Nicotine prevents and reverses paclitaxel-induced mechanical allodynia in a mouse model of CIPN

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Abbreviations: CIPN, chemotherapy-induced peripheral neuropathy; IENF, intra-epidermal nerve fiber; nAChR, nicotinic acetylcholine receptor; NSCLC, non-small cell lung cancer

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Abstract:

Chemotherapy-induced peripheral neuropathy (CIPN), a consequence of peripheral nerve fiber dysfunction or degeneration, continues to be a dose-limiting and debilitating side effect during and/or after cancer chemotherapy. Paclitaxel, a taxane commonly used to treat breast, lung, and ovarian cancers, causes CIPN in 59-78% of cancer patients. Novel interventions are needed due to the current lack of effective CIPN treatments. Our studies were designed to investigate if nicotine can prevent and/or reverse paclitaxel-induced peripheral neuropathy in a mouse model of CIPN, while ensuring that nicotine will not stimulate lung tumor cell proliferation or interfere with the anti-tumor properties of paclitaxel. Male C57BL/6J mice received paclitaxel every other day for a total of four injections (8 mg/kg, i.p.). Acute (0.3-0.9 mg/kg, i.p.) and chronic (24 mg/kg/day, s.c.) administration of nicotine respectively reversed and prevented paclitaxel-induced mechanical allodynia. Blockade of the antinociceptive effect of nicotine with mecamylamine and methyllycaconitine suggests that the reversal of paclitaxel-induced mechanical allodynia is primarily mediated by the α7 nicotinic acetylcholine receptor subtype. Chronic nicotine treatment also prevented paclitaxel-induced intra-epidermal nerve fiber loss. Notably, nicotine neither promoted proliferation of A549 and H460 non-small cell lung cancer (NSCLC) cells, nor interfered with paclitaxel-induced anti-tumor effects, including apoptosis. Most importantly, chronic nicotine administration did not enhance Lewis lung carcinoma tumor growth in C57BL/6J mice. These data suggest that the nicotinic acetylcholine receptor-mediated pathways may be promising drug targets for the prevention and treatment of CIPN.

Introduction:

Chemotherapy continues to play a significant role in the treatment and survival of cancer patients. However, a number of cancer chemotherapeutic drugs can promote either transient or prolonged tissue and organ toxicities, including chemotherapy-induced peripheral neuropathy (CIPN). CIPN, a result of peripheral nerve fiber dysfunction or degeneration, is characterized by sensory symptoms including: numbness, tingling, burning, hyperalgesia, allodynia, and in some cases neuropathic pain. Approximately 68% of cancer patients experience CIPN within a month following the completion of their treatment, whereas 30% suffer from symptoms of CIPN for 6 months or longer after chemotherapy (Seretny et al., 2014). When CIPN manifests during the administration of chemotherapy, it can become dose-limiting and/or delay treatment, thereby interfering with the full course of treatment that may be required for a positive clinical outcome (Hama and Takamatsu, 2016).

Cancer chemotherapeutic drugs and drug classes associated with peripheral neuropathy include the taxanes (paclitaxel), platinum-based compounds (cisplatin, oxaliplatin), vinca alkaloids (vincristine), and bortezomib. Paclitaxel, a taxane commonly used to treat breast, lung, and ovarian cancers, increases both progression-free and overall survival in cancer patients (Dranitsaris et al., 2015). Unfortunately, paclitaxel has been found to cause CIPN both acutely and chronically in 59-78% and 30% of cancer patients, respectively (Beijers et al., 2012).

There are currently no effective preventative or therapeutic treatments for CIPN. Opioids, anti-convulsants, anti-depressants, anesthetics, and muscle relaxants either perform modestly in relieving CIPN-induced neuropathic pain, do not show consistent efficacy in the majority of patients, and/or produce intolerable side effects (Majithia et al., 2016; Hershman et al., 2014; Kim et al., 2015).

Nicotine and nicotine analogues have demonstrated potential utility as analgesic and/or antinociceptive drugs, as well as anti-inflammatory agents in both human and experimental pain studies (Umana et al., 2013; Alsharari et al., 2013; Flood and Damaj, 2014). For example, nicotine elicits analgesic effects in non-smokers suffering from spinal cord injury in a randomized, placebo-controlled, cross-over design experiment (Richardson et al., 2012). Additionally, a recent preclinical study demonstrated that intraperitoneal administration of nicotine at a dose of 1.5 mg/kg reverses allodynia induced by oxaliplatin, a chemotherapeutic agent used to induce peripheral neuropathy in rats (Di Cesare Mannelli et al., 2013).

The studies described in this report characterize the antinociceptive and/or neuroprotective effects of nicotine in a CIPN mouse model, while further evaluating the influence of nicotine on lung tumor cell proliferation and sensitivity to the anti-tumor properties of paclitaxel.

Methods:

Animals. Adult male C57BL/6J mice (8 weeks at the beginning of experiments, 20-30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in an AAALAC-accredited facility in groups of four; the mice in each cage were randomly allocated to different treatment groups. Food and water were available *ad libitum.* Experiments were performed during the light cycle (7:00 am to 7:00 pm) and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were euthanized via CO_2 asphyxiation followed by cervical dislocation. Any subjects that showed behavioral disturbances unrelated to chemotherapy-induced pain were excluded from further behavioral testing.

Drugs. Paclitaxel was purchased from Tocris (1097, Bristol, United Kingdom) and dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ)/18 volumes distilled water]. Paclitaxel injections were administered intraperitoneally (i.p.) every other day for a total of four injections to induce neuropathy, as previously described by Toma et al., (2017). (-)-Nicotine hydrogen tartrate salt and mecamylamine HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline. For acute administration, nicotine was injected i.p. at doses of 0.3, 0.6, or 0.9 mg/kg (Bagdas et al., 2017; Di Cesare Mannelli et al., 2013). Nicotine at doses of 6, 12, or 24 mg/kg/day was also administered chronically via 7-day subcutaneous (s.c.) osmotic minipumps (Alzet, Model 1007D, Cupertino, CA), which were implanted 2 days prior to paclitaxel treatment (Alsharari et al., 2015). Mecamylamine was administered at a dose of 2 mg/kg s.c. 15 minutes before administration of nicotine or saline (Bagdas et al., 2014). Methyllycaconitine (MLA) was purchased from RBI (Natick, MA, USA) and administered at a dose of 10 mg/kg s.c. 10 minutes before administration of nicotine (Freitas et al., 2013). All doses were chosen based on previous works that demonstrated which dose, time of exposure, and route of administration for each drug effectively

6

acted upon the appropriate receptor and was not toxic to the animal. All i.p. or s.c. injections were given in a volume of 1 ml/100 g body weight, whereas the osmotic minipumps released 0.5 µl/hour.

