UNIVERSITA' DEGLI STUDI DI MESSINA
Dottorato di Ricerca in Scienze Biomediche Cliniche e Sperimentali
XXX Ciclo
Dipartimento di Medicina Clinica e Sperimentale
Coordinatore del Dottorato di Ricerca: Prof. Edoardo Spina

ADENOSINE RECEPTORS AS STRATEGIC TARGETS FOR THE DESIGN AND DEVELOPMENT OF INNOVATIVE DRUGS FOR THE MANAGEMENT OF SPINAL CORD INJURY

Tesi di:
Dott.ssa Natasha Irrera

Relatore:
Prof. Francesco Squadrito
SSD: BIO14

Co-Relatore:
Prof.ssa Alessandra Bitto

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INTRODUCTION

Adenosine

Many different intercellular signals preserve homeostasis in tissues and organs. One of the signals involved in the regulation of physiological and pathological processes is adenosine.

Adenosine is a potent vasodilator, identified for the first time by Drury and Szent-Gyorgi in 1929 (Drury AN and Szent-Gyorgi A, 1929). Subsequently, adenosine was shown to mediate its effects on cells via recruitment of specific receptors (A1, A2A, A2B and A3). Adenosine is released from cells in response to hypoxia, metabolic stress or injury and promotes the processes required to reduce harmful stimuli. Moreover, adenosine is an endogenous regulator of inflammatory processes, mediating the resolution of inflammation (Newby *AC et al.*, 1983).

Intracellular ATP is the most abundant molecule in cells and represents one of the main source of adenosine production; however, most adenosine is produced in the extracellular space as a result of the dephosphorylation of adenine nucleotides to adenosine. Some transporters are involved in the export of ATP, including the proteins connexin-43 (also known as gap junction α-1 protein) (Beyer EC and Steinberg TH, 1991), progressive ankylosis protein homologue (ANK) (Rosenthal AK *et al.*, 2013), pannexin-1 and pannexin-3 (Bao L *et al.*, 2004), and others as well, such as P2X7, which affects ATP transport indirectly (Iglesias R *et al.*, 2008). Some ATP is dephosphorylated to adenosine but injury, hypoxia or other

metabolic processes may trigger increased rates of intracellular conversion of ATP to adenosine or may stimulate the release of adenine nucleotides into the extracellular space.

Adenine nucleotides are dephosphorylated to adenosine by ectoenzymes at the cell surface (ecto-5′nucleotidase [CD73] and ecto-nucleoside triphosphate phosphohydrolase [CD39]) and also by enzymes both in blood and other extracellular fluids (for example, alkaline or acid phosphatases, including tissue nonspecific alkaline phosphatase [TNAP]). Once released into the extracellular space, adenosine may be deaminated to inosine and, in humans, ultimately to uric acid or taken up directly by cells by specific nucleoside transporters (ENT1 and ENT2) (Baldwin SA *et al.*, 2004) and re-phosphorylated to ATP (Anselmi F *et al.*, 2008; Beckel JM *et al.*, 2014; Iwamoto T *et al.*, 2010).

Adenosine receptors

Among the many molecular mechanisms regulating tissues homeostasis, the purinergic signalling assumes an important role.

Two main different purinergic receptor families were identified: P1 receptors activated by adenosine and antagonized by methylxantine; P2 receptors that bind both ADP and ATP (Burnstock G *et al.*, 1978). Adenosine is present in the extracellular fluids at basal concentrations ranging between 30 nM and 200nM and can reach 10-100 μM during stress conditions (Ballarin M *et al.*, 1991; Fredholm BB, 2007).

Adenosine formation from ATP transported into the extracellular space by cell surface enzymes rapidly regulates adenosine concentrations in the extracellular space. A1, A_{2A}, A_{2B} and A3 are the 4 transmembrane receptors that once activated by adenosine trigger the molecular signalling (Fredholm BB, 2011).

These receptors belong to the G-protein coupled receptor family: A_{2A} and A_{2B} receptors are coupled to G_s/Golf protein increasing cAMP followed by activation of protein kinase A and EPAC1/2; in contrast, A1 and A3 receptors inhibit cAMP production (Sheth S *et al.*, 2014). These receptors show high sequence similarity and a similar potency in modulating cAMP, except for A_{2B}R which is 50 times less potent than the other receptors (Jacobson KA and Gao ZG, 2006). Adenosine receptor activation, like other G protein coupled receptors, is regulated by mechanisms of desensitization involving receptor phosphorylation mediated by G-protein coupled receptor kinases. Desensitization is rapid for A_{2A}R and A_{2B}R (few minutes) and it is a relatively slow process for A3R and A1R (respectively 1 hour and few hours) (Sheth S *et al.*, 2014).

Adenosine receptors are ubiquitously expressed, engaged in the physiology and pharmacology of many tissues and organs. Moreover, forming heterodimers, for instance with dopamine and cannabinoid receptors, can interfere with and regulate other molecular signalling (Fredholm BB *et al.*, 2011). Expression of adenosine receptors is regulated by a number of stimuli, including inflammatory ones. In particular, A_{2A} receptor is upregulated by agents that stimulate activation of nuclear factor-κB (NF-κB;

a central transcriptional regulator in the inflammatory process), such as tumor necrosis factor (TNF), interleukin (IL)-1, and endotoxin, acting as a feedback inhibitor of inflammation (Nguyen DK *et al.*, 2003; Khoa ND *et al.*, 2001).

