy of both compounds was not different among 1 and 24 h after treatment suggesting a stable improved pain threshold. Repeated administrations of duloxetine did not modify the hypersensitivity induced by FOLFOX.

Behavioural, neurological, and motor functions: effect of duloxetine, pregabalin, and dimiracetam on FOLFOX-treated animals

As evaluated by the open-field test (Table 1), FOLFOX treatment, on day 22, significantly reduced the number of rearings in comparison with the vehicle group (19.4 ± 2.9, FOLFOX vs. 55.2 ± 6.3, vehicle). The spontaneous activity was also reduced (141.2 ± 20.4, FOLFOX vs. 387.1 ± 32.8, vehicle).

On days 22 and 42, behavioural, autonomic, and neurological parameters were evaluated giving an arbitrary score (from 0 to ± 4), by the Irwin test. On day 22, FOLFOX reduced spontaneous activity, reactivity, and curiosity, whereas the frequencies of tremors, ataxia, piloerections, anemia, and hypothermia were increased (Table 2). Other observational categories were not significantly affected. On day 42, following 21 days of twice-daily oral treatment with

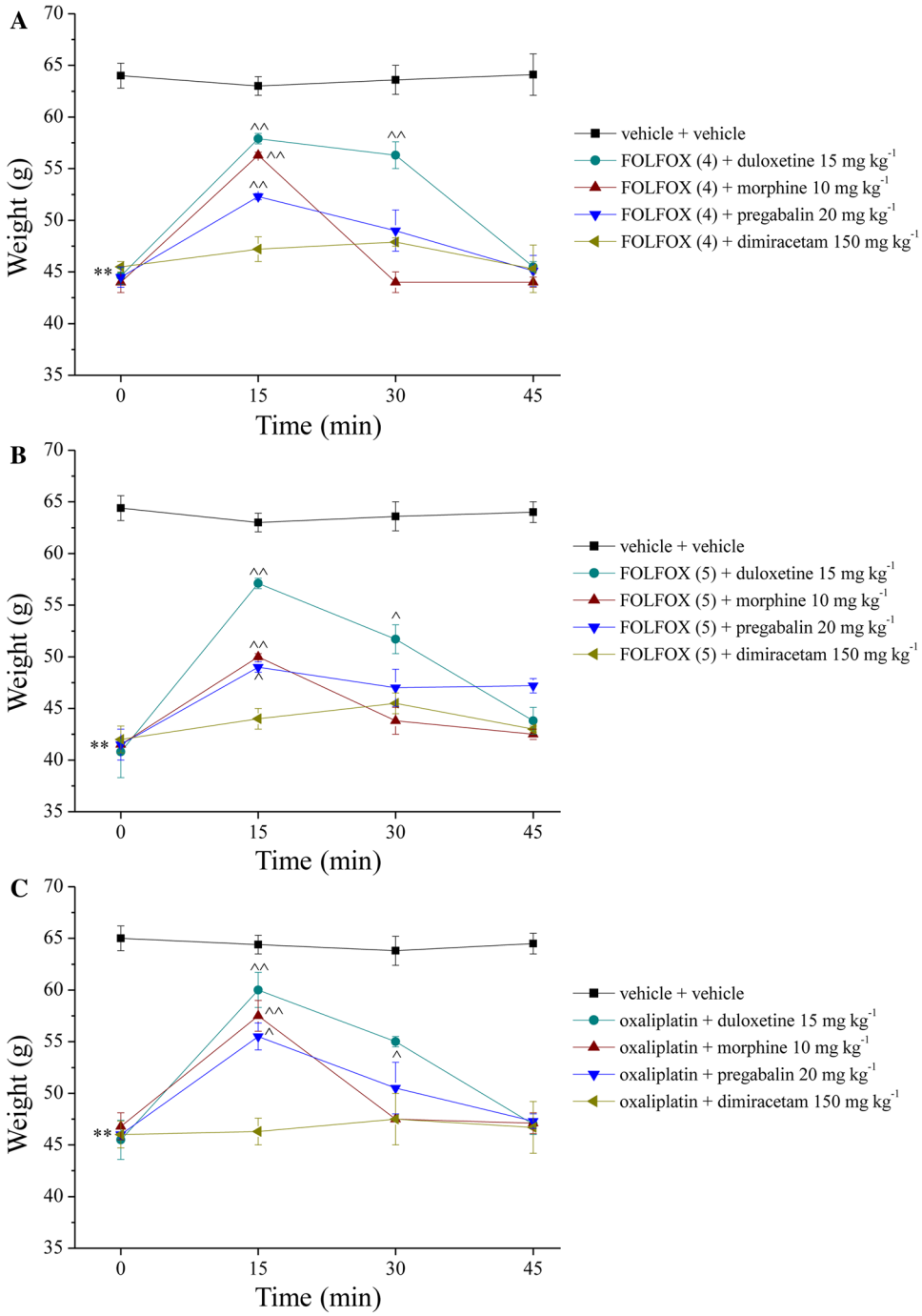

Fig. 2 Mechanical noxious stimulus. Effect of duloxetine, morphine, pregabalin, and dimiracetam. Acute treatment. Duloxetine (15 mg kg^{-1}, p.o.), morphine (10 mg kg^{-1}, s.c.), pregabalin (20 mg kg^{-1}, p.o.), or dimiracetam (150 mg kg^{-1}, p.o.) was administered on day 22 on FOLFOX (4)- (**a**) and oxaliplatin-treated (**b**) animals or on day 28 on FOLFOX (5)-treated (**c**) animals and the response to a mechanical noxious stimulus was measured 0, 15, 30, and 45 min after injection by the paw pressure test. Control rats were treated with vehicles. Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets. $^{**}P < 0.01$ vs. vehicle + vehicle treated rats; $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ vs. time 0 of the same group

dimiracetam, these FOLFOX effects were nearly completely reversed; pregabalin was similarly effective, whereas duloxetine was less effective (Table 3).

Motor coordination was assessed by the Rota-rod apparatus, before and 24 h after FOLFOX administration; during the repeated treatments with dimiracetam, pregabalin and duloxetine measurements were performed once a week. Neither FOLFOX nor compounds altered the number of falls in 600 s in comparison with the control group (Table 4).

Dimiracetam effects on FOLFOX-dependent lethality on colon cancer cells

To evaluate the potential interaction between dimiracetam treatment and the therapeutic property of FOLFOX, the viability of the human colon cancer cell line HT-29 was measured. Table 5 shows the lack of influence of dimiracetam on the concentration-dependent FOLFOX lethal effect after 24 and 48 h incubation.