Immunohistochemistry and quantification of intra-epidermal nerve fibers. The hind paw epidermis was collected from the following groups of mice: vehicle-saline, vehicle-nicotine (24 mg/kg/day), paclitaxel (8 mg/kg)-saline, and paclitaxel (8 mg/kg)-nicotine (24 mg/kg/day). The staining procedure was performed as previously described (Toma et al., 2017). Briefly, the glabrous skin of the hind paw was excised, placed in freshly prepared 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and stored overnight at 4 °C in the same fixative. The samples were embedded in paraffin, sectioned at 25 µm, and stained with PGP9.5 (Fitzgerald - 70R-30722, MA, USA) and goat anti-rabbit IgG (H+L) secondary antibody conjugated with Alexa Fluor® 594 (Life Technologies - A11037, OR, USA). Sections were examined using a Zeiss Axio Imager A1 – Fluorescence microscope (Carl Zeiss, AG, Germany) in a blinded fashion under 63x magnification, but imaged under 40x magnification; the density of fibers is expressed as fibers/mm.

Mechanical allodynia evaluation (von Frey test). Mechanical allodynia thresholds were determined using von Frey filaments according to the method suggested by Chaplan et al. (1994) and as described previously (Bagdas et al., 2015). The mechanical threshold is expressed as log10 (10 £ force in [mg]). For the nicotine-mediated reversal of CIPN experiment, paclitaxel-treated mice were tested for mechanical allodynia following acute nicotine administration on days 7-14 following the initial paclitaxel injection. All behavioral testing on animals was performed in a blinded manner.

Minipump Implantation. The procedure was performed as previously described in Alsharari et al. (2013) with minor modifications. Mice were anesthetized with 2.5% isoflurane/ 97.5% oxygen. The anesthetized mice were prepared by shaving of the back and swabbing with betadine, followed by 70% ethanol pads. Sharp, sterile scissors was used to make a 1 cm incision in the skin of the upper back/neck. The sterile, preloaded minipump (Alzet, Model 1007D, Cupertino, CA) with different doses of nicotine or saline was inserted with sterile forceps by a technician wearing sterile gloves. The wound was closed with sterile 9 mm stainless steel wound clips. The mice were allowed to recover on heated pads and were monitored before returning to their home cages.

Cell culture. All lung cancer cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Serum Source International, FB22-500HI, NC, USA) and 1% (v/v) combination of 10,000 U/ml penicillin and 10,000 µg/ml streptomycin (Pen/Strep, ThermoFisher Scientific, 15140-122, Carlsbad, CA), unless stated otherwise. Cells were incubated at 37°C under a humidified, 5% CO_2 atmosphere. The H460 non-small cell lung cancer (NSCLC) cell line was generously provided by the laboratory of Dr. Richard Moran at Virginia Commonwealth University (VCU), the A549 NSCLC cell line was a gift from the laboratory of Dr. Charles Chalfant at VCU, and the Lewis lung carcinoma (LLC) cells were provided by Dr. Andrew Larner at VCU. In order to establish the T1 primary lung cancer cell line, tissues were obtained from adenocarcinoma tumors in accordance with the VCU IRB protocol. Tissues were minced well and washed multiple times by centrifugation in sterile PBS. Thereafter, the tissues were resuspended in DMEM. Tissue homogenates were layered on collagen (Sigma-Aldrich, C3867, St. Louis, MO)-coated plates. Cell colonies started to appear after 2-3 weeks. Upon confluence, the cells were trypsinized and passaged. The ovarian cancer cell lines, SKOV-3/DDP and OVCAR-3, were generously provided by the laboratory of Dr. Xianjun Fang at VCU and were cultured in RPMI160 supplemented with 10% (v/v) FBS and 1% (v/v) Pen/Strep.

Paclitaxel was dissolved in DMSO, diluted with sterile PBS, and added to the medium in order to obtain the desired concentration. Staurosporine (Sigma-Aldrich, S6942, St. Louis, MO) was purchased as 1 mM in DMSO. Cells were not exposed to greater than 0.1% DMSO in any experiment. (-)-Nicotine hydrogen tartrate salt was dissolved in sterile PBS. All experiments involving these light-sensitive drugs were performed in the dark.

Assessment of cell viability. Cell viability was measured by either the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay or trypan blue exclusion. For the MTT/MTS assay, cells were seeded in 96-well plates and treated with various concentrations of nicotine for 24 hours, at which time the drug was removed and replaced with fresh medium. Depending on the replication rate of the cell line, the cells were allowed 24 or 48 hours to proliferate following drug exposure. For the serum deprivation study, cells were seeded in DMEM (10% FBS) for 24 hours, then the medium was removed and replaced with DMEM supplemented with various concentrations of FBS (0-10%) with or without nicotine (1 µM); cell viability was assessed at either 48 or 96 hours post-treatment without drug removal. At the time of testing, the medium was removed, then the cells were washed with PBS and stained with thiazolyl blue tetrazolium bromide (MTT, 2 mg/ml; Sigma-Aldrich, M2128, St. Louis, MO) in PBS for 3 hours. The MTT solution was aspirated and replaced with DMSO. The color change was measured by a spectrophotometer (ELx800UV, BioTek, VT) at 490 nm. To avoid potentially aspirating cells, the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega, G358C, Madison, WI) was utilized for less adherent cell lines (A549, LLC, and T1); the use of MTS rather than MTT eliminates washing steps before and after staining.

For trypan blue exclusion, cells were incubated with trypsin (0.25% trypsin-EDTA) for 3 minutes, stained with trypan blue (Invitrogen, 15250, Carlsbad, CA), and the viable, unstained cells were counted using a hemocytometer with bright-field microscopy.

Assessment of colony formation. Cells were seeded at a low density in DMEM (10% FBS). After 24 hours, the paclitaxel and paclitaxel + nicotine samples were exposed to paclitaxel (50 nM) for 24 hours, after which the medium was replaced with fresh, drug-free medium. After 24 hours, the nicotine and paclitaxel + nicotine samples were exposed to nicotine (1 µM) for 24 hours, after which the medium was replaced with fresh, drug-free medium. Once the control colonies reached a size of 50 cells per colony (approximately 8-10 days after seeding), the samples were fixed with methanol, stained with crystal violet, and quantified (ColCount, Discovery Technologies International).

Assessment of apoptosis and DNA content. Flow cytometry analyses were performed using BD FACSCanto II (BD Biosciences, San Jose, CA) and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core facility. For all studies, 10,000 cells per replicate within the gated region were analyzed. When collecting samples, both adherent and floating cells were harvested with 0.1% trypsin-EDTA and neutralized with medium after 48 hours of drug exposure. For quantification of apoptosis, cells were centrifuged and washed with PBS, then resuspended in 100 µl of 1x binding buffer with 5 µl of Annexin V and 5 µl of propidium iodide (BD Biosciences, FITC Annexin V Apoptosis Detection Kit, 556547, San Jose, CA). The samples were then incubated at room temperature while protected from light for 15 minutes. The suspension solution was then brought up to 500 µl using the 1x binding buffer and analyzed by flow cytometry. For quantification of DNA content, the cells were resuspended in 500 µl of a propidium iodide (PI) solution (50 µg/ml PI, 4 mM sodium citrate, 0.2 mg/ml DNase-free RNase A, and 0.1% Triton-X 100) for 1 hour at room temperature, while being protected from light (Tate et al., 1983). Before

flow cytometry analysis, NaCl was added to the cell suspensions to achieve a final concentration of 0.20 M.

