Research Paper

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Nicotinamide riboside, a form of vitamin B3 and NAD^+ precursor, relieves the nociceptive and aversive dimensions of paclitaxel-induced peripheral neuropathy in female rats

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Abstract

Injury to sensory afferents may contribute to the peripheral neuropathies that develop after administration of chemotherapeutic agents. Manipulations that increase levels of nicotinamide adenine dinucleotide (NAD^+) can protect against neuronal injury. This study examined whether nicotinamide riboside (NR), a third form of vitamin B3 and precursor of NAD^+, diminishes tactile hypersensitivity and place escape–avoidance behaviors in a rodent model of paclitaxel-induced peripheral neuropathy. Female Sprague-Dawley rats received 3 intravenous injections of 6.6 mg/kg paclitaxel over 5 days. Daily oral administration of 200 mg/kg NR beginning 7 days before paclitaxel treatment and continuing for another 24 days prevented the development of tactile hypersensitivity and blunted place escape–avoidance behaviors. These effects were sustained after a 2-week washout period. This dose of NR increased blood levels of NAD^+ by 50%, did not interfere with the myelosuppressive effects of paclitaxel, and did not produce adverse locomotor effects. Treatment with 200 mg/kg NR for 3 weeks after paclitaxel reversed the well-established tactile hypersensitivity in a subset of rats and blunted escape–avoidance behaviors. Pretreatment with 100 mg/kg oral acetyl-L-carnitine (ALCAR) did not prevent paclitaxel-induced tactile hypersensitivity or blunt escape–avoidance behaviors. ALCAR by itself produced tactile hypersensitivity. These findings suggest that agents that increase NAD^+, a critical cofactor for mitochondrial oxidative phosphorylation systems and cellular redox systems involved with fuel utilization and energy metabolism, represent a novel therapeutic approach for relief of chemotherapy-induced peripheral neuropathies. Because NR is a vitamin B3 precursor of NAD^+ and a nutritional supplement, clinical tests of this hypothesis may be accelerated.

Keywords: NAD^+, Nicotinamide riboside, Paclitaxel, Chemotherapy induced peripheral neuropathy, Place escape avoidance paradigm

1. Introduction

Continuing advances in the detection and treatment of cancer have greatly increased the number of cancer survivors, and with this has come a much better appreciation of the long-term sequelae of treatment. Chemotherapy-induced peripheral neuropathy (CIPN) is a common adverse side effect of many anticancer agents used today, including taxanes such as paclitaxel.[3,39,42,56] In some cases, the neuropathies increase in severity and persist for years. Despite being a major clinical problem for patients with cancer and cancer survivors, there are virtually no evidence-based treatments for CIPN.[25] The need for highly effective treatments for CIPN and the absence of evidence supporting the efficacy of those currently in use has been highlighted in a position article from the American Society for Clinical Oncology.[25]

Multiple mechanisms, some specific to the chemotherapeutic agent, are implicated in the development of CIPN. Candidate mechanisms include mitochondrial dysfunction, peripheral nerve degeneration, nitro-oxidative stress, modulation of ion channels, and glial activation.[12,17,24,28,29] In recent years, evidence has accrued that nicotinamide adenine dinucleotide (NAD^+), an essential redox coenzyme,[48,70] plays an important role in protection against axonal injury from either mechanical or neurotoxic injury.[2,22,52–54] Maintenance of NAD^+ has been shown to be protective in mitochondrial disease.[31] Nicotinamide riboside (NR) is a recently discovered vitamin precursor of NAD^+.[8,65] It is converted to NAD^+ in a manner first initiated by the NR kinases NRK1 and NRK2[8,45] leading to nicotinamide mononucleotide, to which a second adenine is transferred by nicotinamide mononucleotide adenylyl transferase to generate NAD^+. Significantly, the transcript for the tissue-specific enzyme NRK2 is increased between 5 and 20-fold for at least 14 days in dorsal root ganglion neurons of rats after sciatic nerve transection.[52] Transcriptional upregulation of NRK2 suggests that damaged neurons are primed to use NR either to elevate or maintain NAD^+. Indeed, administration of NR was recently reported to protect mice against noise-induced hearing loss and neurite degeneration in the spiral ganglia[10] and to

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normalize deficits in nerve conduction velocity, alleviate heat hypoalgesia, and protect against loss of corneal and intraepidermal nerve fibers in prediabetic and type 2 diabetic mice.[64] We therefore examined the efficacy of NR in a rat model of paclitaxel-induced peripheral neuropathy that entailed intravenous (i.v.) injection of doses of paclitaxel that closely approximate those given to patients. This study not only included a conventional measure of tactile hypersensitivity but also assessed the aversive dimension of nociception using the place escape–avoidance paradigm. Acetyl-L-carnitine (ALCAR) was included as a comparator given the existence of compelling preclinical data supporting its efficacy in rodent models of CIPN.[19,20,30,69]

2. Methods and materials

2.1. Animals

These studies were approved by the University of Iowa Animal Care and Use Committee (protocol # 4101188) and were conducted in accordance with guidelines set forth by the National Institutes of Health and the International Association for the Study of Pain. Every effort was made to minimize animal suffering and the number of animals used in this study. Adult female Sprague-Dawley rats weighing 125 to 150 g (Charles River Laboratories, Raleigh, NC) were purchased for this study. The estrous cycle was not monitored given that the workgroup of the International Association for the Study of Pain considered the use of a hormone depletion/replacement approach to be a more direct and preferable approach to assessing the influence of reproductive hormones than testing female rodents at different stages of the estrous cycle.[23] All rats were acclimated for at least 48 hours in the animal care facility before any experiment. Rats were housed in the animal care facility as two per cage in a temperature and humidity controlled room on a 12 hour light/dark cycle, and with ad libitum access to food and water. Gruel was made available in the cages of paclitaxel-treated rats if it seemed that weight gain was affected in the week of its administration. **Table 1** lists the number of rats in each treatment group and experiment, as well as reasons for exclusion or loss to study.

Table 1

Summary of rat usage and allocation by study.

Treatment	Number entered	Number completed	Number excluded
Effects of prophylactic administration of NR on paclitaxel-induced pain behaviors			
p.o. NR + i.v. paclitaxel	12	12	0
p.o. VEH + i.v. paclitaxel	13	11	2*
p.o. NR + i.v. KES-VEH	9	8	1†
p.o. VEH + i.v. KES-VEH	8	8	0
Effects of therapeutic administration of NR on paclitaxel-induced pain behaviors			
p.o. NR + i.v. paclitaxel	12	12	0
p.o. VEH + i.v. paclitaxel	10	8	2‡
Effects of prophylactic administration of ALCAR on paclitaxel-induced pain behaviors			
p.o. ALCAR + i.v. paclitaxel	8	8	0
p.o. VEH + i.v. paclitaxel	5	5	0
p.o. ALCAR + i.v. KES-VEH	8	8	0
p.o. VEH + i.v. KES-VEH	7	7	0
Myelosuppression by paclitaxel			
p.o. NR + i.v. paclitaxel	8	8	0
p.o. VEH + i.v. paclitaxel	8	8	0
Naive	8	7	1§
i.p. paclitaxel	8	8	0
Locomotor activity			
p.o. NR	8	8	0
p.o. VEH	8	8	0
NAD^+ measurements			
p.o. NR + i.v. paclitaxel	8	8	0
p.o. VEH + i.v. paclitaxel	8	8	0
p.o. NR + i.v. KES-VEH	9	9	0
p.o. VEH + i.v. KES-VEH	8	8	0
Naive	4	4	0

Rats were used once.
* Killed because of weight loss attributed to malfunctioning water lick stick; housed together in one cage.
† Died after second injection of KES vehicle.
‡ Did not develop tactile hypersensitivity.
§ Blood clotted; not submitted for analysis.
ALCAR, acetyl-L-carnitine; i.p., intraperitoneal; i.v., intravenous; NR, nicotinamide riboside; p.o, oral.