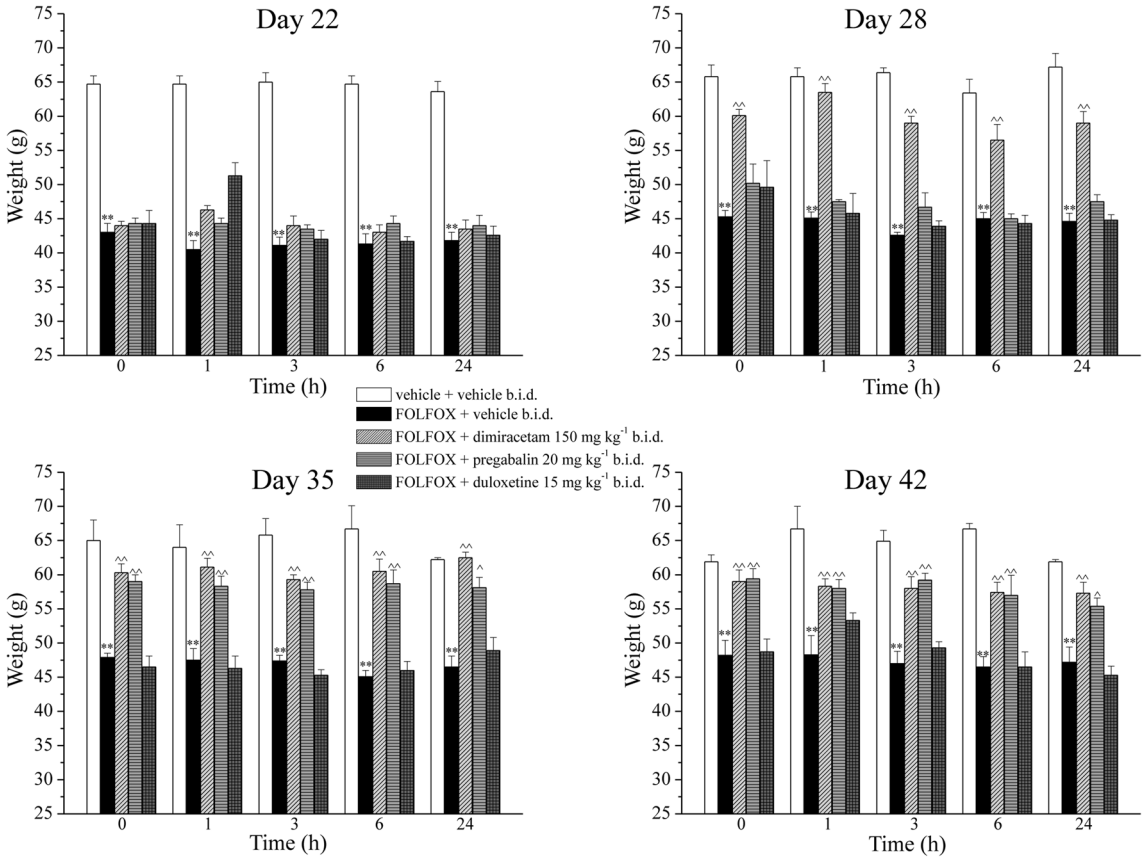

Fig. 3 Mechanical noxious stimulus. Effect of dimiracetam, pregabalin, and duloxetine. Chronic treatment. Starting from day 22, FOLFOX-injected rats (four administrations) were orally administered b.i.d. with dimiracetam (150 mg kg^{-1}), pregabalin (20 mg kg^{-1}), or duloxetine (15 mg kg^{-1}) daily for three consecutive weeks. The response to a mechanical noxious stimulus was assessed by the paw pressure test 0, 1, 3, 6, and 24 h after injection on days 22, 28, 35, and 42. Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets. $^{**}P < 0.01$ vs. vehicle + vehicle-treated animals; $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ vs. FOLFOX + vehicle-treated animals

Discussion

A rat model of FOLFOX-induced neuropathy was developed. Animals weekly treated with the complete antineoplastic regimen, oxaliplatin, 5-FU and LV, received in 4 weeks a cumulative oxaliplatin dose of 24 mg kg^{-1}, corresponding to the clinically relevant dose [20] of 888 mg m^{-2} for the human body, as calculated by the body surface area (BSA) normalization method [21]. The FOLFOX-regimen induced in rats hypersensitivity to mechanical noxious and thermal (cold) non-noxious stimuli, which untreated persists for at least 5 weeks after the last FOLFOX administration. In the present model, we could not detect a change from baseline in the paw-withdrawal threshold in response to a mechanical non-noxious stimulus. Another injection of FOLFOX (the fifth, reaching 30 mg kg^{-1} of cumulative oxaliplatin, corresponding to 1110 mg m^{-2}) enhanced the severity of the hypersensitivity to both mechanical noxious and thermal (cold) non-noxious stimuli, but the course of recovery in the drug-free follow-up period was similar to the FOLFOX (4).

Behavioural and motor parameters were impaired, since FOLFOX strongly reduced the curiosity and the spontaneous activity of animals even if motor coordination was not altered. A deeper investigation on behavioural, neurological, and autonomic functions highlighted reduced reactivity and curiosity concomitantly with increased frequency of tremors, ataxia, anemia, and hypothermia. These alterations were mighty evident after the last administration of FOLFOX and, although reduced, still present 3 weeks after treatment discontinuation.

