Published in final edited form as:

Pain. 2018 June ; 159(6): 1025–1034. doi:10.1097/j.pain.0000000000001177.

Chemotherapy-induced pain is promoted by enhanced spinal adenosine kinase levels via astrocyte-dependent mechanisms

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Keywords

chemotherapy-induced neuropathic pain; adenosine kinase; astrocytes

Introduction

The development of chemotherapy-induced neuropathic pain (CINP) is a major dose limiting neurotoxicity created by widely used chemotherapeutic agents [5], including the platinum-based drug, oxaliplatin. CINP can occur in up to 60% of patients receiving oxaliplatin, which is used as a first-line chemotherapeutic for treatment of colorectal cancer [5]. CINP can linger for years and greatly impacts a patient’s quality of life due to inadequate pain management with currently available analgesics [11]. Finding novel therapies for these patients are hampered by our poor understanding of the cellular and molecular mechanisms involved. Peripheral neuropathological changes in response to chemotherapeutics are considered the provocative processes for the development of CINP [50]; however neuropathological changes within the CNS are critical for establishing the

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Author Contributions Performed experiments: CW, TD, JL, LL, KJ, ZC, EE and SC; Data analysis: CW, TD, JL, LL, KJ, ZC, EE and SC; Provided MRS5698: DT and KAJ. Assisted in manuscript preparation: TD, JL, SC, KAJ and DT; Wrote manuscript: CW and DS; Conceived, designed and lead project: DS.

chronic pain state [50]. We have recently reported that highly selective A₃ adenosine receptor (AR) agonists block and reverse CINP [21; 24], suggesting that chemotherapy causes the dysregulation of adenosine signaling at this receptor subtype. However the underlying mechanisms that drive these pathways and the molecular pathways engaged by A₃AR agonism remain to be defined.

Astrocytes in the spinal cord are emerging as the predominant cellular agent in establishing CINP [18] unlike in other types of neuropathic pain where both astrocyte and microglial cell activation contribute to neuropathological processes [39]. The mechanisms initiated in astrocytes responsible for establishing CINP remain elusive. We hypothesized a role for the dysregulation of adenosine kinase (ADK) in spinal cord. In the adult brain, ADK is found primarily in astrocytes where it is the predominant determinate of extracellular adenosine levels [2; 8; 27]. ADK establishes an adenosine gradient by converting intracellular adenosine to ATP to remove adenosine from the extracellular space down its concentration gradient through equilibrative nucleotide transporters [2; 4; 6]. ADK expression increases in astrocytes upon their activation and contributes to a number of neuropathologies [2; 25; 46]. Increasing ADK expression compromises the beneficial effects of adenosinergic signaling by creating deficits in extracellular adenosine levels [2; 25; 46] and shifting purinergic signaling towards ATP and P2Y/P2X receptor-driven neuroinflammatory processes, which include activating the NLRP3 inflammasome/IL-1 β pathway [13; 19; 49]. Conversely, maintaining adequate extracellular adenosine provides protection to neurons and glial cells during stress through its signaling at its four G protein-coupled adenosine receptors (ARs: A₁AR, A_{2A}AR, A_{2B}AR, A₃AR) [4]. A₃AR is expressed throughout the CNS in neurons and astrocytes and its activation with selective A₃AR agonists has been found to reduce neuronal excitability and attenuate astrocyte activation during neuroinflammation [23].

Here, we reveal for the first time that oxaliplatin causes the dysregulation of extracellular adenosine signaling at the A₃AR in the spinal cord by inducing ADK expression in astrocytes and engaging NLRP3/IL-1 β neuroinflammation. Restoring A₃AR signaling in the spinal cord by inhibiting ADK or activating A₃AR with intrathecal selective A₃AR agonists prevented the establishment CINP. The beneficial effects of A₃AR signaling were associated with a shift in the neuroinflammatory cytokine balance following the attenuation of NLRP3 activation and IL-1 β production and the counteractive engagement of IL-10 signaling pathways. Since an A₃AR agonist shows promise in clinical trials as an anticancer agent [1], these findings provide compelling support for clinical development of A₃AR agonists as adjunct to chemotherapeutic agents [23]. Our work addresses a huge unmet medical need while filling major gaps in our understanding of the role of astrocytes in chronic neuropathic pain associated with CIPN.

Materials and Methods

Experimental animals

Male Sprague Dawley rats (200–250 g starting weight) from Envigo (Harlan) Laboratories (Indianapolis, IN; Frederick, MD breeding colony) were housed 1–4 per cage in a controlled environment (12 hour light/dark cycle) with food and water available *ad libitum*. Male wild-type C57BL/6 or IL-10^{-/-} mice (B6.129P2-*Il10*^{tm1Cgn}/J; 7 weeks old; age and sex-matched)

were purchased from Jackson Laboratory (Bar Harbor, ME) and housed 5–10 per cage in a controlled environment (12 hour light/dark cycle) with food and water available *ad libitum*. All experiments were performed in accordance with International Association for the Study of Pain and National Institutes of Health guidelines, Italian regulations on protection of animals used for experimental and other scientific purposes (Ministerial Decree 16192), the Council Regulation (EEC) (Official Journal of the European Union L 358/1 12/18/1986) on laboratory animal welfare and the recommendations by Saint Louis University Institutional Animal Care and Use Committee and The University of Messina Review Board (41/2016-PR prot 89126.2,11/12/2015). All experiments were conducted with the experimenters blinded to treatment conditions. Animals were randomly placed in groups.