In addition to regulation of A_{2A} expression, TNF and other pro-inflammatory cytokines increase the function of these receptors by preventing receptor desensitization, further downregulating inflammation (Khoa ND *et al.*, 2006). By contrast, interferon-γ (IFNγ) downregulates both the expression and function of A_{2A} (Block ET and Cronstein BN, 2010). This downregulation (which occurs following the increase in both extracellular adenosine levels and A2A expression and function after cellular injury or necrosis) indicates the potent role of adenosine and its receptors as feedback regulators of inflammation and innate immune responses.

In the last few years adenosine receptors have been studied as a target for many diseases including cancer, regulation of the immune system, skin fibrosis, neurodegenerative conditions and bone metabolism disorders (Burnstock G, 2016; Di Virgilio F and Adinolfi E, 2017; Perez-Aso *et al.*, 2016; Cekic C and Linden J, 2016).

Target drug for adenosine signalling

The importance of adenosine receptor signalling on human health indicates that targeting multiple steps and pathways involved in adenosine receptor signalling, such as adenosine generation and metabolism, may be effective for the management of several diseases.

Adenosine receptor agonists represent an attractive and novel alternative to growth factors and they often are small synthetic molecules of longer stability and lower cost than growth factors.

Studies from Squadrito Lab have demonstrated that the use of an adenosine receptor agonist, the Polydeoxyribonucleotide (PDRN), presents the advantages of reducing inflammation and increasing vascularisation and extracellular matrix deposition (Bitto A *et al.*, 2008; Bitto A *et al.*, 2011). One of the most important factors to be considered is the pharmaceutical formulation and the pharmacokinetics of the agonist used, in order to minimize its adverse effects.

PDRN represents a new advancement in pharmacotherapy since the lack of effects on the immune system is one of the most important determinants of the good safety profile of the drug. In fact, adenosine and dipyridamole are non-selective activators of adenosine receptors and they may cause unwanted side effects. Indeed, regadenoson is the only other one A_{2A} receptor agonist available in the market but has been approved by the FDA as a pharmacological stress agent in myocardial perfusion imaging, a well-established non-invasive modality for the diagnosis and prognosis of coronary artery disease (Reyes E, 2016). It displays fewer side effects than

adenosine or dipyridamole, but its potential use in other pathological conditions has not yet explored.

PDRN and its pharmacological properties

PDRN is a proprietary and registered DNA derived drug, consisting of deoxyribonucleotides with molecular weights between 50 and 1,500 KDa. It is derived from *Oncorhynchus mykiss* (Salmon trout) or *Oncorhynchus keta* (Chum Salmon) sperm DNA. The most represented molecular weight is 80–200 KDa, with a peak of the Gaussian distribution at 132 KDa. PDRN has a higher molecular weight compared to defibrotide (16.5 ± 2.5 KDa) (Guglielmelli T *et al.,* 2012; 12:353–361; Richardson PG *et al.,* 2013). PDRN is extracted and purified at high temperature, a procedure that allows to recover a >95% pure active substance with inactivated proteins and peptides. This latter guarantees the safety of the product and the absolute lack of any immunological side effect. Indeed, the source of raw material (cells vs organs) is of particular importance: spermatozoa are the most appropriate cells to provide highly purified DNA without risk of impurity such as peptides, proteins and lipids, which can remain from the somatic cells.

Measurable levels of PDRN were observed 15 min post-injection, and peak levels 1 h after drug administration, with a bioavailability of 90%, following a single PDRN intraperitoneal administration at the dose of 8 mg/kg. Drug levels then decreased progressively, being PDRN still measurable (0.137 μg/ml) 6 h following injection. The half-life is of 3 h and it is not influenced

by dosage. The drug stimulates the initiation of a cascade of events involving a number of transduction effectors that last much more than its plasma half–life. Due to its chemical structure, plasmatic carrier proteins do not bind PDRN, and it may be found free in plasma. The distribution of the free drug depends upon tissue blood flow, being higher in those organs with elevated blood supply. The liver does not metabolize the drug and there is no evidence for a first-passage metabolism. Instead, the drug is mainly degraded by unspecific plasma DNA nucleases, or by nucleases bound to cell membranes leading to oligo and mononucleotides. From a pharmacodynamics point of view, this event is of paramount importance: in fact, PDRN degradation gives rise to the formation of nucleosides and nucleotides that become available for the main activity: the binding to the adenosine receptors. PDRN fragments are then excreted in the urine (~65%) and to a lesser extent in the feces.

The pharmacokinetics of PDRN has been also studied in healthy volunteers after intramuscular administration (5.625 mg). The results of this study showed a pharmacokinetic profile overlapping to that observed in experimental animals: more specifically peak levels at ~1 h; a half-life of ~3.5 h, with a bioavailability in the range of 80/90% (Squadrito F *et al.*, 2017).

The A_{2A} receptor plays a central role in modulating inflammation, oxygen consumption, ischemia, cell growth, and angiogenesis. PDRN was compared to adenosine in primary cultures of human skin fibroblasts (Thellung S *et al.*, 1999): both PDRN and adenosine induced cell growth.

Moreover, PDRN may represent a pro-drug able to generate active deoxyribonucleotides, nucleosides and bases, thus activating the "salvage pathway".

The use of PDRN accelerates the repair and the growth of bone tissue (Guizzardi S *et al.*, 2007). Furthermore, PDRN effects were investigated in a model of diabetes-impaired wound healing (Galeano M *et al.*, 2008). Disorders in wound healing are very common in diabetes and they represent a major clinical challenge: in fact, there is still an unmet need for a safe treatment able to counteract this clinical condition. PDRN improved the skin repair process and enhanced wound-breaking strength in diabetic animals. This effect was supported by a marked increase in the expression of Vascular Endothelial Growth Factor (VEGF), a master regulator of angiogenesis that is impaired in diabetes-related wound disorders (Galeano M *et al.*, 2008). Angiogenesis improvement was confirmed by an increase in CD31, transglutaminase-II, and angiopoietin, factors contributing to new vessel formation.