2.2. Paclitaxel model of chemotherapy-induced peripheral neuropathy

Rats were weighed and lightly anesthetized with isoflurane for i.v. injection of paclitaxel through the tail vein. Immediately before injection, the volume of the generic formulation of paclitaxel (6 mg/mL, Lot 58J5141; Hospira, Inc, Lake Forest, IL) required to deliver a dose of 6.6 mg/kg body weight was drawn by a syringe and mixed with sufficient sterile saline to a final volume of 500 μL. Because the solution becomes viscous if allowed to sit, the clinical formulation was diluted immediately before injection. This approach results in a solution that retains the paclitaxel in solution, is not viscous, and readily passes through a 25-G needle for i.v. injection. Each rat received 3 i.v. injections of 500 μL over 5 days (days 1, 3, and 5), resulting in a final total dose of 19.8 mg/kg. For intraperitoneal (i.p.) injections of paclitaxel, the clinical formulation was diluted with sterile saline to deliver 2 mg/kg to each rat. Four injections were made over 8 days to deliver a final total dose of 8 mg/kg. For control animals, a similar volume of the vehicle for paclitaxel, a 50:50 (vol/vol) mixture of Kolliphor (formerly Cremophor, Lot BCBM8568V; Sigma-Aldrich, St. Louis, MO) and 100% ethanol, based on body weight was drawn into a syringe to which sterile saline was added (KES-VEH or KES vehicle) for a final volume of 500 μL. Control rats received 3 i.v. injections of KES vehicle as for paclitaxel. Rats were returned to their cages and monitored daily. If there was difficulty with the i.v. injection, an ulcer could develop at the site of injection that was readily treated with antibiotic ointment. No abnormal behaviors were noted during or after paclitaxel or vehicle treatment.

Paclitaxel was administered intravenously because it is a common route of administration for patients with cancer. The total paclitaxel dose of 19.8 mg/kg was selected based on conversion factors recommended by the Food and Drug Administration to scale doses among mice, rats, and humans.[21] It is equivalent to a dose of 120 mg/m^2 in humans and within the range of therapeutic doses administered to patients with breast cancer.[38,58] When administered to female rats, this dose produced an 80% reduction in neutrophils, eosinophils, and monocytes compared with values in naive rats (**Table 2**). Of note, the low-dose regimen favored by many investigators[27] in which rats receive 4 i.p. injections of 2 mg/kg paclitaxel (days 1, 3, 5, and 7) did not reduce the number of white blood cells (**Table 2**).

2.3. Experimental design for nociception

All experiments were conducted using cohorts of rats; the experimental unit was a single rat. Rats from each shipment of

Table 2

Complete blood count in time-matched naive and paclitaxel-treated rats.

Treatment	WBC ($\times 10^3$)	RBC ($\times 10^6$)	HGB, g/dL	Neutrophils	Lymphocytes	Monocytes	Eosinophils
Naïve (7)	4.5 ± 0.6	7.1 ± 0.3	14.3 ± 0.4	433.0 (317-710)	3767.1 ± 543.4	118.0 (70-277)	56.6 ± 15.4
VEH + IV PAC (8)	2.8* ± 0.4	6.4 ± 0.3	12.8 ± 0.5	85.0† (49.5-168)	2637.0 ± 343.0	20.5† (13.3-27.8)	9.6† ± 3.2
NR + IV PAC (8)	2.8* ± 0.3	6.3 ± 0.2	12.9 ± 0.4	99.5† (53.5-119)	2665.5 ± 267.4	11.0† (2.5-27.3)	17.1† ± 4.1
IP PAC (8)	4.0 ± 0.5	7.2 ± 0.2	14.4 ± 0.3	357.0 (253.8-491.8)	3415.8 ± 468.7	135.0 (79.3-235.8)	46.1 ± 11.6

Rats were pretreated for 1 week with vehicle (VEH) or 200 mg/kg oral nicotinamide riboside (NR) followed by a cumulative dose of 19.8 mg/kg intravenous paclitaxel (IV PAC). Samples were also obtained from naive rats and those treated with a cumulative dose of 8 mg/kg intraperitoneal paclitaxel (IP PAC). Samples were obtained the day after the last dose of paclitaxel. Values are per microliter blood, with the exception of hemoglobin (HGB). Data are expressed as mean ± SEM when assumptions for statistical analysis by 1-way analysis of variance were met or expressed as median and 25th to 75th percentile when differences in variance warranted analysis by the Kruskal–Wallis test. The number of rats is indicated in parentheses. The number of basophils was zero in the majority of rats. One rat in the naive group had 12 basophils and 2 rats in the IP PAC group had 10 and 11 basophils.

* $P < 0.05$.

† $P < 0.01$ compared with naive rats.

RBC, red blood cells; WBC, white blood cells.

8 to 12 were randomly allocated among the different treatment conditions to form a cohort; the numbers of rats in each treatment group in a cohort were not necessarily identical. With the exception of the study of ALCAR, multiple replicates of each cohort were performed. The experimenter (M.V.H.) was masked to the drug treatment at all times. Rats were assigned permanent codes and were injected with paclitaxel or the KES vehicle by S.R.W. Daily gavage with NR, vehicle, or ALCAR was performed by S.R.W. or the senior author. The day before the behavioral testing, each rat was assigned a temporary identifier and cages were randomized on the rack system. Data were entered by S.R.W. and analyzed by the senior author. Given the longitudinal nature of the study, rats were assigned a different temporary identifier on each occasion that behaviors were tested.

Nicotinamide riboside chloride (Lot # 40C910-15205-21/CDXA-RSS-6269-03/40C910-15202-21; ChromaDex, Irvine, CA) or ALCAR (Lot # SLBL2963V; Sigma-Aldrich) was dissolved in distilled water and administered orally by gavage (1 mL/kg body weight; orally [p.o.]) using a feeding needle. The dose of NR was based on previous reports that supplementation of food with 400 mg/kg/d NR increased NAD^+ in mouse muscle stem cells,[71] skeletal muscle,[11,13] and liver.[11,66] Using conversion tables from the Food and Drug Administration,[21] this dose equated to 200 mg/kg in rats. Additionally, it should be noted that single oral doses of 185 mg/kg to mice and equivalent oral doses to humans are sufficient to elevate liver and blood NAD^+ metabolism, respectively.[66] To control for circadian effects, dosing with NR or ALCAR occurred between 14:00 and 15:00 hours. Behavioral testing occurred between 07:00 and 12:00 hours.

A prophylactic experimental design was used to determine the ability of NR to prevent the development of CIPN. Baseline measurements of tactile sensitivity were taken before paclitaxel or KES vehicle administration. Rats were then orally dosed once per day with 200 mg/kg NR, 100 mg/kg ALCAR, or water for 7 days before the first i.v. injection of paclitaxel or KES vehicle. Drug treatment continued for another 24 consecutive days. Tactile sensitivity was assessed on days 10, 14, and 21, and place escape–avoidance behaviors were assessed on day 24 after initiation of paclitaxel treatment. Additional measures of tactile sensitivity were then made 7 and 14 days after NR treatment ceased, whereas a second assessment of place escape–avoidance behaviors was made at day 42. These latter measures examined whether the beneficial effect of NR was sustained after treatment ceased.