Test Compounds

MRS5698 ((1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide) was synthesized as previously described [48]. MRS1523 (3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sheep anti-rat IL-10 neutralizing antibody and its non-specific control (IgG) were a kind gift from Dr. Linda Watkins (University of Colorado at Boulder). IL-1RA was purchased from R&D Systems (Milan, Italy). ABT-702 dihydrochloride (5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride] and AR-A014418 (*N*-[(4-methoxyphenyl)methyl]-*N'*-(5-nitro-2-thiazolyl)urea) were purchased from Tocris Bioscience (Bristol, United Kingdom). All intrathecal and intraperitoneal test compounds were given after behavior measurements and prior to oxaliplatin. I.th. MRS1523, anti-IL-10 neutralizing antibodies, IL-1RA or IgG was always given before i.th. or intraperitoneal administration of A₃AR agonist or ABT-702.

Chemotherapy-induced neuropathic pain

Oxaliplatin (Oncology Supply; Dothan, AL) or its vehicle (5% dextrose) was administered as an intraperitoneal (i.p.) injection in rats on 5 consecutive days (D0 to 4) for a final cumulative dose of 10 mg/kg [24]. This low dose paradigm does not cause kidney injury, as previously reported. In mice, oxaliplatin (3 mg/kg) or its vehicle (5% dextrose) was administered i.p. for 5 consecutive days (D0-4) and then again D10-14 for a total cumulative dose of 30 mg/kg [47].

Intrathecal catheterization and injection

Rats were catheterized intrathecally using the L5/L6 lumbar approach described previously by our group [32]. Following anesthesia with isoflurane, a guide cannula (20G) was passed between lumbar vertebrae 5 and 6 into the i.th. space. A PE-10 catheter was positioned 3 cm beyond the tip of the cannula near the lumbar enlargement. To prevent displacement of the catheter, the tubing was tunneled s.c. to the occipital region through a small skin incision and secured with 5-0 silk sutures. The catheter was flushed with sterile saline and heat-sealed. Animals received Rimadyl (5 mg/kg; s.c.) and were allowed to recover on a heating pad and then were single-housed. I.th. injections (5 or 10 µl) were administered using a 25 µl or 50 µl Hamilton syringe followed by a 10 µl sterile saline flush. Animals were excluded if they did not have patent catheters, showed signs of infection or adverse reactions to the

surgery (i.e., abnormal posture, grooming, locomotor behaviors, abnormal hair coat, piloerection, ocular porphyrin discharge, and loss of body weight) or exhibited abnormal pain behaviors prior to the start of chemotherapy and drug treatment on D0.

Behavioral testing

Mechano-allodynia: Rats or mice were placed in elevated Plexiglass chambers (28 X 40 X 35-cm) upon a wire mesh floor and allowed to acclimate for 15 min prior to behavioral testing. The mechanical paw withdrawal threshold in grams [PWT, (g)] was measured with manual von Frey filaments according to the up and down method [10] [Stoelting, rats ranging from 4.31 (2 g) to 5.46 (26 g) bending force; mice ranging from 2.36 (0.02g) to 4.31 (2 g) bending force]. The development of mechano-allodynia is evidenced by a significant ($P<0.05$) reduction in mechanical mean absolute PWT (g) at forces that failed to elicit withdrawal responses before chemotherapy treatment (D0). **Mechano-hyperalgesia:** PWTs (g) were measured by the Randall and Sellitto paw pressure test [42] using a Ugo-Basile analgesiometer (Italy model 37215) that applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. The nociceptive threshold was defined as the force (g) at which the rat withdrew its paw (cut off set at 250 g). Chemotherapeutic treatment results in bilateral allodynia and hyperalgesia with no differences between left and right PWTs (g) at any time point in any group; thus, the values from both paws were averaged. Animals receiving chemotherapeutic agents in the presence or absence of the experimental test substances tested did not display signs of any toxicities: i.e., they exhibited normal posture, grooming, locomotor behavior, hair coat was normal, no signs of piloerection or ocular porphyrin discharge, and gained body weight normally and comparable to vehicle-treated rats.