Another clinical condition characterized by a poor skin repair process and impaired angiogenesis is thermal injury. PDRN effects were investigated in mice with a deep-dermal second degree burn injury (Bitto A *et al.*, 2008), where the treatment enhanced burn wound re-epithelialization and decreased time to final wound closure. PDRN also showed a marked systemic effect: in fact, it reduced the serum levels of TNF-α and augmented wound VEGF expression and nitric oxide production. The wound healing properties of PDRN might be the consequence of the stimulation of the altered cell-cycle

machinery that is deeply impaired in several conditions: in a diabetes setting the drug stimulated the proliferation of the granulation tissue by activating cyclins driven cell-cycle progression and turning off the cell-cycle negative regulators p15 and p27 (Altavilla D *et al.*, 2011).

The ability of PDRN to promote therapeutic angiogenesis was also studied in an experimental model of peripheral artery occlusive disease induced by the excision of the femoral artery. PDRN boosted a robust blood flow restoration together with a marked increase in VEGF expression, while DMPX abrogated the beneficial effects of the drug (Bitto A *et al.*, 2008).

Skin flap technique is commonly used in plastic surgery and esthetic medicine for a faster wound coverage, to reduce the risk of infection and restore organ function. In an experimental ischemic skin flap model PDRN increased blood flow, evaluated by laser Doppler, and again boosted a strong VEGF-driven angiogenesis (Polito F e*t al.*, 2012). Overall, all these experimental pre-clinical observations anticipate a marked therapeutic efficacy of PDRN in a clinical setting of impaired wound healing, angiogenesis and disturbed skin repair processes.

A_{2A} receptor activation results in anti-inflammatory effects, so that PDRN was evaluated in collagen-induced arthritis (Bitto A *et al.*, 2011). In this experimental paradigm, PDRN significantly improved the clinical signs of arthritis, reduced the histological damage and blunted the cartilage content and blood levels of several inflammatory cytokine, while increased the expression of the anti-inflammatory cytokine interleukin-10 (IL-10). The immunological and pathological processes occurring in rheumatoid arthritis

and another condition named periodontitis are nearly identical. Both conditions are characterized by chronic inflammation in a soft-tissue site adjacent to bone, and periodontitis is one of the most important cause of teeth loss in adults. Experimentally periodontitis can be induced in rodents by ligation of the lower left first molar cervix. In a rat model, PRDN reduced the histological damage, decreased the tissue levels of several inflammatory cytokines and blunted apoptotic protein expression (Bitto A *et al.*, 2013). The treatment also markedly protected the alveolar bone quality, thus suggesting that PDRN may also promote bone regeneration, as recently confirmed by other studies (Kim I *et al.*, 2016). Chronic inflammation is also deeply involved in the etiology and development of other conditions as inflammatory bowel disease and it is known that the activation of adenosine A_{2A} mitigates the inflammatory cascade in colonic epithelial cells. In agreement with this evidence, PDRN was tested in two experimental models of colitis, the drug was given by intraperitoneal injection and it was able to ameliorate tissue repair and to reduce symptomology (Pallio G *et al.*, 2016). Also clinical studies have demonstrated PDRN efficacy such as in a trial carried out in diabetic patients with poor diabetic skin repair that points out a dramatic efficacy of PDRN in improving hard-to-heal chronic diabetic foot ulcers (Squadrito F *et al.*, 2014).

Adenosine receptors and Wnt Signalling: possible crosstalk

The wingless-type mouse mammary tumor virus integration site family (WNT)/β-catenin pathway regulates several processes during normal organ development and tissue homeostasis (Clevers H, 2006). In fact, alteration of Wnt/β-catenin pathway seems to be involved in various diseases, such as wound healing, fibrosis and osteoporosis (Beyer C *et al.*, 2012; Cheon SS *et al.*, 2006; Guo Y *et al.*, 2012).

The canonical Wnt pathway is activated when Wnt ligand binds one member of the Frizzled (Fz) receptors, of which 10 members have been described (Wang HY *et al.*, 2006). The interaction Wnt protein-Fz receptor requires the co-receptor low-density lipid receptor-related protein 6 (LRP6) and the recruitment of the scaffold protein Disheveled (Dvl). Once activated, the canonical Wnt pathway activates the intracellular β-catenin protein in the cytoplasm, whose levels remain low by the action of a so-called destruction complex formed by several proteins including the scaffold proteins axin, adenomatous polyposis coli (APC), casein kinase I, and the enzyme glycogen synthase kinase 3β (GSK-3β). This complex mediates β-catenin phosphorylation and activation (Niehrs C, 2012).

The β-catenin translocates into the nucleus acting as a transcriptional coactivator of transcription factors belonging to the TCF (T-cell specific transcription factor) /LEF (lymphoid enhancer-binding factor) family (Clevers H and Nusse R, 2012). When Wnt signalling is not activated, β-catenin remains accumulated in the cytoplasm forming a complex. Dickkopf1 (DKK1) is one of the main Wnt/β-catenin signalling inhibitors

and, inhibiting Wnt activation, decreases β-catenin expression (MacDonald BT *et al.*, 2009). β-catenin-dependent Wnt signalling activation may be considered as one of the initial cellular responses to tissue injury. For example, Wnt/β-catenin signalling stimulation i) increased fibroblasts proliferation, migration and also the production of extracellular matrix (Wei J *et al.*, 2011) and ii) is activated in altered wound healing patterns (Cheon SS *et al.*, 2006). In fact, β-catenin may support the contraction of collagen lattices which is an important step during the maturation of scars (Poon R *et al.*, 2009).