A therapeutic treatment paradigm was also used. In this set of experiments, baseline tactile sensitivity was assessed after which paclitaxel administration was initiated. Tactile sensitivity was reassessed on days 10 and 14 to establish baseline values, after which once daily oral treatment with 200 mg/kg NR or its vehicle began for 24 consecutive days. Tactile sensitivity was reassessed on days 7, 14, and 21 after oral treatment began with NR or vehicle. Place escape–avoidance behaviors were assessed on day 24 after NR treatment began.

2.4. Measures of nociception

2.4.1. Tactile hypersensitivity

Rats were acclimated to the behavior testing room for 30 minutes. To assess tactile hypersensitivity, rats were placed on an elevated mesh surface and allowed to acclimate for an additional 15 minutes. Monofilaments of increasing force (Smith & Nephew, Germantown, WI) were applied to the webbing between the third and fourth digits of the hind paw. Paw withdrawal threshold was determined using the up-and-down method of Dixon as described[14] and the following filaments, 1, 1.4, 2, 4, 6, 8, 10, 15, and 28.8 g. The highest filament passively lifted the hind paw of rats of this weight. Rats that did not respond to the 28.8-g filament were assigned this value.

2.4.2. Modified place escape–avoidance paradigm

To assess the affective/aversive nature of paclitaxel-induced peripheral neuropathy, we used a modification of the place avoidance/escape paradigm (PEAP).[33,44,57] Briefly, a 2-chamber box was used in which one half is dark and the other half is brightly lit. The floor of both chambers was mesh. After placement in the box, the rats had 15 minutes to explore both chambers unperturbed. By nature, rats prefer the dark chamber. Thereafter, during the 15-minute testing phase, whenever the rat entered the preferred dark chamber, each hind paw was repetitively stimulated with a von Frey filament (10 g). Stimuli were delivered at 15-second intervals to alternate hind paws until the rat exited the dark chamber for the brightly lit chamber. Stimulation of the hind paws ceased when the rat entered the brightly lit chamber. The amount of time spent in each chamber was recorded. The chambers were washed thoroughly between rats to minimize any olfactory cues.

2.5. Measurements of NAD^+

Measurements of NAD^+ in the blood were made in a separate cohort of rats that did not undergo any behavioral testing. After daily treatment with 200 mg/kg NR or its vehicle for 1 week, rats received 3 injections of paclitaxel or KES vehicle as previously described for the prophylactic experimental design. Two weeks after the first injection of paclitaxel or KES vehicle, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Blood

samples were obtained by cardiac puncture, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. All blood samples were collected at approximately the same time of day. Quantitative analysis of the NAD^+ metabolome from whole blood was performed by LC-tandem MS.[63] Although NAD^+ is entirely intracellular, its concentration is expressed in molar units with respect to whole-blood volume.

2.6. Measures of myelosuppression

A separate cohort of female rats was randomized to 4 treatment groups. Group 1 and group 2 were gavaged with 200 mg/kg NR or with water, respectively, once daily for 1 week after which they received 3 i.v. injections of 6.6 mg/kg paclitaxel as previously described. Group 3 was housed for a week and then received 4 injections of 2 mg/kg i.p. paclitaxel every other day for a total dose of 8 mg/kg. This low-dose regimen is used by many investigators studying paclitaxel-induced peripheral neuropathy in rats.[27] Group 4 was drug naive and housed as a time-matched cohort. All rats were deeply anesthetized with pentobarbital; blood samples were obtained by intracardiac puncture, encoded to blind identity, and submitted to the University of Iowa Comparative Veterinary Pathology core for a complete blood count analysis.

2.7. Measures of locomotor activity

Locomotor activity was assessed in a separate group of female rats that were gavaged with 200 mg/kg p.o. NR or vehicle once daily for 3 weeks. Two tests were used: accelerating rota-rod (Ugo Basile, Varese, Italy) and open field activity (Coulbourn Instruments, Whitehall, PA). For the open field test, an image of the 40 cm × 40 cm open field was overlaid with a 3 × 3 grid for analysis to generate 9 squares. The number of crossings and total distance traveled after placement in the novel environment field were remotely tracked for 30 minutes and analyzed off-line. The field was cleaned between each rat. For the rota-rod test, the rats were first acclimated to sit quietly on the experimenter's arm and were then given 3 opportunities to navigate the rod for 20 seconds while it rotated at 4 rpm. The next day, the rats were placed on the rota-rod and the rod continuously accelerated to 40 rpm and time until they fell off was recorded. Three trials were conducted and results averaged to yield a single value for each rat.

2.8. Statistical analysis

Data were expressed as mean ± SEM or as median with 25th and 75th percentiles as appropriate. $P < 0.05$ was considered significant for all analyses. Data were examined for adherence to assumptions of equal variance.

2.8.1. Paw withdrawal latency

Paw withdrawal threshold values are nonparametric. The Kruskal–Wallis test was therefore used to compare treatment groups at each time point, and comparisons determined a priori were made by the Mann–Whitney test with correction for the number of comparisons. However, for ease of comparison with a large body of prior literature, the data are presented as mean ± SEM. (see also Refs. 46, 47). Considerable variability in response was observed among NR-treated rats in the posttreatment paradigm. Therefore, a secondary analysis was conducted in which the data were stratified into 2 groups: NR responders and NR nonresponders. The criterion value for this quantal analysis was determined using data from the vehicle-paclitaxel treatment group at 35 days for which the median response, 25th, and 75th percentiles were calculated. In this instance, the 75th percentile was 9.3 g. Therefore, rats in the NR treatment group whose paw withdrawal threshold was greater than 9.3 g were considered to have responded to NR treatment.

2.8.2. Place escape–avoidance paradigm

A 2-way repeated-measure analysis of variance (ANOVA) in which treatment was one factor and phase (explore or test) was the other factor was used to compare effects in the PEAP followed by the Holm–Sidak test.

2.8.3. Measures of locomotor activity

The Mann–Whitney test was used to compare time spent on the rota-rod. Repeated-measures 2-way ANOVA was used to compare the distance traveled and the number of grid crossings as a function of time, as two 15-minute epochs, between the 2 treatment groups.

2.8.4. Other measures

The levels of NAD^+ in the blood were compared among treatment groups using a 2-way ANOVA in which oral treatment was one factor and i.v. treatment was the other factor. Post hoc comparisons among mean values for the individual treatment groups were made using the Bonferroni test. For analysis of complete blood counts, a 1-way ANOVA followed by the Holm–Sidak test was used to compare numbers in the treatment groups with those of naive rats. Where assumptions of equal variance were not met, the Kruskal–Wallis test followed by the Dunn test for comparison with naive rats was used.

3. Results

3.1. General observations

Rats were monitored daily. No obvious changes in behavior, grooming, or activity were noted. No rats exhibited alopecia or diarrhea. The i.v. administration of 19.8 mg/kg paclitaxel in 3 divided doses did not induce weight loss. **Figure 1** illustrates findings for the prophylactic study, which are representative of those for the other experiments. It demonstrates that the 4 treatment groups, 2 that received i.v. KES vehicle and 2 that received paclitaxel, did not differ with respect to weight gain at any time ($P > 0.7$; **Fig. 1**).