Western Blot

Western blot analyses were performed as previously described with slight modifications [32]. Dorsal lumbar spinal cord samples were homogenized and protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Thermo-Fisher, Waltham MA). Proteins were denatured in Laemmli buffer and boiled for 5 min. The proteins (20–40 μ g) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene fluoride membranes. Membranes were blocked for 2 hrs at room temperature in 5% non-fat dried milk or 1% bovine serum albumin in 1X PBS, pH 7.4, depending on manufacturer's protocol for antibody and subsequently probed with specific antibodies: anti-pGSK3 β (1:1000; Cell Signaling, MA, USA, #5558), anti-GSK3 β (1:1000; Cell Signaling, MA, USA, #12456), anti-ADK (1:500; ThermoFisher Scientific, Italy, #PA5-27399), anti-NLRP3 (1:400; Santa Cruz Biotechnology, CA, USA, #sc-66846), anti-GFAP (1:1000; Dako, #Z0334) or anti-caspase 1 (1:500; Santa Cruz Biotechnology, CA, USA, #sc-1597) in 1X PBS, 2.5% nonfat dried milk or 0.1% bovine serum albumin and 0.1% Tween-20 at 4°C overnight. The bound antibodies were then visualized following incubation with anti-rabbit IgG DyLight 488 (1:1000, ThermoFisher Scientific, Italy), peroxidase-conjugated bovine anti-mouse IgG secondary antibody (1:3000, Jackson ImmunoResearch, PA, USA), peroxidase-conjugated goat anti-rabbit IgG (1:3000, Jackson ImmunoResearch, PA, USA) or donkey anti-mouse IgG DyLight 650 (1:1000, ThermoFisher Scientific, Italy) for 1 h at room temperature. Peroxidase-conjugated

antibodies were visualized by enhance chemiluminescence (Bio-Rad, Hercules CA). Chemiluminescence and fluorescent-tagged antibody signal were documented using Chemidoc XRS+ documentation system and ImageLab™ software (BioRad, Hercules CA) and quantified for band densitometry. Each membrane was then probed for β -actin (1:5000, Sigma-Aldrich, Italy) for use as endogenous loading controls.

Immunofluorescence and Image Analysis

Immunofluorescence was performed using modifications of previously reported methods [31]. After behavioral measurements, rats were sacrificed according to SLU IACUC regulations. The lower lumbar enlargement of the spinal cord (L4-L6) was harvested, transferred to OCT, and frozen in an isopropanol/dry ice bath. Transverse sections (20 μ m) were cut using a cryostat, collected on gelatin coated glass microscope slides, and stored at -20°C . Spinal cord sections were fixed in 10% buffered neutral formalin (10 min), blocked (10% normal goat serum, 2% bovine serum albumin, 0.2% Triton-X100 in phosphate buffered saline, PBS, for 1 h) then immunolabeled using an 18 h incubation (4°C) with rabbit polyclonal anti-ADK (1:100; ThermoFisher) and mouse monoclonal anti-NeuN (1:1000; Millipore), anti-glial fibrillary acidic protein (GFAP) (1:1000; Sigma-Aldrich), or anti-CD11b antibody (OX42, 1:200; Millipore). Following a series of PBS rinses, sections were incubated for 2 h with a goat anti-rabbit Alexa Fluor 568 conjugated (1:250; Invitrogen) and anti-mouse Alexa Fluor 488 conjugated antibody (1:250; Invitrogen). The coverslips were mounted with Fluorogel II containing DAPI to label nuclei (Electron Microscopy Sciences; Hatfield, PA) and immunolabeled spinal cord sections observed with an Olympus FV1000 MPE confocal microscope (multiline argon lasers with excitation at 405, 488, and 543 nm) using a 10X objective (UPLSAPO; 0.4 NA) for regional fluorescence intensity image analysis. Images were acquired within the dynamic range of the microscope (i.e., no pixel intensity values of 0 or 255 in an 8-bit image). Sections treated with isotype controls at equivalent concentrations to primary antibodies yielded only non-specific background fluorescence. The mean fluorescence intensity (MFI) in the dorsal horn was determined as previously reported [15; 22; 24; 31]. Image analysis was performed using the NIH freeware program ImageJ (version 1.43) [45]. The superficial dorsal horns (laminae I and II) at the L4, L5, and L6 levels (3 sections per animal; n=4 animals per group) were outlined on images bilaterally using the ImageJ region of interest tool. The superficial dorsal horn was determined and confirmed using cresyl violet stained sections of adjacent sections and an atlas [41]. There were no significant differences bilaterally, so MFI was calculated as a combined value and reported as fold-change compared to the vehicle group.