Assessment of tumor growth *in vivo*. Male C57BL/6J adult mice were subcutaneously injected with 1.5 x 10^6 Lewis lung carcinoma (LLC) cells in both flanks. The LLC cells were collected via trypsinization, then neutralized with medium, centrifuged, and washed with PBS. Pellets of 1.5 x 10^6 LLC cells were then resuspended in 30 µl of 80% basement membrane extract (Trevigen, 3632-010-02, Gaithersburg, MD)/20% PBS. Mice were anesthetized with isoflurane via inhalation during tumor cell injection. Palpable tumors formed at approximately 7 days post-tumor cell injection, and on day 11 tumor volumes (*l* x *w* x *h*) were sufficient to be assessed with calipers; subsequent tumor volume measurements were collected every other day. Subcutaneous osmotic minipumps (Alzet, Model 1007D, Cupertino, CA) were implanted as previously described at 13 days post-tumor cell injection to release 24 mg/kg nicotine daily for a total of 7 days. Body weight and tumor volume were observed until humane endpoints were reached, at which time mice were euthanized via CO_2 asphyxiation followed by cervical dislocation.

Statistical analysis. A power analysis calculation was performed with the Lamorte's Power Calculator (Boston University Research Compliance) to determine the sample size of animals for each group (Charan and Kantharia, 2013). For assessing nociceptive behavior and tumor volume, the calculations showed that an n of 5 was required to achieve a power of 90% with an alpha error of 0.05; we used 8 mice per group for the nociceptive assay and 5-6 mice per group for the *in vivo* cancer study. The data were analyzed with GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA) and SPSS, version 24, and are expressed as mean ± SEM. One- and two-way analysis of variance (ANOVA) tests were conducted and followed by the Bonferroni post hoc test, three-way mixed factor ANOVAs were performed and followed by the Sidak post hoc test, and linear mixed models were conducted to account for the loss of tumor-bearing mice

(Little and Rubin, 1987); repeated measures were considered for all *in vivo* studies. Differences

were determined to be significant at $P < 0.05$.

Results:

Nicotine reverses and prevents paclitaxel-induced mechanical allodynia. Initial experiments were designed to determine if acute administration of nicotine reverses paclitaxel-induced mechanical allodynia. **Fig. 1A** demonstrates that nicotine reversed mechanical allodynia in paclitaxel-treated mice in a time- and dose-dependent manner [$F_{\text{time x dose}}$ (18, 126) = 17.10, $P < 0.0001$], with full reversal (mechanical threshold values restored to baseline levels) following administration of 0.9 mg/kg and partial reversal with 0.6 mg/kg. Nicotine did not alter mechanical thresholds in vehicle-treated mice [$F_{\text{time x dose}}$ (18, 126) = 0.6122, $P = 0.88$] (**Supplemental Fig. 1**).

Having demonstrated that nicotine reversed the allodynic effect of paclitaxel, the next series of experiments was designed to investigate whether nicotine also *prevents* the development of paclitaxel-induced nociceptive (allodynic) responses. Seven days of nicotine (24 mg/kg/day) administration prevented the development of mechanical allodynia throughout the entire duration of the experiment, up to 35 days post-paclitaxel injection [F (9, 252) = 6.703, $P < 0.001$; **Fig. 1B**]. As shown in **Supplemental Figure 2**, 6 mg/kg/day and 12 mg/kg/day nicotine did not prevent the development of paclitaxel-induced mechanical allodynia.

To examine the possibility that nicotinic acetylcholine receptors (nAChRs) mediate the antinociceptive effect of nicotine, mecamylamine, a non-selective nAChR antagonist, was administered prior to nicotine. Mecamylamine effectively blocked the antinociceptive effect of nicotine in paclitaxel-treated mice [$F_{\text{time x dose}}$ (6, 42) = 10.38, $P < 0.0001$; **Fig. 2A**]. To begin determining which nAChR subtypes are involved in the reversal of paclitaxel-induced mechanical allodynia, we administered MLA, an α7 nAChR antagonist, before nicotine treatment, which effectively blocked the antinociceptive effect of nicotine in paclitaxel-treated mice [$F_{\text{time x dose}}$ (6, 42) = 15.58, $P < 0.0001$; **Fig. 2B**].

13

Nicotine prevents paclitaxel-induced reduction of intra-epidermal nerve fibers. A decrease in the intra-epidermal nerve fiber (IENF) density in the paw epidermis is a common marker for evaluating CIPN in rodent models (Bennett et al., 2011). To determine if nicotine also protects the IENFs from the toxic effect of paclitaxel, mice were treated with vehicle or paclitaxel (8 mg/kg, i.p.) and implanted with minipumps releasing saline or nicotine (24 mg/kg/day), and sacrificed 35 days following the first paclitaxel injection, when their hind paw epidermis was collected for immunohistochemical analysis. Quantification of IENFs revealed a significant overall interaction between paclitaxel and nicotine treatment [$F_{paclitaxel \times nicotine}$ (1, 28) = 11.58, $P < 0.01$; **Fig. 3A**]. As illustrated in **Fig. 3B**, mice treated with paclitaxel-saline demonstrated a significant decrease in IENF density when compared to vehicle-saline-treated mice ($P < 0.0001$). In contrast, paclitaxel-nicotine-treated mice showed a significant increase in IENF density when compared to paclitaxel-saline-treated mice ($P < 0.01$). Paclitaxel-nicotine-treated mice did not show a change in the IENF density when compared to vehicle-nicotine-treated mice ($P = 0.54$) and vehicle-nicotine-treated mice did not exhibit an alteration in IENF density when compared to the vehicle-saline group ($P = 0.53$).

Collectively, the behavioral and immunohistochemical studies presented in Figures 1-3 indicate that nicotine reverses paclitaxel-induced mechanical allodynia, via the α7 nAChR, but also protects against paclitaxel-induced mechanical allodynia and IENF loss. However, multiple reports have argued that nicotine can stimulate tumor growth or interfere with cancer chemotherapeutic drug-induced apoptosis, which would severely limit the potential utility of nicotine in the clinic (Zhang et al., 2009; Pillai et al., 2011; Wu et al., 2013; Liu et al., 2015; Dasgupta et al., 2006). As a review of the relevant literature revealed a number of inconsistencies (see Discussion), we re-evaluated the effect of nicotine on tumor cell proliferation and paclitaxel-induced apoptosis.

Nicotine fails to stimulate lung cancer cell proliferation or interfere with paclitaxel-induced cytotoxicity. Initial experiments were performed by utilizing the MTT/MTS colorimetric assay with both A549 and H460 cells, two commonly used experimental models of non-small cell lung cancer (NSCLC) that express multiple nAChRs (Tsurutani et al., 2005; Dasgupta et al., 2006; Yoo et al., 2014). Figure 4 indicates that 48 and 96 hour exposure to nicotine (1 μM) did not induce a significant increase in viable cell number when compared to untreated cells under both normal (10% FBS) and serum deprivation (0-5% FBS) conditions in A549 (**Fig. 4A**) and H460 (**Fig. 4B**) cells. The only observed effect was a significant increase in viable cell number under serum starvation (0% FBS) conditions in one cell line at a single time point (**Fig. 4B**).