3.2. Nicotinamide riboside increased levels of NAD^+ in the blood

Levels of NAD^+ were increased by ~50% in rats that received 200 mg/kg NR once daily for 3 weeks (**Fig. 2**). This increase was evident in NR + KES vehicle and NR + paclitaxel-treated rats ($P < 0.05$ for each). The levels of NAD^+ did not differ between the 2 groups ($P = 0.07$). In vehicle-treated rats, the levels of NAD^+ did not differ between paclitaxel- and KES vehicle-treated rats. It should be noted that the blood samples were obtained 9 days after the last injection of paclitaxel to model the behavioral study. These data indicate that paclitaxel does not produce a persistent decrease in NAD^+. Whether it has an immediate effect would necessitate taking samples within 24 hours of the last dose of paclitaxel. The KES vehicle can have neurotoxic effects of its own.[5,57,68] Therefore, levels of NAD^+ were also determined in the blood of naive, untreated rats. These values (51.5 ± 1.7 μM;

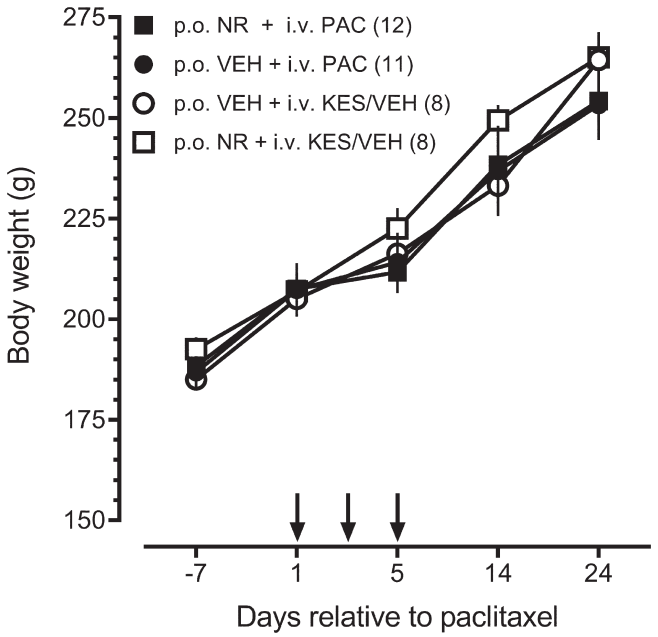

Figure 1. Female rats in all 4 treatment groups gained weight to a similar extent over the course of the prophylactic study. Neither the dose regimen of paclitaxel nor the once-daily oral administration of 200 mg/kg nicotinamide riboside (NR) altered weight gain compared with the corresponding control group. Arrows indicate administration of the 3 divided doses of paclitaxel (PAC) or the Kolliphor:ethanol:saline vehicle (KES/VEH). Data are expressed as mean ± SEM. The ordinate has been cropped and expanded for greater resolution.

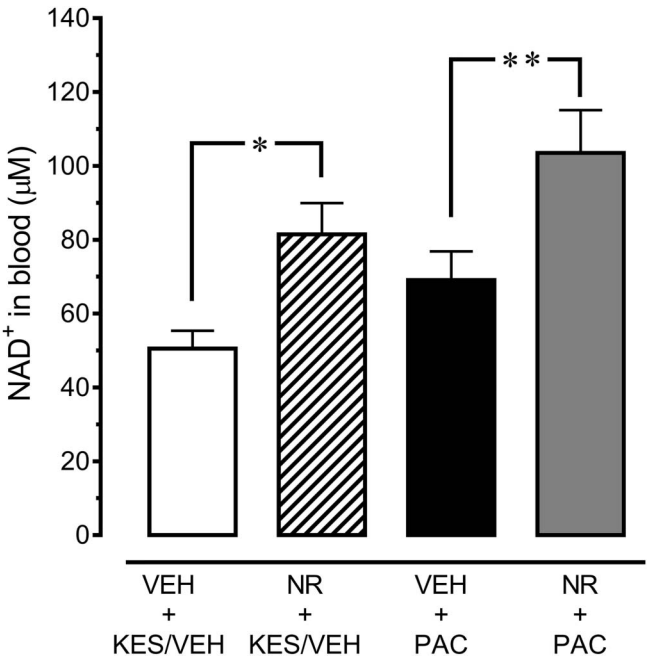

Figure 2. Once-daily oral administration of 200 mg/kg nicotinamide riboside (NR) to female rats for 3 weeks significantly increased levels of NAD^+ in the blood. Rats were treated with NR or vehicle for 7 days before i.v. injection of paclitaxel or its Kolliphor:ethanol:saline vehicle (KES/VEH) and treated for another 2 weeks thereafter. Blood samples were obtained 24 hours after the last dose of NR or its vehicle. In this and subsequent figures, VEH + KES/VEH and VEH + PAC refer to rats gavaged with water and injected i.v. with KES vehicle or paclitaxel, respectively. NR + paclitaxel and NR + KES/VEH refer to rats gavaged with NR and injected i.v. with paclitaxel or KES vehicle, respectively. Data are mean ± SEM of determinations in 8 to 9 rats. *$P < 0.05$, **$P < 0.01$ compared with the corresponding vehicle-treated group.

n = 4) were indistinguishable from those of vehicle + KES vehicle-treated rats ($P > 0.4$; Mann–Whitney *U* test).

3.3. Pretreatment with NR prevented paclitaxel-induced neuropathy

Figure 3A reiterates the dose regimen for prophylactic administration of NR. In vehicle-treated rats, i.v. administration of paclitaxel produced a significant and lasting tactile hypersensitivity that was maximal at 21 days and lasted at least 38 days after its first injection (**Fig. 3B**). Prophylactic oral administration of 200 mg/kg NR for 7 days before and continuing until 24 days after i.v. paclitaxel prevented the development of paclitaxel-induced tactile hypersensitivity (**Fig. 3B**). Moreover, the prophylactic effect of NR was sustained for at least 2 weeks after treatment with NR had ceased.

Pretreatment with NR also significantly blunted escape–avoidance behaviors in paclitaxel-treated rats. **Figure 3C** illustrates the time spent in the light chamber during the exploration and testing phases. On the first occasion of PEAP testing, rats in all 4 treatment groups spent about 3 minutes in the brightly lit chamber, preferring the dark chamber during the 15-minute period of exploration. No differences were observed among the treatment groups. During the subsequent 15-minute test phase, vehicle-paclitaxel-treated rats spent significantly more time in the brightly lit chamber compared with the exploration phase, which suggests that repetitive stimulation of the hind paws in the dark chamber was aversive or nociceptive. In NR + paclitaxel-treated rats, repetitive stimulation of the hind paws did not increase the time spent in the brightly lit chamber. Similarly, repetitive stimulation of the hind paws did not increase the time spent in the brightly lit chamber in either rats treated with vehicle + KES vehicle or NR + KES vehicle. Indeed, in the NR + KES vehicle and the NR + paclitaxel treatment groups, the rats actually spent less time in the brightly lit chamber than during the exploration phase. The vehicle + KES vehicle-treated rats did not exhibit this decrease, which may reflect the presence of mild tactile hypersensitivity.