Co-localization studies were performed for ADK expression with astrocytes (anti-GFAP), microglia (anti-CD11b, OX-42), and neurons (anti-NeuN) using analysis of three confocal Z-stacks from the medial, middle, and lateral portions of the lumbar superficial dorsal horn (L4) in oxaliplatin and vehicle treated rats. Higher magnification co-localization studies were performed using an Olympus FV 1000 confocal microscope (multiline argon lasers with excitation at 405 nm, 488 nm, and 543 nm) with 60X oil-immersion objective (1.42 NA) and 2.4X zoom to provide 0.1 μ m pixel dimensions in the X-Y plane and the pinhole set at 1 Airy unit. Z-stack images (3 Z-stacks per animal) were obtained using sequential scanning at a 0.2 μ m Z-step with a minimum of 50 total steps (10 μ m). The images were

acquired within the dynamic range of the microscope (i.e., no pixel intensity values of 0 or 255 in an 8-bit image). Z stacks underwent deconvolution with the deconvolution wizard tool using Huygens Essential (Scientific Volume Imaging v14.10) and were analyzed for 3D co-localization. Manders' overlap coefficient (M) was used to assess ADK co-localization with NeuN, GFAP, or OX42. Manders' coefficient [36] was calculated to compare the proportion of NeuN, GFAP, or OX42-labeled voxels that are also labeled for ADK. In addition to co-localization, 3D rendered images were analyzed in ImageJ using the 3D viewer plugin to count ADK-positive (ADK⁺) cell profiles that are co-expressed with NeuN, GFAP, or OX42 positive (NeuN⁺, GFAP⁺ or OX42⁺) cells as previously described [46]. A positive cell profile was defined as one that labeled with ADK and/or cell markers with a DAPI-labeled nucleus. Total ADK⁺ cell profiles and those ADK⁺ cell profiles that co-localized with NeuN⁺, GFAP⁺ or OX42⁺ cell profiles were counted and reported as a percentage (number of co-expressed ADK⁺ cells/total ADK⁺ cells and normalized to total DAPI nuclei). Images are shown at one level of the z stack and in a 3D rendered Z stack in maximum intensity projection mode in XYZ with orthogonal ZY and YZ slices (Huygens Essential v.14.10).

Cytokine determination

The levels of cytokines within the dorsal horn of the lumbar spinal cord were assessed using a commercially available ELISA kit (Thermo Fisher Scientific, Italy).

Statistical Analysis

Data are expressed as mean $\pm$ SD for n animals. Data were analyzed by Student's t-test or two-tailed, two-way repeated measures or one-way ANOVA with Holms Sidak *post hoc* comparisons. Significant differences were defined as $P < 0.05$. All statistical analyses were performed using GraphPad Prism (v6.04, GraphPad Software, Inc.).

Results

ADK Expression In The Spinal Cord Is Increased With Oxaliplatin Treatment

Using an established model of oxaliplatin-induced neuropathy [20; 24; 53], we found that the development of mechano-allodynia and mechano-hyperalgesia (neuropathic pain) (Fig. 1A, B) and astrocyte activation (increased expression of GFAP; Fig. 1C) were associated with time-dependent increases in ADK expression in the dorsal horn of the spinal cord (DH-SC; Fig. 1D). Immunofluorescence analyses of the superficial DH-SC from oxaliplatin-treated rats demonstrated similar increases in ADK expression on D25 (Figs. 1E–G). Co-localization analyses revealed that ADK was expressed in both astrocytes (GFAP⁺; Fig. 2) and neurons (NeuN⁺; Figs. 3A–I), but not microglia (OX-42⁺, Figs. 3J–R), consistent with a previous study [40]. Further analyses showed that while the percentage of ADK⁺ astrocytes did not change (vehicle: 44.97% $\pm$ 12.23 vs. oxaliplatin: 44.49% $\pm$ 5.72; $P = 0.95$; *t*-test; n=3/group), the cellular volume of astrocytes occupied by ADK (ADK⁺ voxels/GFAP⁺ voxels) increased 2-fold in the oxaliplatin-treated rats (vehicle: M2=0.20 $\pm$ 0.09 vs. oxaliplatin: M2=0.42 $\pm$ 0.08; $P = 0.048$; *t*-test; n=3/group). Increased ADK signal was found in the astrocyte nucleus and cytoplasm in somas that expanded into processes (Fig. 2E–G). In contrast to astrocytes, both the percentage of ADK⁺ neurons (vehicle: 51.74% $\pm$ 11.04 vs.

oxaliplatin: $62.30\% \pm 3.78$; $P=0.19$; t -test; $n=3/\text{group}$) and volume of neuronal nuclei occupied by ADK (ADK⁺ voxels/NeuN⁺ voxels) did not significantly increase (1.18-fold change; vehicle: $M2=0.49 \pm 0.16$ vs. oxaliplatin: $M2=0.58 \pm 0.08$; $P=0.41$; t -test; $n=3/\text{group}$). Cytoplasmic ADK is closely associated with regulation of the extracellular adenosine levels in the brain that are responsible for adenosine receptor signaling [2; 8; 27]. Thus, the increased ADK expression observed in the cytoplasm of astrocytes could be sufficient to suppress spinal extracellular adenosine and contribute to oxaliplatin-induced neuropathic pain.