Hino et al. demonstrated a possible crosstalk between A_{2A} receptor stimulation and Wnt/β-catenin in increasing cAMP levels. Moreover, AKT may directly promote β-catenin phosphorylation, representing a possible A_{2A} receptor-mediated non-canonical pathway (Hino S *et al.*, 2005).

Shaikh et al. demonstrated that A_{2A}R stimulation with CGS21680, an A_{2A}R agonist, promoted β-catenin activation in normal human dermal fibroblasts. This effect was blocked by SCH58261, an A_{2A}R antagonist (Shaikh G *et al.*, 2016).

Adenosine signalling in spinal cord injury

Spinal cord injury (SCI) is a complex clinical and progressive condition characterized by neuronal loss, axonal destruction and demyelination, in particular at the site of impact (Maegele M *et al.*, 2005). Injuries on spinal cord affect neurons which suffer alterations such as atrophy and shrinkage (Brock JH *et al.*, 2010; Nielson JL *et al.*,2011).

Spinal cord atrophy is related to the decrease of cortico-spinal tract integrity and cortical grey matter volume (Freund P *et al.*, 2013), suggesting that spinal cord injuries activate degenerative process spreads towards the brain.

When traumatic and mechanical injuries hit spinal cord, neurons start to die and tissue loses neuronal cells which can't be recovered or regenerated. Demyelinating processes occur (Hagg T and Oudega M, 2006) and acute injury may develop in chronic injury. In fact, a "secondary" phase generally follows the mechanical injury which is characterized by microvascular dysfunction and enrolment of inflammatory cells. Once activated, inflammatory cells release pro-inflammatory cytokines, thus contributing to further spinal cord damage. Local and systemic inflammation is responsible for neurodegenerative processes both during acute and chronic phases of SCI, and consequently leads to death of glial cells and neurons, producing glial scar and a cavity in the spinal parenchyma (Fleming JC *et al.*, 2006; Kigerl KA *et al*., 2009). In particular, microglia/macrophages associated with inflammatory process seems to be an important mechanism of neuronal degeneration and regeneration (Beuche W and Friede RL, 1984; Zhou X *et al.*, 2014). Macrophages are dynamic cells and depending on their phenotype, they initiate secondary damage and also initiate repair mechanisms (Stout RD and Suttles J, 2004).

Currently, drugs prescribed to treat spinal cord injury, such as corticosteroids, try to prevent secondary inflammatory neuronal damage (Moreno-Flores MT and Avila J, 2006) and to improve behavioural outcomes (Hurlbert RJ, 2006). These medical strategies are not 100%

effective in treating SCI, but they are useful to ameliorate symptoms; for this reason, the researchers need to focus their attention on others molecules and targets to manage patients suffering consequences of spinal cord injury. Adenosine receptors may modulate several pathophysiologic mechanisms characterizing SCI which let us hypothesize that the use of adenosine receptor analogues might represent a promising group of candidate compounds.

Leukocyte infiltration and production of cytokines such as tumor necrosis factor alpha (TNF-α) are markedly increased following SCI (Zhou X *et al.*, 2014). Adenosine A_{2A} receptor activation reduced both neutrophil recruitment and TNF-α release (Cassada DC *et al.*, 2002). Furthermore, Adenosine A_{2A} receptor stimulation may protect injured spinal cord by its vasodilatory effects on neural microvasculature (Vinten-Johansen J *et al.*, 2003).

A previous study demonstrated that the use of a selective adenosine A_{2A} receptor agonist protected against locomotor dysfunction and improved neurological function following SC ischemia-reperfusion and traumatic injury (Cassada DC *et al.*, 2002).

The use of a selective adenosine A_{2A} receptor agonist for the management of spinal cord injury may have advantages compared to the use of corticosteroids. For example, the mechanism of action of methylprednisolone is based on its anti-inflammatory and antioxidant properties, activating transcriptional processes that may require too long time. Instead, A_{2A} receptor activation increases cAMP levels and exerts anti-

inflammatory effects by seconds (Linden J, 2001), inactivating neutrophils and reducing pro-inflammatory cytokines (Sullivan GW *et al.*, 1990).

Spinal cord injury and Wnt-β catenin pathway

Wnt signaling may regulate critical processes such as neurogenesis and regeneration (David MD *et al.*, 2010; Varela-Nallar L *et al.*, 2010). Some reports have described an improved recovery of the CNS after injury through the activation of the Wnt/β-catenin signalling suggesting that Wnt signalling may be considered a promising therapeutic strategy. SCI is the consequence of a mechanical trauma characterized by the activation of a complex network of cellular processes, such as microglial and astroglial cell reactivity, leukocyte infiltration and mobilization of neural precursors regulated by a wide range of molecules, such as cytokines, chemokines, growth factors, and inflammation-related enzymes. On the other hand, this pathologic condition usually leads to an extensive neural cells death and the disruption of neural circuits.

In fact, Wnt proteins and mRNA are constitutively expressed in the healthy adult spinal cord of rats and mice (Fernandez-Martos CM *et al.*, 2011).