The influence of various concentrations of nicotine on tumor cell proliferation was further investigated under full serum (10% FBS) conditions that are the standard for cancer cell studies. Again, exposure to a range of nicotine concentrations (0.1-10 μM) for 24 hours under full serum conditions did not significantly increase numbers of viable A549, H460, Lewis lung carcinoma (LLC), or T1 (primary lung cancer) cells (**Supplemental Fig. 3**).

Additional studies were designed to more closely mimic the potential use of nicotine following chemotherapy treatment in the clinic. NSCLC cells were first exposed to paclitaxel (50 nM) for 24 hours, followed by a 24-hour drug-free period, and subsequent treatment with nicotine (1 μM) for 24 hours. Paclitaxel significantly decreased the number of A549 and H460 colonies, and the impact of paclitaxel was not altered by nicotine; nicotine alone did not stimulate colony formation (**Fig. 5**).

In previous work (Alotaibi et al., 2016; Emery et al., 2014; Jones et al., 2005; Roberson et al., 2005; Efimova et al., 2010; Webster et al., 2015), we and others have reported that growth arrest induced by cancer therapy is transient and that tumor cells recover proliferative capacity within 7-

10 days post-treatment. In order to determine if prior exposure to nicotine stimulates proliferation and/or promotes early tumor cell recovery from paclitaxel-induced growth arrest, NSCLC cell proliferation was monitored for a period of 7 days after treatment with nicotine (1µM, 48 hours), paclitaxel (50 nM, 24 hours), or a combination of the two drugs, which consisted of a 24-hour nicotine pretreatment period preceding 24-hour co-treatment. Nicotine did not stimulate the proliferation of either the A549 or H460 cells throughout the duration of the assay, and even induced a slight but significant decrease in H460 cell number on Day 5 (**Fig. 6**). Most importantly, nicotine did not interfere with the paclitaxel-induced decrease in viable cell number at any time point (**Fig. 6**). Furthermore, nicotine did not promote an early proliferative recovery in either cell line (**Insets of Fig. 6**).

Although we failed to detect any interference with the anti-tumor activity of paclitaxel in two different assays, other studies have argued that nicotine suppresses paclitaxel-induced apoptosis (Tsurutani et al., 2005; Dasgupta et al., 2006). Consequently, additional experiments were performed to evaluate the effects of nicotine on paclitaxel-induced apoptosis. Paclitaxel (100 nM) induced significant apoptosis in both the A549 and H460 cells after 48 hours of treatment (**Fig. 7A**). Most importantly, nicotine (1 µM) did not interfere with the promotion of paclitaxel-induced apoptosis in either NSCLC cell line following co-treatment (**Fig. 7A**); staurosporine (2 µM), a non-selective protein kinase inhibitor, induced significant apoptosis and was used as a positive control. Similarly, cell cycle analysis revealed that paclitaxel (100 nM) induces significant sub-G1 fragmented DNA content, an indicator of late-stage cell death, in both A549 and H460 cells, and that nicotine does not attenuate this effect (**Fig. 7B**).

In order to ensure that our observations applied to other cancer types commonly treated with paclitaxel, we also evaluated the effects of nicotine on cancer cell proliferation in two human

16

ovarian cancer cell lines. As was the case with the lung cancer cells, nicotine did not stimulate ovarian cancer cell proliferation in SKOV-3/DDP and OVCAR-3 cells (**Supplemental Fig. 4**).

To investigate whether the *in vitro* findings are indicative of tumor cell responses *in vivo*, immunocompetent C57BL/6J mice were subcutaneously injected with LLC cells in the flank, a commonly used syngeneic model of lung cancer (Kellar et al., 2015). Once the tumors formed, the mice were treated with nicotine at a dose of 24 mg/kg/day for 7 days via a subcutaneous osmotic minipump in order to mimic the nicotine treatment regimen administered in the peripheral neuropathy studies. In accordance with the *in vitro* findings, chronic administration of nicotine failed to enhance LLC tumor growth (**Fig. 8**).

Discussion:

Effects of nicotine on chemotherapy-induced peripheral neuropathy. To the best of our knowledge, this is the first study to report that nicotine reverses paclitaxel-induced mechanical allodynia, as well as prevents paclitaxel-induced peripheral neuropathy when administered prior to and during paclitaxel treatment in the mouse. Our work also indicates that nAChRs mediate the antinociceptive effects of nicotine, based on interference by mecamylamine, a non-selective nAChR antagonist, and MLA, an α7 nAChR antagonist. Our studies further demonstrate that chronic nicotine infusion prevents the loss of IENFs in the epidermis of the hind paw following paclitaxel treatment. Taken together, these findings suggest that nicotine could have potential utility for the prevention and/or treatment of chemotherapy-induced peripheral neuropathy.

Mice treated with 8 mg/kg of paclitaxel developed significant mechanical allodynia, which is consistent with our recent report (Toma et al., 2017) and previous studies (Deng et al., 2015; Slivicki et al., 2016; Neelakantan et al., 2016). Acute administration of nicotine reverses the mechanical allodynia induced by paclitaxel, which is consistent with studies by Di Cesare Mannelli et al., (2013) in a rat model of oxaliplatin-induced peripheral neuropathy. As nicotine has a very short half-life of approximately 15 minutes in mice (Damaj et al., 2007), it was also administered chronically via 7-day osmotic minipumps in order to achieve and maintain steady-state levels. We have previously reported that subcutaneous minipump administration of 12 and 25 mg/kg/day nicotine leads to plasma nicotine concentrations of approximately 56 and 97 ng/ml, or 0.121 and 0.210 µM, respectively (Alsharari et al., 2015; Alsharari et al., 2013). Chronic administration of nicotine (24 mg/kg/day) prior to and during paclitaxel injections significantly prevents both the development of mechanical allodynia and the reduction of intra-epidermal nerve fibers induced by paclitaxel, as previously described by our group (Toma et al., 2017); others have also shown protection from paclitaxel-induced IENF loss with pifithrin-µ (Krukowski et al., 2015).

18

The antinociceptive and antiallodynic properties of nicotine have been demonstrated in numerous animal and human studies (Flood and Damaj, 2014), including neuropathic pain in humans (Richarson et al., 2012; Rowbothan et al., 2009). Randomized, double-blind, placebo-controlled trials have reported that intranasal or transdermal administration of nicotine pre- or post-operatively results in significantly decreased pain scores and lower morphine consumption, respectively (Yagoubian et al., 2011; Flood and Daniel, 2004; Habib et al., 2008). Similarly, laboratory animal studies have revealed that nicotine acts as an antinociceptive drug in a variety of acute and chronic pain models in rodents (Alsharari et al., 2015; Alsharari et al., 2012). More specifically, the α7 nAChR subtype has been reported to mediate the antinociceptive effects of nicotine in a mouse model of postoperative pain (Rowley and Lu, 2008).