It is unlikely that NR decreased time in the brightly lit chamber during the test phase because of sedative, anxiolytic, or motoric effects, and more likely that the decline observed in the NR + KES vehicle and NR + paclitaxel groups reflects further acclimation to the environment during the testing phase. First, in an ancillary experiment, 5 naive rats showed a similar decrease in time spent in the brightly lit chamber during the testing phase (data not shown). Second, neither group that received NR differed in time spent in the brightly lit chamber during the exploration phase compared with the respective vehicle group. Third, the number of crossovers between chambers by NR + paclitaxel (22.3 ± 3.0) and NR + KES vehicle treatment groups (19.6 ± 2.5) did not differ from those of vehicle + KES vehicle-treated rats (18.7 ± 2.2). Fourth, in a separate group of rats that received vehicle or NR for 3 weeks, no differences were observed with respect to time on the accelerating rota-rod (**Fig. 4A**, $P > 0.9$), or the number of grid crossings ($P > 0.4$; **Fig. 4B**) and the distance traveled ($P > 0.4$; **Fig. 4C**) after placement in a novel open field environment for 30 minutes. Both treatment groups were less active in the second 15-minute epoch than in the first 15 minutes in terms of grid crossings and distance traveled ($P < 0.01$ for both tests). These data indicate that long-term administration of NR does not adversely affect exploratory or locomotor activity, and that the rats acclimated to the environment in the second 15-minute epoch.

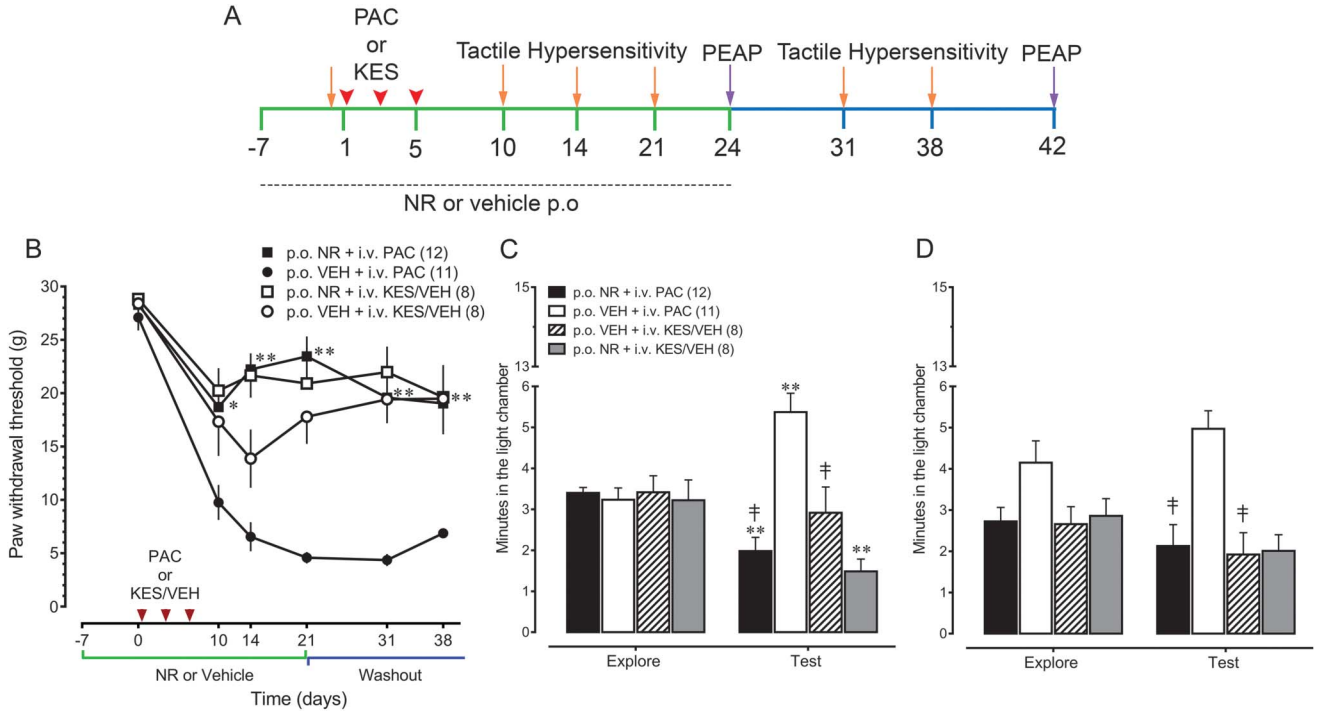

Figure 3. Pretreatment with 200 mg/kg nicotinamide riboside (NR) prevented the development of tactile hypersensitivity and diminished place escape–avoidance behaviors in female paclitaxel-treated rats. (A) Schema depicting the dosing regimen for the prophylactic study. (B) Effects of NR on tactile hypersensitivity. The ordinate is the paw withdrawal threshold in grams. Abscissa is time relative to the first of 3 injections of 6.6 mg/kg i.v. paclitaxel or KES vehicle (arrowheads). Days −7 to 0 represent the 1 week oral pretreatment period with NR or its vehicle. NR and vehicle treatment continued until day 24, at which time NR and vehicle administration ceased and a 2-week washout period began. *$P < 0.05$, **$P < 0.01$ NR + PAC compared with VEH + PAC at the corresponding time point. Place-escape behaviors determined (C) at the conclusion of NR treatment and (D) after a 2-week washout of NR. The ordinate is time spent in the brightly lit chamber and is broken to indicate the full period that behavior was monitored. (C and D) Explore refers to the initial 15-minute period of acclimation to the 2 chambers. Test refers to the subsequent 15 minutes during which the rat's hind paws were repetitively stimulated when in the dark chamber. **$P < 0.01$ compared with the corresponding treatment group during the exploration phase. ‡$P < 0.01$ compared with VEH + PAC. Data are mean ± SEM. Numbers of rats are indicated in parentheses.

On the second occasion of PEAP testing, after a 2-week washout period, vehicle + paclitaxel-treated rats appeared agitated when reintroduced to the apparatus. During the exploration phase, they tended to spend more time in the brightly lit chamber than the other groups, although this did not reach statistical significance. During the testing phase, they continued to avoid the dark chamber and remain in the brightly lit chamber, spending significantly more time in that chamber than vehicle + KES vehicle-treated rats (**Fig. 3D**). As observed in the first test session, vehicle + KES vehicle and NR + KES vehicle rats did not increase their time in the brightly lit chamber upon repetitive stimulation of the hind paw. The NR + paclitaxel-treated rats also did not increase their time in the brightly lit chamber, and spent significantly less time in the brightly lit chamber than vehicle + paclitaxel-treated rats.

3.4. Posttreatment with NR alleviated paclitaxel-induced neuropathy

Figure 5A reiterates the design of the therapeutic trial. Tactile hypersensitivity was well established 14 days after paclitaxel treatment began (**Fig. 5B**). Daily oral administration of 200 mg/kg of NR beginning 14 days after the first dose of paclitaxel and continuing for 3 weeks progressively blunted paclitaxel-induced tactile hypersensitivity. However, this trend did not achieve statistical significance ($P > 0.9$ at 21 and 28 days, $P > 0.1$ at 35 days). Given that there was greater than average variability among the 12 rats, a secondary analysis was conducted in which rats were stratified as NR nonresponders or NR responders based on whether paw withdrawal threshold exceed the criterion value of 9.3 g. **Figure 5B** illustrates the mean effect all 12 rats in the treatment group, as well as the 2 stratified groups. Interestingly, posttreatment with NR significantly blunted PEAP behaviors in nearly all paclitaxel-treated rats, irrespective of their response to von Frey filaments (**Fig. 5C**).