Increased Astrocyte ADK Through Deficient A₃AR Signaling Promotes Neuropathic Pain

To test the functional significance of such increased ADK expression, rats were administered intrathecal (i.th.) injections of ABT-702 (30 nmol/day) during oxaliplatin treatment. ABT-702 is a potent ($IC_{50}=1.7$ nM) and highly selective non-nucleoside ADK inhibitor [28]. When compared to its vehicle, ABT-702 attenuated mechano-allodynia and mechano-hyperalgesia (Figs. 1A and B). Moreover, the beneficial effects of ABT-702 were blocked by daily i.th. injections of the A₃AR antagonist, MRS1523 [32] (Figs. 1A and B). This suggested that oxaliplatin-induced ADK expression depressed beneficial A₃AR signaling. Indeed, when i.th. injections of the highly selective A₃AR agonist MRS5698 (3 nmol/day) [32] were given to rats concurrently with oxaliplatin treatment, the development of mechano-allodynia was blocked through day 25 (Figs. 4A and B).

Restoring Deficient Adenosine Signaling Attenuates Oxaliplatin-Induced NLRP3/IL-1 β Neuroinflammation

Activation of astrocytes and increased formation of IL-1 β are significant contributors to the neuroinflammatory processes that promote CINP [21; 22; 24; 44]. Supplementing A₃AR signaling with intrathecal injection of MRS5698 during oxaliplatin treatment significantly reduced IL-1 β levels in the spinal cord (b). To investigate the potential mechanisms through which supplemental A₃AR signaling attenuated neuroinflammation, we examined two well known pathways that provide tight regulation of the neuroinflammatory balance and are critical in the regulation of pro- and anti-inflammatory cytokines: GSK3 β and NLRP3-inflammasome [35; 38].

GSK3 β activity has recently been linked to the development of CINP following paclitaxel [16]. GSK3 β is a constitutively active serine/threonine protein kinase and its activity is attenuated primarily through AKT-mediated phosphorylation of serine-9 [26]. However, as shown in Fig. 4E, the levels of GSK3 β serine-9 phosphorylation (p-GSK3 β) and total GSK3 β in spinal cord dorsal horn at time of peak neuropathic pain were similar in oxaliplatin- and vehicle-treated rats (Fig. 4E). Moreover, i.th. injection of the GSK3 β inhibitor, AR-A014418 (100 ng/day) [51], did not block oxaliplatin-induced neuropathic pain (Fig. 4F).

In contrast, we found significant activation of the NLRP3 inflammasome. NLRP3 is an intracellular signaling molecule that senses many endogenous and pathogen-derived danger signals [49]. Upon activation, NLRP3 forms the inflammasome complex by binding and stimulating the autoactivation of the cysteine protease caspase-1 that catalyzes the cleavage

of pro-IL-1 β to the active form, IL-1 β [49]. Results in Figs. 4G and H demonstrate significantly increased expression of the NLRP3 subunit and maturation of caspase 1 (p20) in animals treated with oxaliplatin when compared to vehicle groups. Blocking NLRP3-inflammasome activity with daily i.th. injections of the potent, selective, small molecule NLRP3 inhibitor, MCC950 [5 μ mol/day, a dose previously shown not to inhibit other inflammasomes [7]], significantly attenuated oxaliplatin-induced neuropathic pain (Figs. 4A and B). MCC950 inhibits NLRP3-dependent oligomerization of apoptosis-associated speck-like protein containing a CARD (ASC), a key event in NLRP3 inflammasome activation [7]. Collectively, these results suggest that oxaliplatin induces the activation of NLRP3 and not GSK3 β during the development of chronic pain. Moreover, supplementing A₃AR signaling in rats with i.th. MRS5698 during oxaliplatin treatment significantly reduced NLRP3 protein expression and caspase 1 maturation in the DH-SC compared to those receiving vehicle (Figs. 4G and H). These data suggest that A₃AR attenuates neuroinflammation by interfering with the activation of NLRP3 signaling.

Restoring Deficient Adenosine Signaling Promotes IL-10 Signaling

The inhibitory actions of MRS5698 on IL-1 β were associated with significant increases in the levels of IL-10, a potent neuroprotective and anti-inflammatory cytokine [30], in the DH-SC (Fig. 4D); suggesting that the effects of A₃AR agonists could be driven via IL-10. In support, MRS5698 lost its ability to block oxaliplatin-induced neuropathic pain in mice with genetic ablation of IL-10 when compared to their sex- and aged-matched wild-type controls (Figs. 5A and B) or in rats when a neutralizing anti-IL-10 antibody was co-injected i.th. (α IL-10; 0.2 μ g/day) (Figs. 5C and D). Intrathecal α IL-10 also blocked the beneficial effects of ABT-702 on neuropathic pain (Figs. 6A and B). Moreover, the administration of the neutralizing anti-IL-10 antibody prevented the attenuation NLRP3 activation and IL-1 β production in the rats DH-SC by MRS5698 (Fig. 4C, G and H) and restored IL10 levels to those found in oxaliplatin-treated animals; suggesting that α IL-10 prevented the ability of A₃AR agonists to attenuate IL-1 β -driven neuroinflammation. Indeed, mechano-hypersensitivities were blocked in rats treated i.th. with interleukin 1 receptor antagonist (IL-1RA; 100 μ g/day; i.th.) in the presence of MRS5698 and α IL-10 (Fig. 5D).