Others have also investigated targeting nAChRs for the treatment of CIPN. For example, a recent study indicated that pharmacological and genetic blockade of the α9α10 nAChR subtype prevents the development of neuropathic pain induced by oxaliplatin in mice (Romero et al., 2017), suggesting that nicotinic acetylcholine receptors play a significant role in the development and potentially the treatment of CIPN. Furthermore, nicotine reduces the ratio of pro-inflammatory monocytes compared to anti-inflammatory monocytes in murine bone marrow via the α7 nAChR subtype, thus significantly decreasing the level of pro-inflammatory cytokines, including TNFα and IL-1β, and enhancing the release of anti-inflammatory cytokines, such as IL-12 (St-Pierre et al., 2016). Moreover, nicotine exhibits a neuroprotective effect in animal models of neurodegenerative diseases, such as Alzheimer's disease, an action that is predominantly mediated through the α7 nAChR subtype (Ferrea and Winterer, 2009). Overall, it appears that the α7 nAChR may be one of the predominant nAChR subtypes involved in the neuroprotective actions of nicotine.

Although the current work clearly demonstrates the potential for nicotine to both prevent and reverse paclitaxel-induced peripheral neuropathy, there is an extensive body of literature suggesting that nicotine may stimulate tumor growth and/or interfere with the effectiveness of chemotherapy. This untoward effect is thought to occur via the binding of nicotine to an nAChR on the plasma membrane of the tumor cell, thereby promoting proliferative and anti-apoptotic signaling via the ERK and PI3K/Akt pathways, respectively (Grando, 2014). However, the only experimental condition under which we identified an effect of nicotine was on tumor cell proliferation in serum-free media, in which cells are deprived of nutrients, cytokines, and other growth factors, which is a non-physiological environment. Under standard cell growth conditions, nicotine did not enhance viability, colony formation, or proliferation of a number of experimental tumor cell lines or interfere with apoptosis induced by paclitaxel.

It is somewhat difficult to make direct comparisons between our studies and those in the literature focusing on human NSCLC cell lines as the concentrations of paclitaxel and nicotine vary widely in these experiments, with paclitaxel concentrations ranging between 0.1 μM and 20 μM, and nicotine ranging from 0.1 μM to 10 μM. The human steady-state plasma concentration of paclitaxel falls between 5 nM and 200 nM, while nicotine in cigarette smokers ranges from 20 to 60 ng/ml, or 0.1 to 0.4 μM (Blagosklonny and Fojo, 1999; Benowitz et al., 2009). In addition to the lack of consistency in the concentrations of paclitaxel and nicotine, the duration of drug exposure (18 hours – 7 days) and serum concentration (0-10%) also cover a wide range. In our work, we used 1 μM nicotine for 24 to 96 hours, a treatment regimen that involves both acute and chronic exposure to a nicotine concentration that is slightly higher than peak human plasma levels in order to utilize a clinically relevant dose of nicotine. Similarly, we used paclitaxel concentrations of 50 nM and 100 nM because the former is appropriate for experiments involving low cell numbers, such as the clonogenic assay, and the latter induces substantial apoptosis; yet, most importantly, both concentrations are within the range of human plasma levels.

20

Given the various experimental conditions, it is perhaps not surprising that the reported effects of nicotine vary widely as well. For example, some studies have shown increases of 23-200% in NSCLC cell proliferation (Zhang et al., 2009; Pillai et al., 2011; Wu et al., 2013; Liu et al., 2015), whereas others demonstrate modest increases of 7-18% (Chen et al., 2002; Jarzynka et al., 2006; Puliyappadamba et al., 2010) and in one case decreases of 40-72% (Gao et al., 2016). Nicotine has been reported to reduce paclitaxel-induced apoptosis by a significant 50% (Dasgupta et al., 2006) or only by a modest 8% (Tsurutani et al., 2005). These studies utilized sub-G1 DNA content, the TUNEL assay, and PARP cleavage to assess the impact of nicotine on paclitaxel-induced apoptosis, whereas we included quantification of early and late apoptotic populations with the Annexin V/Propidium Iodide assay. In part, this may provide a rationale for the inconsistencies in outcomes since sub-G1 DNA content alone does not distinguish between apoptotic and necrotic cell death (Mattes, 2007). The observation of substantially more apoptosis than sub-G1 DNA content following 48 hours of paclitaxel treatment likely reflects the possibility that early apoptotic cells had not yet become fragmented.

With regard to *in vivo* studies, while nicotine exposure has been reported to significantly increase lung tumor incidence, volume, weight, and Ki-67+ populations (Jarzynka et al., 2006; Heeschen et al., 2001; Improgo et al., 2013; Liu et al., 2015; Iskandar et al., 2012), other reports have shown that chronic nicotine treatment does not significantly stimulate lung tumor growth in mice (Warren et al., 2012; Maier et al., 2011; Murphy et al., 2011). Similarly, we found that chronic nicotine administration did not enhance LLC tumor growth in immunocompetent mice, suggesting that nicotine may be a potential therapy for CIPN prior to or following chemotherapy with the aim of preventing and reversing peripheral neuropathy, respectively.

In summary, our results provide a proof of concept that nicotine is efficacious in preventing and reversing CIPN, actions that may enhance the quality of life of cancer patients and survivors. In

addition, our findings suggest that nicotine does not significantly promote tumor cell proliferation or interfere with chemotherapy in lung cancer cell lines. In this context, we are encouraged by the report that nicotine replacement therapy is not a significant predictor of cancer in humans (Murray et al., 2009).

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Authorship Contributions:

Participated in research design: S.L.K, W.T., D.B., X.F., M.I.D., D.A.G.

Conducted experiments: S.L.K., W.T., D.B., J.A.M., X.F.

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Wrote or contributed to the writing of the manuscript: S.L.K., W.T., L.D.S., A.H.L., Z.C., E.D., M.I.D., D.A.G.

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Footnotes:

Figure Legends:

Figure 1. Antinociceptive and preventative effect of nicotine in a mouse model of paclitaxel-induced peripheral neuropathy. Reversal of mechanical allodynia by acute administration of nicotine at doses of 0.3, 0.6, and 0.9 mg/kg i.p. in paclitaxel-treated mice at day 7-14 post-initial paclitaxel injection (A). *$P < 0.0001$ vs Saline (0 mg/kg); #$P < 0.0001$ vs nicotine (0.3 mg/kg); $$P < 0.0001$ vs nicotine (0.6 mg/kg). Prevention of mechanical allodynia by chronic administration of nicotine at a dose of 24 mg/kg/day (B). Arrows indicate vehicle/paclitaxel injections on days 0, 2, 4, and 6. Minipumps with nicotine were implanted s.c. in the mouse, starting 2 days before the vehicle/paclitaxel treatment cycle and ending on day 5. Baseline measurements were taken at BL before saline/nicotine minipump implantation and on day 0 before paclitaxel/vehicle administration. ***$P < 0.001$ Pac-Nic vs Pac-Sal; ###$P < 0.001$ Pac-Sal vs Veh-Sal; $$$$P < 0.001$, $$ $P < 0.01$ Veh-Nic vs Veh-Sal. BL, baseline; Veh, vehicle; Sal, saline; Nic, nicotine; Pac, paclitaxel. n = 8 per group; data expressed as mean ± SEM. *Statistical analysis:* For mice treated with 24 mg/kg nicotine, a 2 x 2 x 10 Mixed Factor ANOVA of chemotherapy drug (paclitaxel or vehicle) in nicotine- or saline-treated mice by day showed a significant 3-way interaction [$F_{(9, 252)} = 7.851$, $P < 0.001$]. A subsequent 2 x 10 Mixed Factor ANOVA of paclitaxel or vehicle treatment by day was calculated for each level of treatment (nicotine or saline). Saline-treated mice demonstrated a significant interaction of chemotherapy drug (paclitaxel or vehicle) by day [$F_{(9, 252)} = 15.054$, $P < 0.001$], where a Sidak post hoc test revealed lower threshold responding in paclitaxel-treated mice compared to vehicle-treated mice on days 0 - 35 ($P < 0.001$). A separate 2 x 10 Mixed Factor ANOVA calculated where nicotine or saline treatment by day differed at each level of chemotherapy drug (paclitaxel or vehicle). Paclitaxel-treated mice demonstrated a significant interaction of drug pre-treatment (nicotine or saline) by day [$F_{(9, 252)} = 6.703$, $P < 0.001$], where a Sidak post hoc test revealed higher threshold responding in nicotine-treated mice compared to saline-treated mice on days 0 - 35 ($P < 0.001$). Vehicle-treated mice also