3.5. NR did not interfere with myelosuppressive effects of paclitaxel

Intravenous administration of a cumulative dose of 19.8 mg/kg paclitaxel to vehicle-treated rats significantly decreased the number of white blood cells. Neutrophils, monocytes, and eosinophils were each reduced to 20% of values in naive rats (**Table 2**). Numbers of red blood cells and hemoglobin trended less but were not statistically different from naive rats. In NR-treated rats, this dose of paclitaxel similarly decreased the numbers of white blood cells and their different classes and did not affect numbers of red blood cells or levels of hemoglobin. Intraperitoneal administration of a cumulative dose of 8 mg/kg paclitaxel did not produce myelosuppression (**Table 2**).

3.6. Pretreatment with acetyl-L-carnitine did not prevent paclitaxel-induced neuropathy

The dose regimen for ALCAR was identical to that of NR (**Fig. 3A**), and the dose and route were as used by others.[20,30,69] Daily oral pretreatment with 100 mg/kg of ALCAR did not alleviate

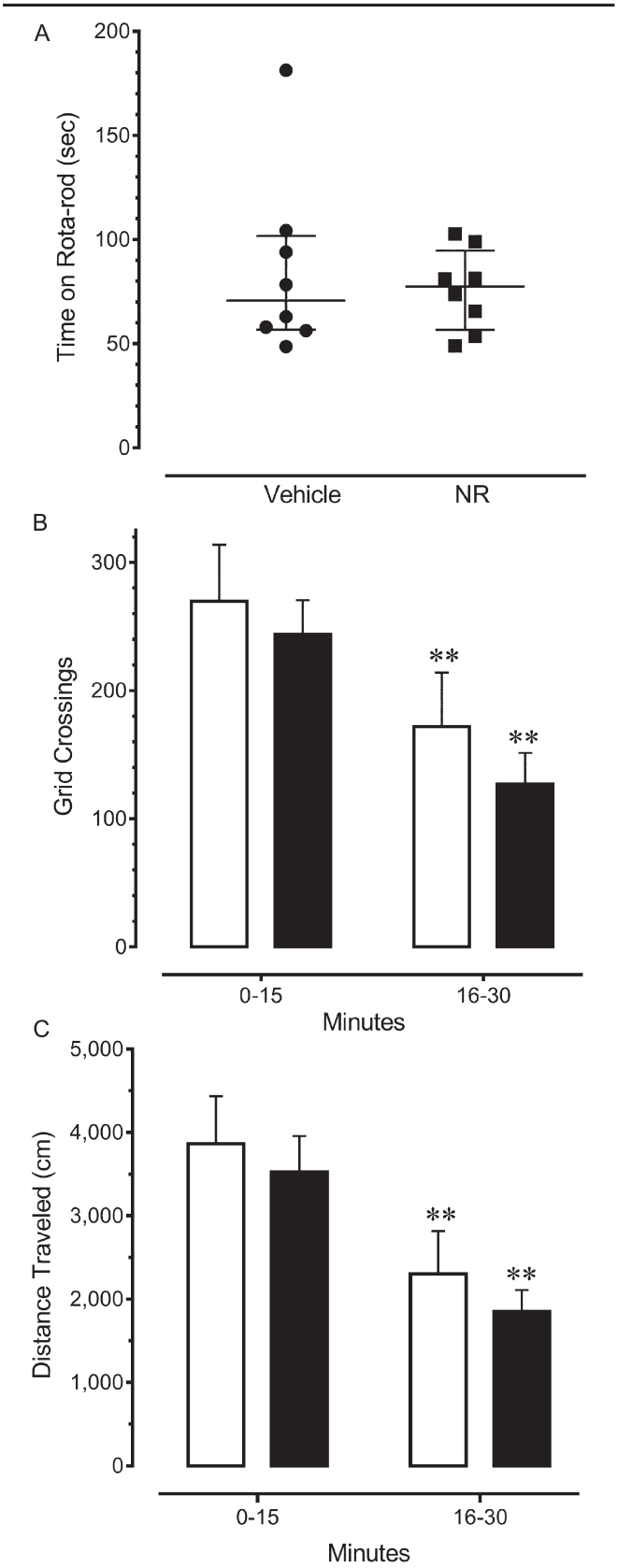

Figure 4. Treatment with 200 mg/kg nicotinamide riboside (NR) does not produce motor impairment. (A) Length of time the rat remained on an accelerating rota-rod. (B) Number of grid crossings and (C) distance traveled after placement in a novel 100-cm^2 open field. (A) Data for individual animals as a scatter plot. (B and C) Data as mean ± SEM. Open bars represent vehicle and solid bars represent NR-treated rats; N = 8 rats for each treatment group. $^{**}P < 0.01$ compared with corresponding treatment group at 0 to 15 minutes.

paclitaxel-induced tactile hypersensitivity (**Fig. 6A**). Of note, daily administration of ALCAR to KES vehicle-treated rats decreased paw withdrawal threshold compared with vehicle + KES vehicle-treated rats; this effect achieved statistical significance at days 10, 14, and 31 days. Prophylactic treatment with ALCAR also did not blunt place escape–avoidance behaviors (**Fig. 6B, C**).

4. Discussion

Nicotinamide riboside is a form of vitamin B3, and a naturally occurring, newly identified precursor of NAD^+.[8,9,45,65,66] Prophylactic administration of a dose that increased blood NAD^+ levels by ~50% prevented tactile hypersensitivity and blunted place escape–avoidance behaviors in a rodent model of paclitaxel-induced peripheral neuropathy. Therapeutic administration of NR also reversed established tactile hypersensitivity in a subset of rats and blunted place escape–avoidance behaviors, which suggests that the mechanisms responsible for paclitaxel-induced tactile hypersensitivity are reversible. Nicotinamide riboside did not interfere with the myelosuppressive effects of paclitaxel or suppress locomotor activity.

These results focus attention on therapeutic approaches that can correct bioenergetic deficits and mitochondrial dysfunction.[6,18,28,49] Experimental manipulations that increase NAD^+ are neuroprotective in in vitro and in vivo models of peripheral axotomy.[2,22,52–54] Doses of NR that increase NAD^+ levels delay mitochondrial myopathy in a mouse genetic model of mitochondrial respiratory chain disease,[31] and protect against noise-induced hearing loss and death of spiral ganglion neurons.[10] Furthermore, addition of NR to the high-fat diet of prediabetic and diabetic male mice protected against deficits in nerve conduction velocity, heat hypoalgesia, and loss of corneal and intraepidermal nerve fibers.[64] Obvious sites of action that warrant investigation include the peripheral terminals and soma of sensory afferents, although actions in the dorsal horn or supraspinal sites cannot be excluded.

Multiple mechanisms, some specific to the chemotherapeutic agent, are implicated in the development and maintenance of CIPN.[12,17,18,24] In the case of paclitaxel, these mechanisms include (1) mitochondrial dysfunction associated with axonal degeneration, slowing of nerve conduction velocity and epidermal nerve fiber loss; (2) activation of inflammatory processes resulting from activation of macrophages in the dorsal root ganglia and astrocytes and microglia in the spinal cord; and (3) alterations in the activity of Ca^{++} signaling and ion channel activity. Further studies will be required to determine whether the NR rectifies any of these dysfunctions and establish causality of the increase in NAD^+, as well as to characterize its effects in other models of peripheral nerve injury.