Neuropathy is not a common complication of 5-FU therapy [20]. However, some patients have experienced 5-FU-induced neurotoxicity, caused by the accumulation of the fluoropyrimidine metabolites. The alteration of the neurophysiological profile induced by 5-FU may make nerve fibers prone to further degeneration [7]. Otherwise, as evidenced by Andrè and colleagues in the

Table 1 Open-field test: effect of FOLFOX on behavioural and locomotor activity

	Treatments	
	Vehicle + vehicle	FOLFOX + vehicle
Time spent in the corners (s)	977.8 ± 22.0	1053.4 ± 36.3
Time spent in the center (s)	21.5 ± 4.4	11.8 ± 4.3
No. of rearings	55.2 ± 6.3	19.4 ± 2.9**
No. of crossings to the center	6.6 ± 1.5	2.4 ± 1.2
No. of crossings to the periphery	155.3 ± 21.4	90.2 ± 20.8
Spontaneous inactivity (s)	812.9 ± 32.8	1099.6 ± 40.3**
Spontaneous activity (s)	387.1 ± 32.8	141.2 ± 20.4**

On day 22, after four administrations of FOLFOX, behavioural and locomotor activity functions were assessed using the open-field test. Time spent in the corners and in the center; number of rearings; number of crossings to the center or to the periphery; spontaneous activity; and spontaneous inactivity of each rat were monitored for 20 min and analyzed using the X-plo-rat software system. Each value represents the mean ± S.E.M. of ten rats per group performed in two different experimental sets

**$P < 0.01$ vs. vehicle + vehicle-treated animals

MOSAIC trial, the addition of the platinum-derivative to the combination of 5-FU/LV increased the rate of patients manifesting chronic neuropathy [22]. The present FOLFOX model shows few, if any, differences over the simpler oxaliplatin-only model, except that recovery of in the drug-free follow-up period was more complete, confirming the main neurotoxic role of the platinum derivative. In humans, the association with 5-FU and LV allows a decrease of oxaliplatin cumulative dosage [23], however, introducing new variables in the mechanisms of neuropathy as well as more possibilities of interactions with pain relieving drugs. To note, the pathophysiology of FOLFOX neurotoxicity has not been investigated yet and molecular mechanisms occurring in the chronic form of oxaliplatin-induced neuropathy remain unclear. Morphological examination revealed that the primary target of oxaliplatin, and more in general of platinum compounds, is the dorsal root ganglia (DRGs), where the accumulation of the antineoplastic agent triggers nuclear damage [10, 24]. Molecular modifications occurred both in DRGs and in peripheral nerves [10, 25, 26]. To note, despite the low oxaliplatin capability to cross the blood brain barrier [27, 28], dramatic alterations of the CNS were shown. Oxidative damages [29], increased hyper-excitability of the nociceptive-specific neurons in the dorsal horn of the spinal cord [8, 9], were related to oxaliplatin-induced pain. A maladaptive plasticity involves also the glial cells, strongly involved in the development and maintenance of neuropathic pain states [30, 31]. The activation of astrocytes continuously accompanies the treatment with the antineoplastic agent,

Table 2 Irwin test, day 22: effect of FOLFOX on behavioural, autonomic, and neurological parameters