Spinal cord injury may cause Wnt signaling down-regulation. The inhibitor Wif1 is highly expressed following SCI, in particular from hours to days post injury. Frizzled 4, one of the Wnt receptor, is over-expressed in astrocytes during the acute phase of the disease, and in neurons and oligodendrocytes in a late phase (Gonzalez-Fernandez C *et al.*, 2014). Frizzled and Ryk receptors are altered during SCI (Gonzalez P *et al.*, 2012);

furthermore, increased expression of some components such as Dvl-3, the Wnt co-receptor, LRP6, and β-catenin protein was observed.

Wnt pathway is also involved in neuronal repair through axonal regeneration, in fact induction of Ryk receptor, Wnt1, Wnt5a and Wnt4 was observed (Lambert C *et al.*, 2016).

AIMS

The aims of this study were to investigate PDRN mode of action on adenosine receptors and its anti-inflammatory and neural protective effects in an experimental model of SCI.

Moreover, since adenosine receptors stimulation may also activate Wnt pathway, we wanted to study PDRN effects on Wnt signalling during spinal cord injury.

EXPERIMENTAL PROTOCOLS

Animals

Adult male C57BL6/J mice (25–30 g; Charles River, Calco, Italy) were used in this study. Animals were housed in plastic cages, maintained under controlled environmental conditions (12 h light–dark cycle, 24°C), and provided with standard food and water *ad libitum* in the Animal Facility of the Department of Clinical and Experimental Medicine of the University of Messina.

All experiments were carried out according to the standards for care and use of animals as stated in the Directive 2010/63/EU, and the ARRIVE guidelines (Kilkenny C *et al.,* 2010).

Animals were randomly assigned to the following groups: Sham (n = 7), SCI (n = 7), SCI + PDRN (8 mg/kg/i.p.; n = 7).

Experimental Spinal Cord Injury

Mice were anesthetized with ketamine/xylazine at the dose of 80 and 10 mg/kg/ip respectively. The experimental model of SCI was firstly described by Rivlin and Tator in the "clip compression model" (Rivlin AS and Tator CH, 1978). SCI was induced by extradural compression of a section of the spinal cord exposed through a four-level T5-T8 laminectomy and the prominent spinal process of T-5 was used as a surgical guide. An aneurysm clip was extradurally applied with a closing force of 24 g at T5-T8 level. Compression time of spinal cord was approximately 60 seconds. Sham-injured animals were subjected only to laminectomy. Following surgical

procedure, saline solution was administered to replace the blood volume. Sham-injured mice were only subjected to surgical procedure but aneurysm clip was not applied.

Spinal cord tissues were collected 24 hours after trauma.

Locomotor performance

Locomotor performance was analyzed using the Basso mouse scale (BMS) open-field score (Basso DM *et al.*, 1995). The test was performed to assess the degree of motor dysfunction following SCI. All mice were allowed to walk in an open field box before SCI induction, to accustom animals to the new space. Mice were tested before injury and each day following SCI (for 4 minutes by two blinded observers), for 10 days.

Scores were assigned on a scale of 0–9: 0, completely paralyzed; 1–2, slight (<90°) to extensive (>90°) ankle movement; 3–4, ranges from occasional to frequent dorsal stepping to occasional plantar stepping; 5, frequent to consistent plantar stepping and absence of or some coordination with rotated paw placement; 6–7, frequent to consistent stepping with some to mostly coordinated stepping with paw rotation and severe trunk instability; 8, almost normal stepping with points taken off for trunk instability and whether the tail was up or down; 9, normal coordination, paw placement and trunk stability.

Quantification of circulating IL-1β and TNF–α

Blood was collected from animals 24 hours following SCI to evaluate IL-1β and TNF–α levels in serum, using specific ELISA kits (Cusabio, College Park, MD, United States). Samples were run in duplicate, and the absorbance was read at 450 nm; the results were compared to the standard curves and expressed in pg/mL.

Measurement of Myeloperoxidase activity

Myeloperoxidase (MPO), a marker of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane KM *et al.*, 1985). Equal amounts of spinal cord tissue were homogenized mechanically with the MICCRA D-1 homogenizer (Miccra Gmbh, Müllheim, Germany), in a solution containing 0.5% hexa-decyl-trimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0). Lysates were then centrifuged for 30 minutes at 15000 rpm at 4°C. An aliquot of the supernatant was allowed to react with a solution of 1.6 mM tetra-methyl-benzidine and 0.1 mM H_2O_2. The absorbance was measured with a spectrophotometer at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1μmol hydrogen peroxide/min at 37° and was expressed in units per g of tissue.

Histology

Spinal cord tissues were fixed in 10% buffered formalin, embedded in paraffin, cut in longitudinal sections (5 μm) and stained with hematoxylin and eosin to observe morphologic changes.

Damaged neurons were counted, and the histopathology changes of the gray matter were scored on a 6-point scale (Sirin BH *et al.*, 2002): 0, no lesion observed; 1, gray matter contained 1 to 5 eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction (one third to one half of the gray matter area); and 6, large infarction (more than half of the gray matter area). An average of the scores for each group was recorded to give a cumulative score. At least three slides for each animal were examined by a blinded pathologist.

Immunofluorescence

Consecutive sections of spinal cord tissues, prepared for histological analysis, were deparaffinised twice in xylene (5 min each), hydrated twice in 100% ethanol (3 min), 95% ethanol (1 min), 90% ethanol (1 min) and 80% ethanol (1 min), and rinsed in distilled water. To retrieve the antigen sections were immersed in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) at 750W in microwave 3 minutes for 3 times. Then, they were immersed in phosphate buffered saline, 0.2 mol/L, pH 7.6 with 0.9% NaCl (PBS) for 20 minutes at room temperature. Sections were

pre-incubated with 1% bovine serum albumin, 0.3% Triton X-100 in PBS, for 20 min, at room temperature. Sections were incubated with mouse monoclonal anti SMI-32 RT antibody (1:1000 dilution; Covance, Eteryville, CA, USA) for 1 hour.