To the best of our knowledge, this study is the first to document suppression of the affective dimension of nociception in a rodent model of CIPN. Measures of the affective dimension of nociception are increasingly incorporated into studies of inflammatory and neuropathic pain.[32,40] However, with one exception,[41] they have not been used in studies of CIPN. After spared nerve injury[37] and L5 spinal nerve ligation,[33,35,36] rats spend ~60% of their time in the light chamber whereas sham-operated rats spend 15% to 30%. Direct comparison with the PEAP as modified for CIPN is hampered by differences in testing. Here, the effect size may be underestimated because this study used a lower force filament than others, and the light chamber was brightly lit to make it even less preferred. Finally, testing was limited to 15 minutes rather than 30 minutes; aversiveness escalates over time with repeated application of the filaments (data not shown; see Ref. 34).

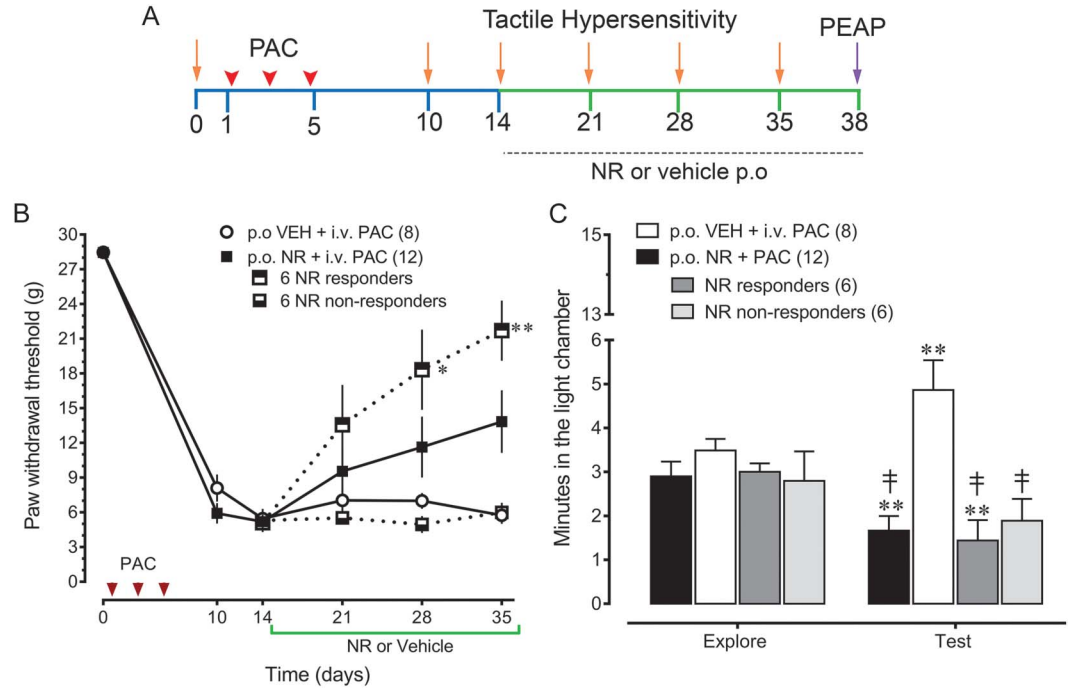

Figure 5. Therapeutic treatment with 200 mg/kg oral nicotinamide riboside (NR) ameliorates established tactile hypersensitivity and place escape–avoidance behaviors induced by paclitaxel. (A) Schema depicting the dosing regimen for the therapeutic study. (B) Effects of NR on tactile hypersensitivity. The ordinate is paw withdrawal threshold in grams. Abscissa is time relative to the first of 3 injections of 6.6 mg/kg i.v. paclitaxel (arrowheads). Data illustrate values for all rats in the NR treatment group, and after stratification of these animals into those that responded to NR (threshold >9.3 g) and those that did not (threshold <9.3 g). $^{*}P < 0.05$, $^{**}P < 0.01$ NR + PAC compared with VEH + PAC at the corresponding time point. (C) Place-escape behaviors determined after 3 weeks of NR or its vehicle treatment. Data are presented as pooled for all 12 rats, and according to response status to NR. Explore refers to the initial 15-minute period of acclimation to both chambers. Test refers to the subsequent 15 minutes when hind paws were repetitively stimulated when rats entered the dark chamber. The ordinate is broken to indicate the full period that behavior was monitored. $^{**}P < 0.01$ compared with exploration phase for that treatment group; ‡$P < 0.01$ compared with the VEH + PAC treatment group. Data for all panels are mean ± SEM. Numbers of rats are indicated in parentheses.

Place escape–avoidance behaviors are elicited by repeated stimulation of the hind paw in rats that exhibit tactile hypersensitivity. Drug treatments that suppress tactile hypersensitivity and thereby render the stimulus innocuous are likely to also suppress place escape–avoidance behaviors. It is therefore interesting that, in the therapeutic protocol, NR suppressed place escape–avoidance behaviors in rats that did not exhibit suppression of tactile hypersensitivity. This observation suggests that NR may

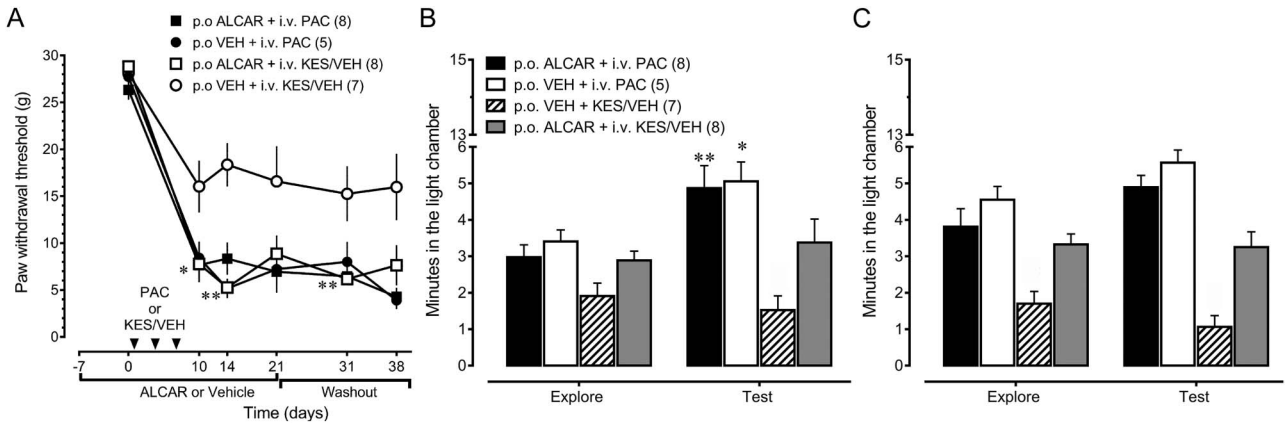

Figure 6. Pretreatment with 100 mg/kg acetyl-L-carnitine (ALCAR) does not prevent the development of tactile hypersensitivity or diminish place escape–avoidance behaviors in paclitaxel-treated rats. (A) Effects of ALCAR on tactile hypersensitivity. Ordinate is paw withdrawal threshold in grams. Abscissa is time relative to the first of 3 injections of 6.6 mg/kg i.v. paclitaxel (arrows). Days −7 to 0 represent the 1 week pretreatment period with ALCAR or vehicle. ALCAR or vehicle treatment continued until day 24, at which time ALCAR administration ceased and a 2 week wash out period commenced. $^{*}P < 0.05$, $^{**}P < 0.01$ for comparison of ALCAR + KES vehicle with VEH + KES vehicle at the corresponding time point. Place-escape behaviors determined (B) at the end of ALCAR treatment and (C) after a 2-week washout of ALCAR. (B and C) Explore refers to the initial 15 minutes period of acclimation to both chambers. Test refers to the subsequent 15 minutes when hind paws were repetitively stimulated when rats entered the dark chamber. The ordinate is broken to indicate the full period that behavior was monitored. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with exploration phase for that treatment group. Comparisons with the VEH + PAC treatment group are not shown because the large difference in the exploration time of this group confounded comparisons. Data for all panels are mean ± SEM. Numbers of rats are indicated in parentheses.

truly suppress the aversive dimension of nociception independent of changes in sensory threshold.