demonstrated a significant interaction of drug pre-treatment (nicotine or saline) by day [F (9, 252) = 37.064, P < 0.001], where a Sidak post hoc test revealed higher threshold responding in nicotine-treated mice compared to saline-treated mice, but only on days 0, 1, and 3 (P < 0.001).

Figure 2. The antinociceptive effect of nicotine is mediated by nicotinic acetylcholine receptors. Mecamylamine, a non-selective nAChR antagonist, was injected at a dose of 2 mg/kg s.c. 15 minutes prior to nicotine to block the nicotine-mediated (0.9 mg/kg, i.p.) reversal of mechanical allodynia in paclitaxel-treated mice (A). MLA, an α7 nAChR antagonist, was administered at a dose of 10 mg/kg s.c. 10 minutes before nicotine treatment (0.9 mg/kg, i.p.) in paclitaxel-treated mice. *P < 0.0001 Veh-Nic at 10 min vs 0 min; #P < 0.0001 Mec-Nic or MLA-Nic vs Veh-Nic at 10 min. Veh, vehicle; Nic, nicotine; Mec, mecamylamine. MLA, methyllycaconitine. n = 8 per group; data expressed as mean ± SEM.

Figure 3. Paclitaxel induces a decrease in IENF density at 35 days post-paclitaxel injection, which is prevented by nicotine administration at a dose of 24 mg/kg/day s.c. Paclitaxel at a dose of 8 mg/kg i.p. significantly decreased IENF density compared to vehicle-saline and paclitaxel-nicotine groups (A). *P < 0.05 paclitaxel-saline vs vehicle-saline; #P < 0.05 paclitaxel-nicotine vs paclitaxel-saline. Immunostained sections of hind paw epidermis represent the reduction of IENF density by paclitaxel and protection by nicotine (B). Bar represents 20 microns in all images, which were captured under 40x magnification. IENF, intra-epidermal nerve fiber; Veh, vehicle; Sal, saline; Nic, nicotine; Pac, paclitaxel. n = 8 per group; data expressed as mean ± SEM.

Figure 4. Nicotine fails to enhance NSCLC viable cell number under normal and serum-deprivation conditions. A549 and H460 cells were treated with nicotine (1 μM) for 48 or 96 hours in DMEM supplemented with various concentrations of FBS. Viability was determined with an
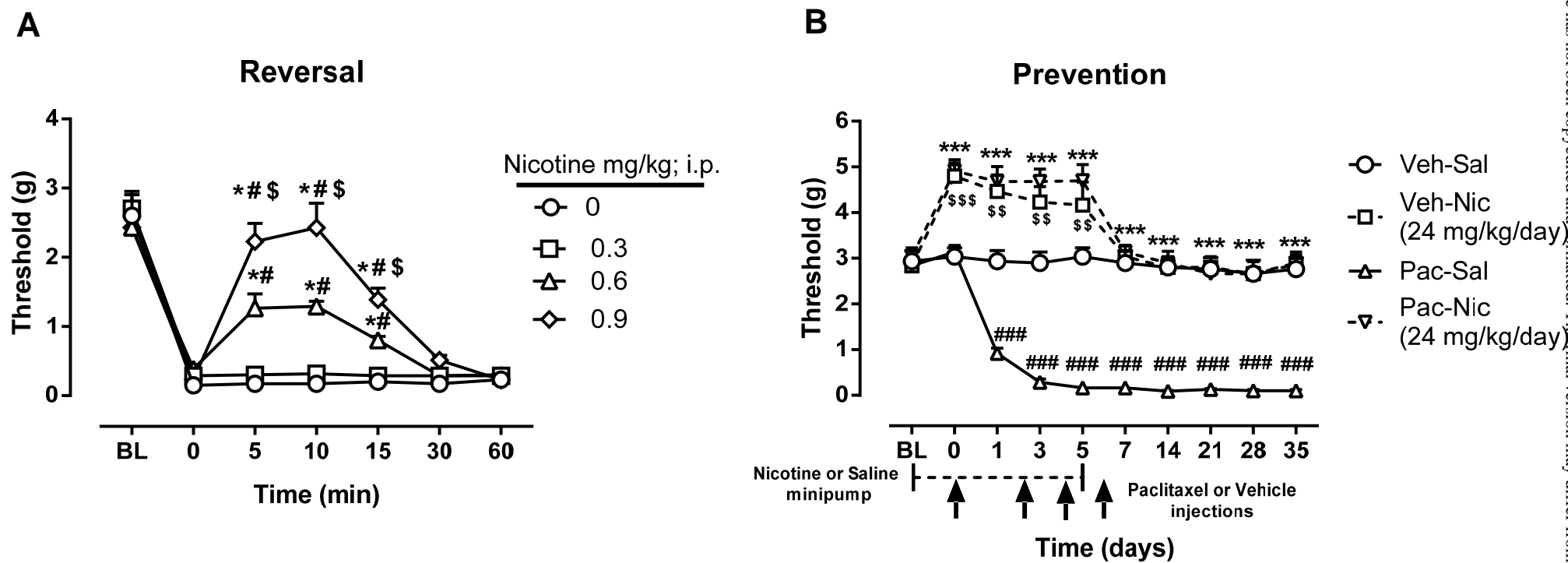
MTT or MTS colorimetric assay. *$P < 0.05$ vs control with 0% serum. Data are expressed as the mean + SEM of three independent experiments.

Figure 5. Nicotine fails to stimulate NSCLC colony formation alone or following paclitaxel treatment. For the single drug treatment conditions, A549 and H460 cells were exposed to nicotine (1 µM) or paclitaxel (50 nM) for 24 h. For the combination treatment, cells were first exposed to paclitaxel for 24 h, followed by a 24 h drug-free period, then treatment with nicotine for 24 h. Colony number was determined by crystal violet staining. ****$P < 0.0001$ vs control; n.s., not significant. Data are expressed as the mean + SEM of three independent experiments.