Primary antibody was detected using Texas Red-conjugated IgG anti mouse (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour in the dark, at room temperature. To label nuclei, after incubation with fluorochrome, samples were incubated with DAPI (1:1000 dilution; Sigma Chemicals, St Louis, MO, USA) for 10 min in the dark, at room temperature. Sections were finally washed in PBS and sealed with mounting medium.

Samples were observed with a Zeiss LSM 510 confocal microscope equipped with Argon laser (458 nm and 488 nm λ) and two HeNe laser (543 nm and 633 nm λ). All images were digitized at a resolution of 8 bits into an array of 2048 x 2048 pixels. Optical sections of fluorescence specimens were obtained at 488 nm λ, at 62/s scanning speed with up to 8 repetitions on average. The pinhole size was set for optimal resolution. Contrast and brightness were established by examining the most brightly labelled pixels and choosing settings that allowed clear visualization of structural details while keeping the highest pixel intensities near 200.

PCR Assays

Total RNA was extracted from spinal cord tissue 24 hours following SCI using Trizol LS reagent (Invitrogen, Carlsbad, CA, United States),

quantified with a spectrophotometer (NanoDrop Lite, Thermo Fisher) and 2.5 μg was reverse transcribed using the Superscript VILO kit (Invitrogen) in a volume of 20 μl. The qPCR analysis was performed as follow: 1 μl of cDNA was added to the EvaGreen qPCR Master Mix (Biotium Inc., Fremont, CA, United States), in a total volume of 20 μl per well. Samples were loaded in duplicate, GADPH was used as housekeeping gene; the reaction was performed using the 2-step thermal protocol suggested by the manufacturer.

We tested three different primer concentrations (100, 300, and 900 nM) and 300 nM was selected and used to perform the analysis. Target genes were BAX, BCL2, Wnt3a, DKK-1 and β-catenin.

Primers used for both target and reference genes are listed below:

Gadph

Fw:5′-GTCAAGGCTGAGAATGGGAA3′

Rv:5′ATACTCAGCACCAGCATCAC3′;

Wnt3a

Fw:5′TTCTTACTTGAGGGCGGAGA3′

Rv:5′CTGTCGGGTCAAGAGAGGAG3′

β-catenin

Fw:5′CGGCACCTTCCTATTTCTTCT3′

Rv:5′TCTGGAAATTAACTTCAGGCAAAC3′

BAX

Fw:5′CGAGCTGATCAGAACCATCA3′

Rv:5′CTCAGCCCATCTTCTTCCAG3′

BCL2

Fw:5′ATACCTGGGCCACAAGTGAG3′

Rv:5′TGATTTGACCATTTGCCTGA3′

DKK-1

Fw:5′AATCGAGGAAGGCATCATTG3′

Rv:5′GCTTGGTGCATACCTGACCT3′;

Results were calculated using the $2^{-\Delta\Delta C}{}_t$ method, and expressed as *n*-fold increase in gene expression using the CTRL group as calibrator.

PDRN mode of action: further in vitro study

Preliminary data on a glucocorticoid-induced osteoporosis (GIO) experimental model demonstrated that PDRN (8 mg/kg) markedly improved bone formation by the activation of both canonical and non-canonical Wnt pathways through the activation of Wnt10b and Wnt5a (Pizzino G *et al*., 2017).

Considering these previous observations, it was investigated whether adenosine receptor stimulation might activate β-catenin translocation using adenosine receptors agonists and antagonists. To characterize PDRN effects on β-catenin translocation human dermal fibroblasts (NHDFs) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. NHDF cells were stimulated with the A_{2A} and the A_{2B} well known receptor-selective agonists CGS21680 and BAY60-6583 (1 μM each). NHDFs were also treated with PDRN at the dose 1 μM. Two sets of cells treated with PDRN were also treated with the A_{2A} and the A_{2B} receptor-selective antagonists SCH58261 and PSB (1 μM each). SCH58261 was added to the culture medium 30 min before PDRN.

Immunofluorescence on fibroblasts

Cells were plated in 8-well chamber slides and, after the appropriate treatments, were washed with cold PBS and fixed with cold methanol (10 min). Cells were permeabilized using a solution of PBS containing Triton 0.25% for 10 min. After 3 washes for 5 min each, a blocking solution (FBS 5%, BSA 1% in PBST) was added to the cells for 1 h. Cells were

incubated with primary antibody against phospho- β-catenin (Ser552) (Cell Signalling Technology; Danvers, MA, USA) overnight. Cells were washed 3 times for 5 min each with PBS and incubated with the secondary antibody FITC conjugate (1:200 in PBST) for 1 h. After 3 washes of 5 min each, a cover slide was applied to the slide with a mounting media containing DAPI to stain nuclei. Immunfluorescence was revealed by the Nikon Eclipse Ni fluorescence compound microscope.

Statistical Analysis

All data are expressed as means ± SD. Comparisons between different treatments were analyzed by one-way ANOVA for non-parametric variables with Tukey's post-test for intergroup comparisons. The possibility of error was set at $p<0.05$ and it was considered statistically significant. Graphs were drawn using GraphPad Prism (version 5.0 for Windows).

RESULTS

PDRN promoted recovery of motor function

Motor function was evaluated with the Basso Mouse Scale and was significantly reduced in all SCI animals, as a consequence of the loss of hindlimb movement. PDRN treatment resulted in a significant improvement in motor function 10 days following SCI, compared to SCI group (Figure 1).