	Treatments		Limits
	Vehicle + vehicle	FOLFOX + vehicle	
Behaviour			
Spontaneous activity	4 ± 0	1.2 ± 0.2*	4–0
Passivity	0 ± 0	3.1 ± 0.3*	0–4
Cleaning	4 ± 0	3.1 ± 0.3	4–0
Curiosity	4 ± 0	2.2 ± 0.3*	4–0
Reactivity	4 ± 0	0.9 ± 0.1*	4–0
Vocalization	0 ± 0	0 ± 0	0–4
C.N.S. excitement			
Straub tail	0 ± 0	0 ± 0	0–4
Tremors	0 ± 0	3.3 ± 0.4*	0–4
Convulsions	0 ± 0	0 ± 0	4–0
Movement			
Ataxia	0 ± 0	2.3 ± 0.1*	0–4
Stereotipies	0 ± 0	1.1 ± 0.4	0–4
Straightening reflex	4 ± 0	4 ± 0	4–0
Muscular tone			
Physical strength	4 ± 0	2.4 ± 0.8	4–0
Reflexes			
Palpebral reflex	4 ± 0	3 ± 0.4	4–0
Autonomic signs			
Piloerection	0 ± 0	3.2 ± 0.2*	0–4
Exolphthalmos	0 ± 0	1.8 ± 0.5	0–4
Cyanosis	0 ± 0	0 ± 0	0–4
Flush	0 ± 0	0 ± 0	0–4
Pallor	0 ± 0	3.8 ± 0.2*	0–4
Palpebral opening	4 ± 0	4 ± 0	4–0
Salivation	0 ± 0	0 ± 0	0–4
Lacrimation	0 ± 0	0 ± 0	0–4
Hypo-hyperthermia	0 ± 0	− 2.1 ± 0.1*	− 4/+ 4
Writhing	0 ± 0	0 ± 0	0–4
Toxicity			
Immediate death	0 ± 0	0 ± 0	0–4
Delayed death (48 h)	0 ± 0	0 ± 0	0–4

On day 22, after four administrations of FOLFOX, the Irwin test was performed in the rat; it involves subjective assessment of behavioural, autonomic, and neurological manifestations in spontaneous, freely moving animals. Skin colour was evaluated qualitatively; other signs were evaluated semi-quantitatively, according to the trained observer's personal scale (0 to +4, − 4 to 0, or −4 to +4). Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets

*$P < 0.05$ vs. vehicle + vehicle treated rats

Table 3 Irwin test, day 42: effect of dimiracetam, pregabalin, duloxetine on FOLFOX-induced behavioural, autonomic and neurological alterations

	Treatments					Limits
	Vehicle + vehicle	FOLFOX + vehicle	FOL-FOX + dimiracetam	FOL-FOX + pregabalin	FOL-FOX + duloxetine	
Behaviour						
Spontaneous activity	4 ± 0	2 ± 0.1*	4 ± 0^	4 ± 0^	2.8 ± 0.3	4–0
Passivity	0 ± 0	2.1 ± 0.2*	0 ± 0^	0 ± 0^	1.4 ± 0.5	0–4
Cleaning	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4–0
Curiosity	4 ± 0	2.7 ± 0.1*	4 ± 0^	4 ± 0^	4 ± 0^	4–0
Reactivity	4 ± 0	2.8 ± 0.1*	4 ± 0^	4 ± 0^	3.1 ± 0.2	4–0
Vocalization	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
C.N.S. excitement						
Straub tail	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Tremors	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Convulsions	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4–0
Movement						
Ataxia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Stereotipies	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Straightening reflex	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4–0
Muscular tone						
Physical strength	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4–0
Reflexes						
Palpebral reflex	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4–0
Autonomic signs						
Piloerection	0 ± 0	2.1 ± 0.2*	1.1 ± 0.1^	1 ± 0.2^	1.3 ± 0.1^	0–4
Exolphthalmos	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Cyanosis	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Flush	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Pallor	0 ± 0	3 ± 0.2*	1.2 ± 0.1^	1.2 ± 0.1^	3 ± 0.3	0–4
Palpebral opening	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4–0
Salivation	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Lacrimation	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Hypo-hyperthermia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	− 4/+ 4
Writhing	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Toxicity						
Immediate death	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4
Delayed death (48 h)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0–4

On day 42, following 21 days of twice daily oral administration of dimiracetam (150 mg kg^{-1}), pregabalin (20 mg kg^{-1}), or duloxetine (15 mg kg^{-1}), the Irwin test was performed on FOLFOX-injected rats (four administrations). Skin colour was evaluated qualitatively; other signs were evaluated semi-quantitatively, according to the observer's personal scale (0 to +4, − 4 to 0, or −4 to +4). Each value represents the mean ± S.E.M. of ten rats per group, performed in two different experimental sets

*$P < 0.05$ vs. vehicle + vehicle; ^$P < 0.05$ vs. FOLFOX + vehicle

suggesting a complex involvement of astrocytes in pain chronicization [10, 11, 32].