Discussion

Our studies provide the first molecular insights into adenosinergic mechanisms that contribute to the development of pain associated with oxaliplatin regimens. Our findings suggest that oxaliplatin treatment significantly increases the expression of ADK within astrocytes of the superficial DH-SC where pain is actively processed and modulated. This increase in ADK is sufficient to promote the development of neuropathic pain, since its inhibition during oxaliplatin treatment blocks mechano-allodynia and mechano-hyperalgesia from ever developing. Moreover, our findings that A₃AR antagonists block the beneficial effects of ADK inhibition or that supplementing A₃AR signaling using A₃AR agonist suggests that oxaliplatin-induced ADK activity creates deficiencies in protective A₃AR signaling. Collectively these data allow us to propose a novel mechanistic model whereby oxaliplatin spurs astrocytic ADK activity in the DH-SC to reduce extracellular adenosine

that otherwise would provide protection through A₃AR signaling against maladaptive changes in DH-SC.

Upregulation of ADK in the astrocytes and the dysregulation of extracellular adenosine signaling is a common feature in numerous pathologies within the CNS, including epilepsy, traumatic brain injury, stroke, cognitive impairment [2]. Nuclear and cytoplasmic isoforms of ADK have been described in cells [2; 27; 46]. The expression of these two isoforms is transcriptionally regulated by distinct mechanisms that have been suggested to be associated with their distinct physiological functions [2; 8]. The ADK isoform sequestered within the nucleus is often associated with regulation of the intracellular levels of adenosine [2; 27]; whereas the ADK sequestered in the cytoplasm offers tight regulation of extracellular adenosine levels [2]. Within the adult brain, ADK expression is predominantly located in astrocytes and largely composed of cytoplasmic ADK [2; 46]. Its overexpression is linked to increased dysregulation of synaptic signaling and inflammation through the loss of AR signaling [2; 9]. Thus, it is likely that the increase in cytoplasmic ADK in astrocytes that is largely responsible for the effects observed in our model. However, we also detected astrocyte and neuronal expression of nuclear ADK, which has been observed in neurons of CNS regions known to undergo greater levels of neuroplasticity (granular cell layer neurons of the dentate gyrus, olfactory neurons, etc.) [2; 17; 46]. Nuclear ADK is used in intracellular processes like the DNA transmethylation pathway [2; 8; 17; 27; 46; 52]. DNA methylation is a dynamic epigenetic process that can occur in post-mitotic neurons [12; 33; 34; 52] wherein intracellular adenosine is created as a hydrolysis product of S-adenosyl-homocysteine; nuclear localized ADK salvages this major source of intracellular adenosine to maintain methylation [3]. Previous work in epilepsy demonstrated that in addition to astrocyte-dependent processes, increased nuclear ADK levels can also result in adenosine-reversible DNA hypermethylation in neurons to promote such pathophysiological events, such as enhanced synaptic plasticity [52]. Currently, it is unclear what, if any, role nuclear ADK may play in CINP. We are currently examining the functional role of enhanced spinal dorsal horn ADK levels in epigenetic modifications including DNA methylation on gene expression during pain.

Our findings reveal that among the maladaptive responses in the DH-SC provoked by oxaliplatin, the dysregulation of ADK expression in astrocytes and consequent reductions in extracellular adenosine signaling are significant contributors to oxaliplatin-induced spinal neuroinflammation through activation of NLRP3/IL-1 β neuroinflammation. IL-1 β contributes to neuronal excitability and central sensitization through several mechanisms [43] including dampening the release of anti-inflammatory/neuroprotective molecules, such as interleukin 1 receptor antagonist (IL1RA) and IL-10, which are typically produced in an attempt to counterbalance and attenuate an exaggerated neuroinflammatory response [29]. In contrast, the restoration of adenosine signaling with a selective A₃AR agonist blocked NLRP3/IL-1 β pathways and prevented neuropathic pain from ever developing. Moreover, our findings using both pharmacological and genetic approaches to block IL-10 signaling suggest that A₃AR agonists disengage the inhibition on IL-10 production and/or signaling during the development of CINP. This is anticipated to allow IL-10 to engage its powerful anti-inflammatory and neuroprotective signaling pathways, which include attenuating the activation of NLRP/IL-1 β pathways [30]. These results underscore the dependency of IL-10

downstream of A₃AR activation, and support findings in unrelated pathologies. For example, beneficial effects exerted by A₃AR agonists in a model of experimental autoimmune uveitis were also found to be driven by IL-10 [14].

In addition to NLRP3 inflammasome, our examination of the GSK3 β pathway indicates that its role in CINP is nominal, despite a reported link between A₃AR agonism and the GSK3 β pathway ischemia/reperfusion injury in cardiomyocytes [14]. However, this may depend more upon which chemotherapeutic is used than on A₃AR agonism. GSK3 β appears to be engaged with paclitaxel [16], but not with oxaliplatin (our study and another) [37]. These findings may suggest underlying differences in the mechanisms engaged chemotherapeutics during the development of CINP and/or how the beneficial effects of A₃AR agonism are exerted with different chemotherapeutics. These differences may have significant importance to future development of CINP therapies and require further study.