The low-dose paclitaxel regimen induces delayed tactile hypersensitivity, loss of intraepidermal nerve fibers, and mitochondrial dysfunction.[27] However, this dose does not produce myelosuppression. A similar observation made by Salvemini's group was not sufficiently powered for statistical analysis.[29] Paclitaxel inhibits β-tubulin and microtubule function. In bone marrow the result is an inhibition of the division and differentiation of hematopoietic stem cells. As such, myelosuppression serves as a biomarker for an antitumor dose of paclitaxel. The production of tactile hypersensitivity by a dose of paclitaxel that does not produce myelosuppression in the marrow indicates that the neuropathy that develops is independent of inhibition of microtubule function. This finding supports previous reports that low doses of paclitaxel produce tactile hypersensitivity without affecting the number or morphology of microtubules in sensory afferents.[19] Vincristine also produces very modest changes in microtubule number and morphology.[61,62] This study administered a total dose of 19.8 mg/kg paclitaxel intravenously as 3 divided doses, which were well tolerated. This dose, which closely approximates on a mg/m^2 basis that administered weekly to patients with breast cancer,[38,58] decreased neutrophils, eosinophils, and monocytes as expected of an efficacious antitumor dose of paclitaxel. This observation supports the clinical relevance of this higher dose for translational studies.

Several studies in male[20,30,69] and one study in female[43] rats indicated that ALCAR suppressed paclitaxel-induced tactile hypersensitivity, as well as prevented loss of intraepidermal nerve fibers, normalized conduction velocity, and prevented mitochondrial dysfunction. A 1-arm phase II study of 25 patients reported that it alleviated CIPN in 60% of patients.[7] However, a phase III trial of 409 patients concluded that ALCAR not only lacked efficacy but also actually exacerbated CIPN in some patients.[26] In our hands, prophylactic administration of ALCAR did not alleviate paclitaxel-induced tactile hypersensitivity. Although it did not worsen CIPN, this effect may not have been visible because of the significant hypersensitivity induced by paclitaxel ("floor effect"). The administration of a higher dose of paclitaxel, differences in timing between dosing and testing, an influence of the estrous cycle, or the use of a different method to assess tactile hypersensitivity may contribute to our inability to replicate findings of prior reports.

Administration of ALCAR to KES vehicle-treated rats by itself induced robust tactile hypersensitivity. The KES vehicle is not innocuous.[5,57,68] It can cause hypersensitivity reactions in patients,[57] and repeated injection can induce mechanical hyperalgesia and robust tactile hypersensitivity in rats.[4,5] Indeed, moderate tactile hypersensitivity developed after 3 i.v. injections of the KES vehicle in this study, an effect that was also blunted by NR. Previous studies of ALCAR apparently did not examine its effects in KES vehicle-treated rats or include a vehicle + KES vehicle-treatment group for comparison. Thus, unexpected exacerbation of hypersensitivity was likely overlooked. The present results with ALCAR, ie, both the lack of efficacy in CIPN and its exacerbation of tactile hypersensitivity, align well with the findings of the large phase III trial.

Drugs that alleviate CIPN must do so without interfering with chemotherapy. There has been concern that agents that increase NAD^+ will promote tumorigenesis. First, proliferating cells appear to have higher demands for NAD^+, and increased availability of NAD^+ could support tumorigenesis. Second, high concentrations of nicotinamide inhibit poly-(ADP-ribose) polymerases, which serve as DNA damage sensors and participate in DNA repair. Poly-(ADP-ribose) polymerase inhibitors are being pursued as an anticancer therapy.[16] Third, inhibitors of nicotinamide phosphoribosyltransferase, a key enzyme in the salvage pathway for synthesis of NAD^+ from nicotinamide, exert potent anticancer properties through an increase in nicotinamide and decrease in NAD^+ in tumors.[48,55] Finally, in vitro studies of germ cell-derived diffuse large B-cell lymphoma cell lines indicate that nicotinamide is cytotoxic, and when combined with a pan-deacetylase inhibitor, functions synergistically to kill cells and to inhibit tumor growth in vivo.[1] That noted, the effects of altering NAD^+ levels seem to be organ specific.[59] Other studies suggest that an increase in NAD^+ can be beneficial.[16] Several mechanistic studies of interventions that decrease metastases, increase survival, and interfere with cancer progression in models of liver and breast cancer attribute the beneficial effects to increased levels of NAD^+ downstream.[50,51,60,67] Two phase 2 studies[60] and one phase 3 study[15] have now demonstrated that daily oral administration of up to 1 g of the NAD^+ precursor nicotinamide for 1 year decreased the rate of new nonmelanoma skin cancers by 23% and that of new actinic keratoses by 12%. Whether this outcome is due to increased levels of nicotinamide or NAD^+ is unknown. Clearly, studies of the effect of NR on tumorigenesis with a rodent model are necessary.

Relief of CIPN remains a significant unmet health care need for millions of people undergoing chemotherapy and significant numbers of cancer survivors. The persistence of the beneficial effects of prophylactic NR for 2 weeks after treatment ceased suggests that provision of NR before or concurrent with paclitaxel chemotherapy may be sufficient to protect against injury. The ability of NR to reverse established tactile hypersensitivity and blunt PEAP behaviors suggests that it may be of benefit to a subset of those 30% of cancer survivors who continue to experience CIPN after chemotherapy has ended. The ready availability of NR as a nutritional supplement may facilitate translation of these preclinical findings to the clinic, and the possible development of a new class of therapeutic agent for the prevention and relief of CIPN.

Conflicts of interest statement

D. L. Hammond reports nonfinancial support (provision of NR) from ChromaDex during the conduct of the study; nonfinancial support and other from ChromaDex outside the submitted work. C. Brenner is a member of the Scientific Advisory Board and stockholder of ChromaDex, Inc, which distributes NR, and is Chief Scientific Adviser and cofounder of ProHealthspan, LLC, which sells NR. C. Brenner reports grants and personal fees from ChromaDex, Inc. and stock interest in ProHealthspan, LLC, outside the submitted work. C. Brenner has invented intellectual property concerning NR that has been licensed by ChromaDex, Inc. The remaining authors have no conflicts of interest to declare.

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Author contributions: D.L.H. designed the study, assisted with testing, analyzed data, dosed rats, and edited the manuscript. M.V.H. performed the behavioral experiments and drafted the manuscript. S.R.W. dosed rats and assisted with data collection. R.Y.W. contributed to discussions, assisted with data collection, and edited the manuscript. M.S.S. performed analysis of NAD^+

metabolome in samples. C.B. contributed to discussions, oversaw NAD^+ analyses, and edited the manuscript.

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