Although the evidences collected up to now about the neurotoxicity evoked by the platinum derivative, effective treatments against FOLFOX-induced neuropathy are lacking. Duloxetine is the only recommended intervention. Nevertheless, it is not completely effective and did not work for everyone [33]. Tricyclic antidepressants are not recommended but clinicians may use them after discussion with patients about the limited effectiveness. The antiepileptic drugs, pregabalin and gabapentin, have not well-established efficacy as resulted from clinical trials, but given the limited options for managing neuropathy, their clinical use is encouraged [13].

Table 4 Rota-rod test: evaluation of motor coordination

Day	Treatments				
	Vehicle + vehicle	FOLFOX + vehicle	FOL-FOX + dimiracetam	FOL-FOX + prega-balin	FOL-FOX + dulox-etine
0	4.0 ± 1.0	4.4 ± 0.4			
1	1.3 ± 0.3	0.7 ± 0.2			
7	1.0 ± 0.6	0.7 ± 0.2			
8	0.0 ± 0.0	0.0 ± 0.0			
14	2.0 ± 1.5	1.9 ± 0.5			
15	0.0 ± 0.0	0.5 ± 0.3			
21	2.0 ± 1.5	1.9 ± 0.6			
22	1.2 ± 0.5	1.5 ± 0.5	1.2 ± 0.3	1.0 ± 0.9	1.5 ± 0.6
28	0.8 ± 0.3	1.0 ± 0.3	0.8 ± 0.5	1.0 ± 0.7	1.2 ± 0.8
35	1.0 ± 0.6	0.5 ± 0.3	1.4 ± 1.2	1.3 ± 0.4	1.4 ± 1.2
42	0.7 ± 0.7	0.8 ± 0.3	0.0 ± 0.0	0.5 ± 0.4	0.2 ± 0.2
49	0.0 ± 0.0	1.2 ± 1.3	0.6 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
63	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

From day 0, every week, the integrity of the animals' motor coordination was assessed using a Rota-rod apparatus measuring the number of falls from the rotating rod in a fixed time (600 s). Measurements were performed before and 24 h after FOLFOX administration (four administrations) during the first 4 weeks, and once a week, during treatments with dimiracetam, pregabalin and duloxetine. Each value represents the mean ± SEM of ten rats per group, performed in two different experimental sets

Table 5 HT-29 cell viability after 24 and 48 h incubation

FOLFOX concentration (μM)		Cell viability %			
		24 h incubation		48 h incubation	
		Control	Dimiracetam (100 μM)	Control	Dimiracetam (100 μM)
Oxaliplatin	0	100 ± 3.95	100 ± 3.63	100 ± 3.95	100 ± 3.63
5-Fluorouracil	0				
Leucovorin	0				
Oxaliplatin	0.3	97.50 ± 3.80	105.33 ± 3.98	104.96 ± 4.74	96.32 ± 5.21
5-Fluorouracil	7.7				
Leucovorin	3.5				
Oxaliplatin	1	91.22 ± 2.46	106.20 ± 3.22	108.49 ± 3.54	95.83 ± 4.80
5-Fluorouracil	25.6				
Leucovorin	11.6				
Oxaliplatin	3	107.17 ± 4.45	100.13 ± 5.24	102.99 ± 6.36	92.40 ± 3.05
5-Fluorouracil	76.8				
Leucovorin	35				
Oxaliplatin	10	91.76 ± 2.89	63.27 ± 3.02***	96.68 ± 4.20	61.55 ± 2.06***
5-Fluorouracil	256				
Leucovorin leucovorin 136 μM	116				
Oxaliplatin	30	66.52 ± 2.60***	36.92 ± 1.54***	72.90 ± 2.57***	35.14 ± 0.41***
5-Fluorouracil	768				
Leucovorin leucovorin 410 μM	348				