Figure 6. Nicotine fails to stimulate NSCLC cell proliferation alone or interfere with paclitaxel-induced growth inhibition of NSCLC cells. The "start" time point represents the initial number of cells after seeding. A 24-hour nicotine pretreatment period occurred from Start to Day 0 for the Nicotine and Pac + Nic conditions, then all subsequent treatments lasted 24 hours; no drugs were present after Day 1. The number of cells was determined via trypan blue exclusion. ****$P < 0.0001$ vs control. Data are expressed as the mean ± SEM of three independent experiments.

Figure 7. Nicotine fails to interfere with paclitaxel-induced apoptosis (A) and sub-G1 DNA content (B) of NSCLC cells. A549 and H460 cells were treated with nicotine (1 µM), staurosporine (2 µM), paclitaxel (100 nM), or the combination of paclitaxel and nicotine for 48 h. Quantification of apoptotic cells and sub-G1 DNA content was determined by the Annexin V/PI assay and propidium iodide staining, respectively, followed by flow cytometry analysis. ***$P < 0.001$, ****$P < 0.0001$ vs control; n.s., not significant. Data are expressed as mean + SEM of three (A) or two (B) independent experiments.

36

Figure 8. Nicotine fails to enhance Lewis lung carcinoma tumor growth *in vivo*. C57BL/6J mice were subcutaneously injected with 1.5 x 10^6 LLC cells in both flanks. Once tumors formed, subcutaneous osmotic minipumps were implanted on day 0 to release 24 mg/kg nicotine daily for a total of 7 days. The left and right flank tumor volumes (*l* x *w* x *h*) were compared to the respective baseline tumor volumes to calculate fold change (A); the fold change values were averaged for each mouse. A linear mixed model analysis revealed a significant effect of time [$F_{(4, 39)} = 25.747$, $P < 0.001$] and treatment [$F_{(1, 39)} = 15.683$, $P < 0.001$], but no interaction between time and treatment [$F_{(4, 39)} = 2.560$, $P = 0.054$]. n = 5-6 per group; data are expressed as mean + SEM.
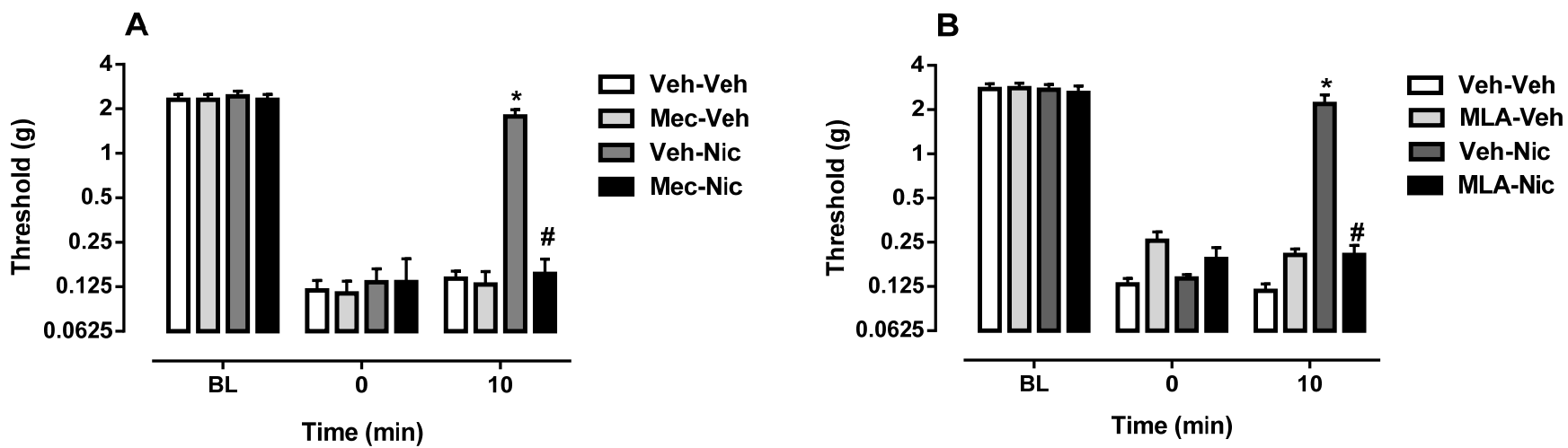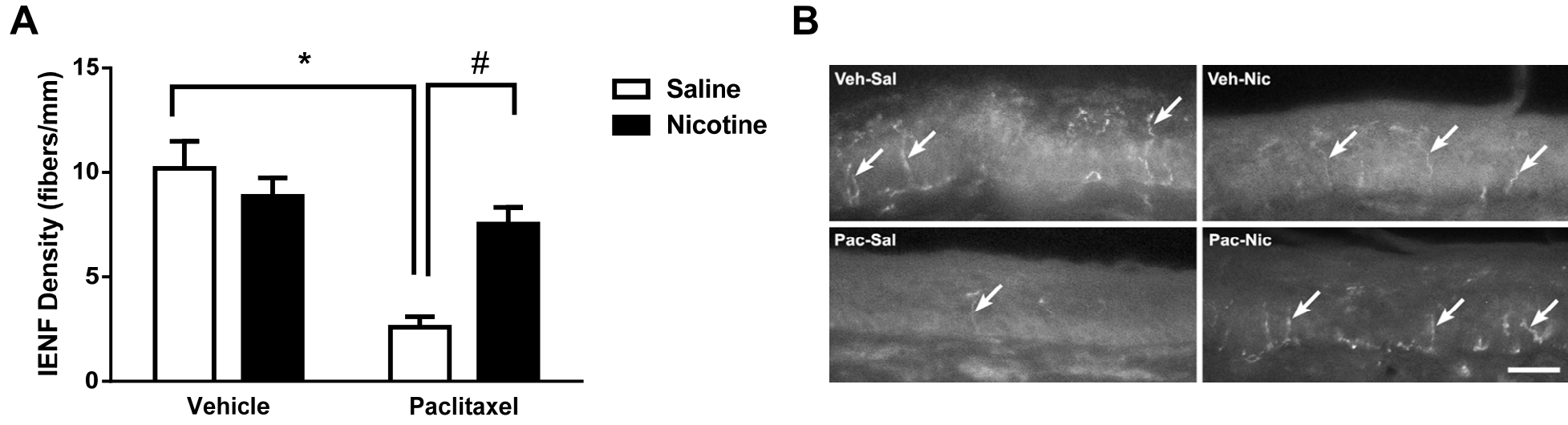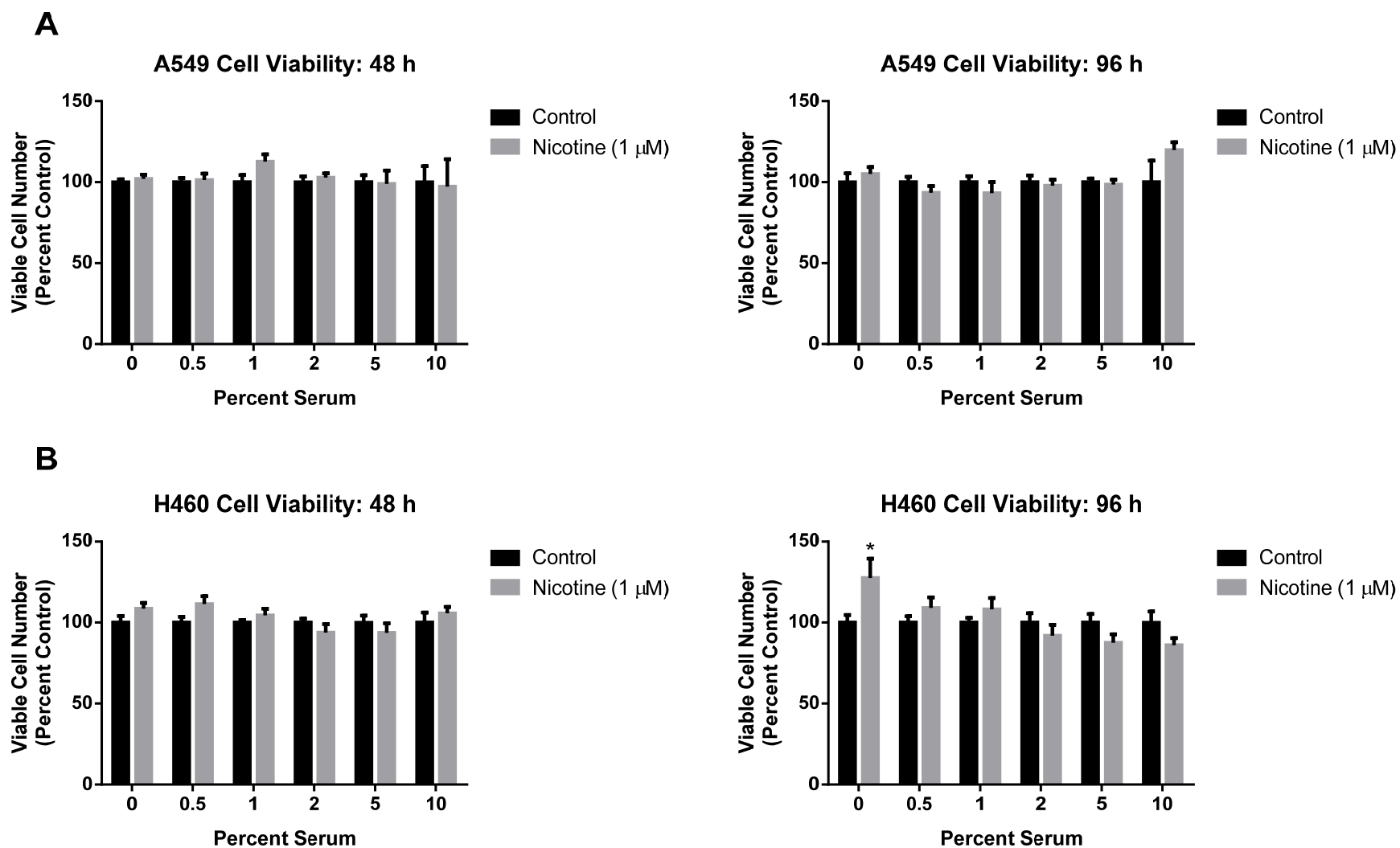
Figure 1