Anti-inflammatory effects of PDRN on cytokines release

IL-1β and TNF-α levels were increased in serum of animals 24 h following SCI compared to Sham mice, which showed almost undetectable levels of these cytokines. PDRN administration markedly decreased both IL-1β and TNF-α levels in SCI-treated animals (Figure 2 A and B) ($p<0.05$), thus confirming PDRN anti-inflammatory property.

Effects of PDRN on neutrophil infiltration

The severity of spinal cord injury is related to leukocytes influx into the spinal cord. Therefore, PDRN effect was also evaluated on neutrophil infiltration by measuring tissue MPO activity.

MPO activity was increased in SCI group. PDRN administration significantly reduced both lipid peroxidation and polymorphonuclear granulocytes heap (Figure 2 C) ($p<0.05$), confirming the protective effects of PDRN on MPO activity also during spinal cord injury.

PDRN reduced the severity of secondary spinal cord trauma

SCI group showed a widespread damage both in the site of injury and in the perilesional area, as confirmed by the presence of oedema and inflammatory features, compared to Sham mice (Figures 3 A and B). Histological results in terms of inflammation confirm the results of MPO activity, which indicated granulocyte infiltration. Moreover, spinal cord of SCI animals presented signs of neuronal suffering (vacuolization) with pyroptotic areas and axon degeneration.

Treatment with PDRN reduced oedema, granulocyte infiltration, and preserved neurons from cell death (Figure 3 C), suggesting that the treatment might be protective decreasing both inflammatory and cell death processes, at least 24 h following injury (Figure 3D).

PDRN preserved neuron structure

Sham animals showed a normal microscopic structure of the spinal cord with several neurons in the microscopic field which are connected to each other by long axonal and dendritic projection; neurons showed a well defined soma, long and thin axons providing long distance connections with other neurons as evidenced by SMI (Anti-Neurofilament H Mouse) antibody (red channel; Figures 4 A and D).

SCI-group showed the absence of SMI staining pattern (red channel) along the soma and along the axonal and dendritic projections in the perilesional area of the spinal cord. Several fragments of interrupted axonal and

dendritic projection were observed in high magnification images and no neuronal soma in the microscopic field was detectable (Figures 3 B and E).

SCI group treated with PDRN showed few neuronal somas positive for SMI (red channel) in the perilesional area of spinal cord but fragments of interrupted axonal and dendritic projections were still detectable. PDRN preserved neuron structure, which showed a conserved soma (as evidenced by SMI red fluorescence) with axons shortly interrupted from their origin. No dendritic projections were detectable (Figures 3 C and F).

SCI induced apoptosis and PDRN preserved neuronal death

SCI promoted cell death by apoptosis in addition to inflammation. SCI animals showed increased apoptotic rate compared to Sham mice. Treatment with PDRN significantly reduced the extent of apoptosis in the spinal tissue (Figure 5).

In fact, the expression of the pro-apoptotic BAX protein was markedly increased following SCI, reducing the number of neurons (Figure 5 A). By contrast, a significant decrease of Bcl-2 anti-apoptotic protein expression was observed in the spinal cord tissue 24 h following SCI. PDRN administration preserved the number of neurons, in fact BAX expression was reduced, whereas Bcl-2 expression was increased following treatment with PDRN (Figures 5 A and B).

PDRN modulated Wnt signalling

Wnt3a mRNA expression and consequently β-catenin mRNA expression was dropped in SCI animals compared to Sham group, whereas treatment with PDRN significantly increased mRNA expression of both Wnt3a and β-catenin (Figures 6 A and B).

In contrast, DKK-1, which is considered one of the most important Wnt inhibitors, demonstrated an increased mRNA expression in SCI animals compared to Sham mice. PDRN reduced DKK-1 mRNA expression in spinal cords of treated animals, confirming its effect on Wnt pathway activation (Figure 6 C).

These results revealed that Wnt signaling is activated by adenosine receptor stimulation, confirming the hypothesis that PDRN may preserve neurons following spinal cord injury and perhaps might also stimulate both neurogenesis and neuronal repair through axonal regeneration, as demonstrated by immunofluorescence staining.

PDRN promoted β-catenin translocation in HDF

To confirm PDRN mode of action on Wnt signalling, β-catenin translocation was evaluated in human dermal fibroblasts (NHDFs).

Nuclear translocation of β-catenin (green channel) was observed by fluorescence microscopy following PDRN incubation for 10 minutes (Figures 7 F and L). Nuclear translocation was also observed by pretreatment and co-treatment with the A_{2A} and A_{2B} receptor-selective antagonists SCH58261 and PSB (Figures 7 G-M-H-N).

The increase of β-catenin levels in the nuclear fraction is consistent with the translocation of β-catenin from the cytosol to the nucleus following both A_{2A} and A_{2B} receptor activation. These results show that adenosine receptor activation not only may induce β-catenin nuclear translocation, additionally it was demonstrated, for the first time, that PDRN may activate both A2A and A2B receptors.