HT-29 cells were treated with increasing concentrations of FOLFOX components in the presence or in the absence of 100 μM dimiracetam. Incubation was allowed for 24 h or 48 h. Cell viability was measured by MTT assay. Control condition was arbitrarily set as 100% and values are expressed as the mean ± SEM of three experiments

***$P < 0.001$ in comparison with control (FOLFOX 0 μM)

Noteworthy, current pharmacotherapy of FOLFOX-dependent neuropathy was developed by modelling pain using oxaliplatin alone (in vitro or in vivo) without considering the complications introduced in humans by the presence of 5-FU and LV. In the present preclinical model of FOLFOX-induced neuropathy, duloxetine, and at a lesser extent morphine or pregabalin, was active after an acute administration. Duloxetine exerted the longest action in comparison with the other two compounds, while morphine is the less effective, confirming the low effectiveness of opioids in CINs [34]. Interestingly, in the present experiments emerged that the effect of duloxetine is symptomatic only since a repeated twice daily treatment does not evoke a prolonged, all day long, pain relieving action. Pregabalin took a "preventive" effect (evaluated as a relief maintained over 24 h) after 14 days of treatment. Morphine was not repeatedly administered, since the well-known development of tolerance to the anti-nociceptive effect [35, 36].

The racetam derivative dimiracetam was not effective after a single administration. On the contrary a repeated treatment fully reduced the FOLFOX-induced hypersensitivity starting after 7 days of treatment. The effect was long lasting (at least 24 h) without the development of tolerance. Moreover, dimiracetam reduced the behavioural, neurological, and autonomic alterations evoked by the anti-neoplastic agents. Racetam, even called nootropics, are a family of 2-pyrrolidinone derivatives designed in the sixties and profiled as cognition enhancers. Racetam compounds, such as nefiracetam and levetiracetam, have shown anti-hyperalgesic effect in animal models of neuropathic pain [37, 38]. Dimiracetam itself has been already reported to be active against neuropathy induced by the chronic constriction injury of the sciatic nerve and diabetic neuropathy [39]. Moreover, dimiracetam was effective in preclinical models of chemotherapy-induced neuropathic pain triggered by the anticancer compounds oxaliplatin or sorafenib or by antiretroviral drugs [14, 40]. To note, the racetam derivative as well as pregabalin act throughout the glutamatergic system, since pregabalin reduces the release of synaptic vesicles from glutamatergic neurons [41], and dimiracetam decreases the NMDA-induced release of glutamate in synaptosomal preparations from rat spinal cord [14]. Recently, we reported that the glutamate release was enhanced in cerebrocortical nerve terminals of oxaliplatin-treated rats [42]. Furthermore, astrocytes, major players in the oxaliplatin-induced chronic neuropathy, once activated, amplify glutamate signals acting on presynaptic AMPA receptors [43]. These evidences suggest that the central nervous system is a primary target of the racetam compound. It is relevant to note the absence of interaction between dimiracetam and the lethal effect exerted by FOLFOX on the human colon cancer cells HT-29, suggesting a safety profile when used in combination with anticancer agents. Dimiracetam is currently in clinical development for treatment and/or prevention of CIN.

Conclusion

Our results describe for the first time a protocol of FOLFOX administration able to induce in the rat the onset of a chronic neuropathy characterized by hypersensitivity to mechanical and thermal (cold) stimuli as well as impairment of behavioural, neurological, and autonomic parameters. Dimiracetam, repeatedly administered, completely counteracts FOLFOX-induced neuropathic alterations. The optimal safety profile of this compound suggests its possible use as treatment of the FOLFOX-induced neuropathy.

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Author contributions MM and LM performed the experimental tests; LDCM, MM, and CF performed the statistical analysis of data; LDCM, CG, CF, and MS participated in the design of the study; LDCM, MM, CG, CF, and MS conceived of the study, and participated in its coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest CG, MS, and CF have a patent (patent n. EP2857017) issued. MS and CF report personal fees, outside the submitted work, from Metys Pharma as CEO and as employee, respectively. LDCM, MM, and LM declare that have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Procedures were approved by the Italian Ministry of Health, decree No. 54/2014B.

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