In summary, our results unravel a previously unrecognized link whereby chemotherapy-induced upregulation of ADK in astrocytes causes neuropathic pain by disrupting adenosine signaling at the A₃AR and engaging NLRP3/IL-1 β neuroinflammation. Chemotherapy-induced alteration of this molecular axis is rescued by A₃AR agonists. These results suggest that A₃AR agonists and NLRP3 inflammasome inhibitors offer a potential strategy to mitigate CINP potentially allowing treatment with escalating doses of chemotherapy to fully eradicate the cancer.

Acknowledgments

We thank Dr. Gary Bennett (University of California, San Diego) and Dr. Todd Vanderah (University of Arizona, Tucson) for their helpful input in the preparation of this manuscript. This study was funded by the NIH/National Cancer Institute (NIH RO1 CA169519; D.S.) and the NIDDK Intramural Research Program (Z01 DK031117-26; K.J.). Dr. Salvemini is a co-founder of Bio-Intervene which has licensed A₃AR agonists used in this study. All other authors claim no conflicts of interest.

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Figure 1. CINP neuropathic pain is dependent on the dysregulation of adenosine to A_3AR signaling via increased adenosine kinase in the DH-SC

Oxaliplatin-induced mechano-allodynia (A) and mechano-hyperalgesia (B) was inhibited by intrathecal (i.th) administration of the ADK inhibitor, ABT-702 (30 nmol/day).

Administration of i.th. A_3AR antagonist, MRS1523 (1 nmol/day), prevented the effects of ABT-702. (C) GFAP expression increased in the DH-SC on D25 in oxaliplatin-treated rats.

(D) Time-dependent increase in ADK expression in the DH-SC of oxaliplatin-treated rats.

(E,F) Immunofluorescence micrographs of ADK (red) of the DH-SC in vehicle- and oxaliplatin-treated rats on D25. (G) Image analysis was performed in the superficial DH-SC

(i.e., laminae I & II) to assess mean fluorescence intensity (MFI) of ADK immunolabeling. MFI was normalized to the vehicle-treated group and expressed as fold change. Micrographs represent one image for n=4 animals/group (three sections per animal from L4, L5, and L6 spinal cord levels). Yellow box = region of interest for Figures 2 and 3. (B) Y-axis was cropped for display. Results are expressed as mean $\pm$ SD for (A: Veh n=4, Ox n=5, Ox + MRS1523 n=3, Ox + ABT702 n=4, and Ox + ABT702 + MRS1523 n=6), (B: Veh n=4, Ox n=5, Ox + MRS1523 n=3, Ox + ABT702 n=5, and Ox + ABT702 + MRS1523 n=6), (C,D: n=6) or (G: n=4). Two-way (A, B) or one-way (C, D) ANOVA with Holms Sidak comparisons or Student's *t*-test (G). *P 0.05 compared to D0 or vehicle; † P 0.05 vs. oxaliplatin. (—) =duration of treatment days 0–4

Figure 2. Increased ADK expression in the superficial DH-SC occurs in astrocytes (A–D) Immunofluorescence micrographs of ADK alone (*red*; A,B) and merged with GFAP (*green*; C,D) on D25 in the superficial DH-SC (i.e., laminae I & II; *yellow box in Figure 1*) of vehicle- and oxaliplatin-treated rats. (E) Higher magnification (*yellow box in D*) of the 3D-rendered image (layered channel mode) from oxaliplatin-treated rat showing ADK in astrocytic somas (*white arrowheads*) with DAPI (*blue*) and processes (*white arrows*). (F–H) Confocal Z-stack 3D rendering of area in (D) demonstrating increased ADK immunolabeling co-localized (*yellow*) with astrocytes (GFAP, *green*) within superficial DH-SC in oxaliplatin-treated rats at D25. Z-stack with 20° rotation (X-axis) in maximal intensity projection mode with orthogonal views (dotted lines) in the YZ (G) and XZ (H) planes demonstrate co-localization in the same voxel (*yellow*) between ADK and GFAP immunolabeling. Micrographs represent one image for n=3 animals/group for Z-stack analysis (3 Z-stacks/animal; L4 level).