A

Reversal

Nicotine mg/kg; i.p.
- 0
- 0.3
- 0.6
- 0.9

B

Prevention

- Veh-Sal
- Veh-Nic (24 mg/kg/day)
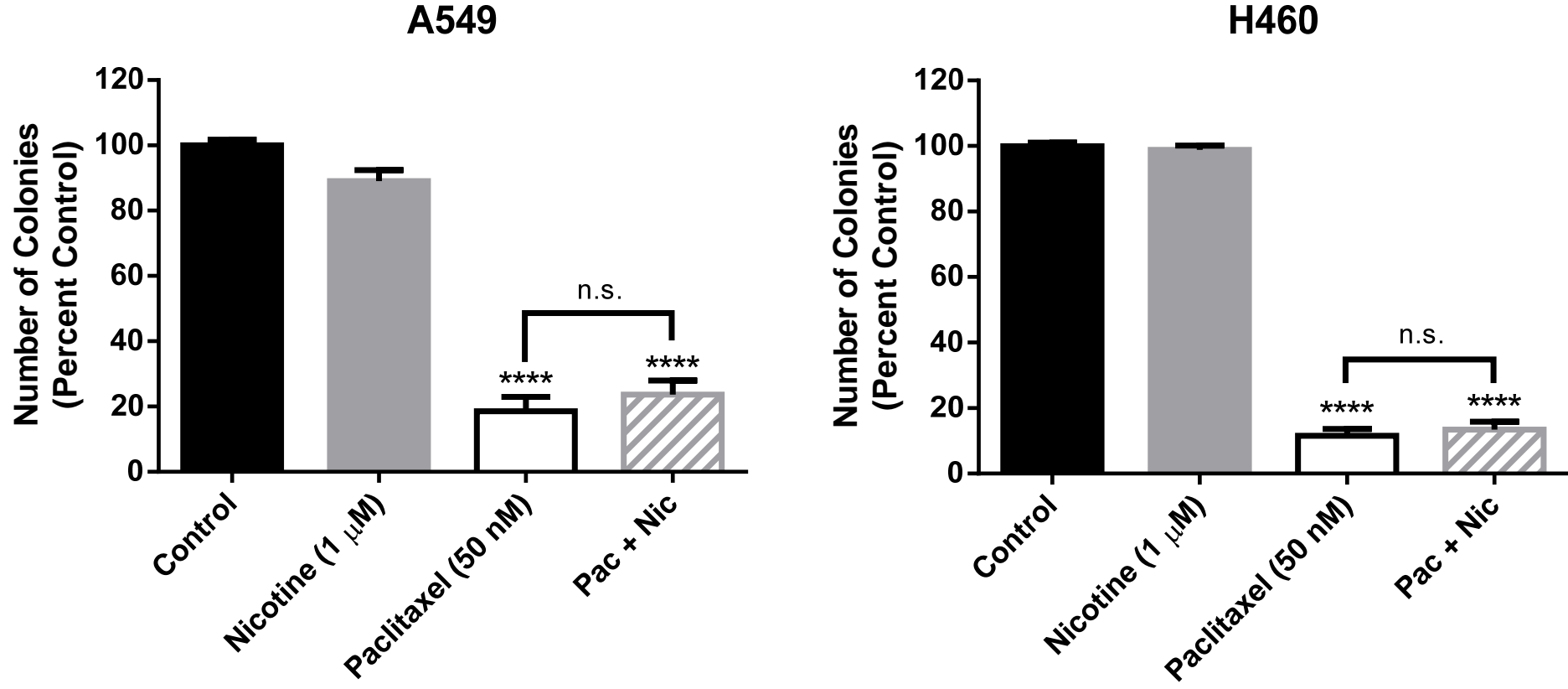- Pac-Sal
- Pac-Nic (24 mg/kg/day)

Nicotine or Saline minipump

Paclitaxel or Vehicle injections

Figure 2

Figure 3

Figure 4

A

A549 Cell Viability: 48 h

A549 Cell Viability: 96 h

B

H460 Cell Viability: 48 h

H460 Cell Viability: 96 h

Figure 5

A549

H460

Figure 6

Figure 7

Figure 8