Figure 3. ADK expression in neurons and microglia of the rat superficial DH-SC
 (A–F) Immunofluorescence micrographs of ADK (*red*; A,D) in neurons (NeuN; *green*; B,E) on D25 in the superficial DH-SC (i.e., laminae I & II; *yellow box in Figure 1*) of vehicle- and oxaliplatin-treated rats and subsequent merged images showing co-localization (*yellow*; C,F). (G–I) Confocal Z-stack 3D rendering demonstrating increased ADK immunolabeling co-localized (*yellow*) with NeuN (*green*) within superficial DH-SC in oxaliplatin-treated rats at D25. (J–O) Immunofluorescence micrographs of ADK (*red*; J,M) in microglia (OX-42; *green*; K,N) on D25 in the superficial DH-SC (i.e., laminae I & II; *yellow box in Figure 1*) of vehicle- and oxaliplatin-treated rats and subsequent merged images showing co-localization (*yellow*; L,O). (P–R) Confocal Z-stack 3D rendering demonstrating increased ADK immunolabeling with OX42 (*green*) within superficial DH-SC in oxaliplatin-treated rats at D25. Z-stack with 20° rotation (X-axis) in maximal intensity projection mode with orthogonal views (dotted lines) in the YZ (H,Q) and XZ (R,I) planes demonstrates co-localization in the same voxel (*yellow*) between ADK and NeuN immunolabeling, but not with OX-42. Micrographs represent one image for n=3 rats/group for Z-stack analysis (3 Z-stacks/animal; L4 level).

Figure 4. GSK3β, NLRP3 inflammasome and IL-10

(A,B) Intrathecal administration of the NLRP3 inhibitor, MCC950 (5 μmol/day) or A₃AR agonist, MRS5698 (3nmol/day), prevented oxaliplatin-induced mechano-allodynia (A) and hyperalgesia (B). (C,D) In rat DH-SC harvested on D25, the expression of IL-1β increased in oxaliplatin-treated rats (C). Intrathecal MRS5698 (3 nmol/day) attenuated IL-1β (C) and increased IL-10 (D) expression. The effects of MRS5698 on cytokine expression were attenuated with i.th. administration of αIL10 (0.2μg/day). (E) Total and phosphorylated GSK3β levels in DH-SC on D25 were similar between vehicle- and oxaliplatin-treated rats. (F) Intrathecal administration of the GSK3β inhibitor, ARA014418 (100 ng/day), had no effect on oxaliplatin or vehicle-treated animals. (G,H) When compared to vehicle, intrathecal MRS5698 (3 nmol/day) attenuated oxaliplatin-induced expression of NLRP3 (G) and maturation of caspase 1 (H) in the DH-SC. The effects of MRS5698 (3 nmol/day) were attenuated with i.th. administration of αIL10 (0.2μg/day). Y-axis was cropped for display (B). All lanes in gel images (E, G, H) were cropped from single blots that were repeated at least twice. Results are expressed as mean ± SD for (A,B: Veh n=5, Ox n=4, Ox + MCC950 n=5 and Ox + MRS5698 n=5), (C–E: n=6/group), (F: Veh n=5, Veh + ARA014418 n=5, Ox n=5 and Ox + ARA014418 n=4) and (G,H: n=6/group). Two-way ANOVA with Holms Sidak post hoc comparisons (A,B,F) one-way ANOVA with Dunnett's (C,D,G,H) or Student's t-test (E). *P<0.05 vs. D0 or vehicle; †P<0.05 vs. oxaliplatin; #P<0.05 vs. oxaliplatin + MRS5698. (—) =duration of treatment days 0–4.

Figure 5. Beneficial effects of MRS5698 in CINP depend upon IL-10; evidence from a pharmacological and genetic approach

Oxaliplatin-induced mechano-allodynia in IL-10^{-/-} and WT control mice to a similar extent. However, MRS5698 (1 mg/kg/day; i.p.) given with oxaliplatin blocked the development of mechano-allodynia in WT (A), but not IL-10^{-/-} mice (B). Intrathecal delivery of a neutralizing αIL-10 antibody, but not its non-specific IgG (0.2 μg/day), given just before i.th. MRS5698 (3 nmol/day) prevented MRS5698's effects (C). Intrathecal IgG or αIL-10 had no effect on behavior in rats treated with vehicle or oxaliplatin (C). Pretreatment with spinal IL-1RA (100 μg/day; i.th.) prior to αIL-10, abrogated αIL-10's inhibitory actions on MRS5698 (D). Results are expressed as mean ± SD for (A,B: n=3/group), (C: Veh + αIL-10 or IgG n=3/group, Ox + αIL-10 or IgG n=3/group, Ox + MRS5698 + αIL-10 or IgG n=6/group) and (D: n=6/group). Two-way ANOVA with Holms Sidak comparisons *P<0.05 compared to D0; †P<0.05 vs. oxaliplatin; #P 0.05 vs. oxaliplatin + MRS5698. (—) =duration of treatment.

Figure 6. Neutralization of spinal IL-10 blocked the beneficial effects of spinal ABT-702
 Intrathecal delivery of a neutralizing α IL-10 antibody, but not its non-specific IgG (0.2 μ g/day), given just before i.th. ABT-702 (30 nmol/day) prevented ABT-702 from inhibiting the development of mechano-allodynia (A) and mechano-hyperalgesia (B). Y-axis has been cropped for display (B). Results are expressed as mean $\pm$ SD for n=6/group. Two-way ANOVA with Holms Sidak comparisons *P<0.05 compared to D0; †P 0.05 vs. oxaliplatin. (—) =duration of treatment days 0–4.