DISCUSSION

PDRN systemically administered 1 hour following spinal cord injury, protected from tissue damage, demyelination, and reduced motor deficits in addition to the release of the pro-inflammatory cytokines TNF-α and IL-1β. These results demonstrated that the use of systemically administered adenosine receptor agonists might have protective effects following SCI. In fact, adenosine receptors are located in astrocytes, microglial cells, myelinated fibers, and neurons and their expression increases in injured spinal cord. One of the first consequences following spinal cord injury is functional impairment characterized by loss of motor and/or sensory function in the upper and lower limbs and often also in the trunk (Kirshblum SC *et al.*, 2011). A_{2A} agonists administration may be effective in protecting motor deficits; in fact, mice lacking A_{2A} receptors on bone marrow-derived cells (BMDCs) show motor dysfunctions and recover the function following reconstitution with A_{2A} receptor and systemic administration with A_{2A} agonists. Moreover, A_{2A} receptor stimulation is used as anti-inflammatory approach in several diseases. The use of PDRN played an effective anti-inflammatory response in various experimental models, demonstrating that adenosine receptors, both A_{2A} and A_{2B} receptors, might be considered as important targets for anti-inflammatory action. Consistent with this point of view, PDRN has been effective following spinal cord injury, reducing pro-inflammatory cytokines and ameliorating spinal cord structure. Twenty-four hours after SCI, clear signs of cell suffering were present, with fragmented and interrupted axonal and dendritic projection. PDRN preserved cell

structure and reduced apoptotic rate, as demonstrated by the decreased expression of the pro-apoptotic protein, in favour of the anti-apoptotic one. However, we have hypothesized that PDRN not only might preserve cell death but also might stimulate neurogenesis. Wnt signalling, involved in developmental processes, may also regulate neurogenesis and regeneration in the nervous system (David MD *et al.*, 2010). In particular, within Wnt family, Wnt-3a controls spinal cord dorsal interneuron specification and regulates neurogenesis (Murashov AK *et al.*, 2005). Moreover, Wnt-3a displays mitogenic activities, promoting spinal cord growth (Megason SG and McMahon AP, 2002). Some studies showed the possible crosstalk between adenosine receptor activation and Wnt signalling, in fact, A_{2A} receptor blockade reduced Wnt signalling in a murine model of bleomycin-induced fibrosis (Zhang J *et al.*, 2017), whereas A_{2A} receptor activation, by using an agonist, stimulated Wnt pathway (Pizzino G *et al.*, 2017). So, the question was whether PDRN might also activate Wnt/β-catenin following adenosine receptor activation, treating experimental SCI. PDRN increased both Wnt3a and β-catenin mRNA expression, whereas DKK-1 mRNA expression, as expected, was reduced in PDRN treated group 24 h following SCI. These results let us hypothesize that PDRN might have a role not only reducing apoptosis but also promoting neurogenesis. To characterize PDRN mode of action, β-catenin translocation was studied in human dermal fibroblast following PDRN stimulation together with A_{2A} and A_{2B} receptor antagonists. We showed, for the first time, that PDRN may act both through A_{2A} and A_{2B} receptors stimulation, also activating Wnt/β-catenin signalling.

In conclusion, this study describes the protective effect of PDRN following SCI: PDRN treatment significantly reduced the SCI-induced spinal cord tissue alteration, also improving motor function. The results of the present study enhance our understanding of PDRN mode of action, confirming PDRN anti-inflammatory activity and demonstrating that a crosstalk between Wnt/β-catenin signalling is possible by activation of both A_{2A} and A_{2B} receptors. Moreover, these data let us hypothesize that PDRN might promote neural repair through axonal regeneration and/or neurogenesis. However, further studies are required to elucidate PDRN mechanism of action in neurological disorders.

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FIGURE LEGEND

Figure 1. Recovery of motor function after SCI in mice. The degree of motor impairment was assessed in a blinded manner on each day of the 10-d study, using the BMS. Scores range from 0 (complete paralysis) to 9 (normal movement of the hindlimbs). Scores were assigned, also in a blinded manner, for each of the hindlimbs, and the 2 scores were then averaged for each mouse. Daily treatment with PDRN significantly enhanced hindlimb recovery following SCI, compared to SCI animals. Data are means ± SD of 7 mice per group. *p<0.05 versus Sham, #P<0.05 versus SCI.

Figure 2. IL-1β and TNF-α levels were significantly reduced by PDRN treatment compared to untreated SCI animals (A and B). PDRN administration also decreased MPO activity compared to SCI mice (C). Each bar represents the mean ± DS of 7 mice per group. *p<0.05 versus Sham, #P<0.05 versus SCI.

Figure 3. Hematoxylin-eosin staining of spinal cord tissue from Sham (A), SCI (B) and SCI+PDRN (C) animals 24 hours following SCI. Blinded histologic scoring (F) performed on a 6-point scale. Each bar represents the mean ± DS of 7 mice per group. *p<0.05 versus Sham, #P<0.05 versus SCI.

Figure 4. Immunofluorescence staining for the detection of SMI at low magnification (20x) of Sham (A), SCI (B) and SCI+PDRN (C) groups. Immunofluorescence staining for the detection of SMI at high magnification (63x) of Sham (D), SCI (E) and SCI+PDRN (F) groups. Red channel represents SMI staining pattern.

Figure 5. BAX (A) and Bcl-2 (B) mRNA expression in spinal cord tissues 24 hours following SCI. Each bar represents the mean ± DS of 7 mice per group. *p<0.05 versus Sham, #P<0.05 versus SCI.

Figure 6. Wnt3a (A), β-catenin (B) and DKK-1 (C) mRNA expression in spinal cord tissues 24 hours following SCI. Each bar represents the mean ± DS of 7 mice per group. *p<0.05 versus Sham, #P<0.05 versus SCI.

Figure 7. Immunofluorescence staining for the detection of β-catenin translocation (green channel) in human dermal fibroblasts not treated (Ctrl, A-E-I) and treated with i) PDRN (B-F-L), ii) PDRN+SCH58261 (C-G-M), iii) PDRN+PSB (D-H-N).

Figures

Figure 1

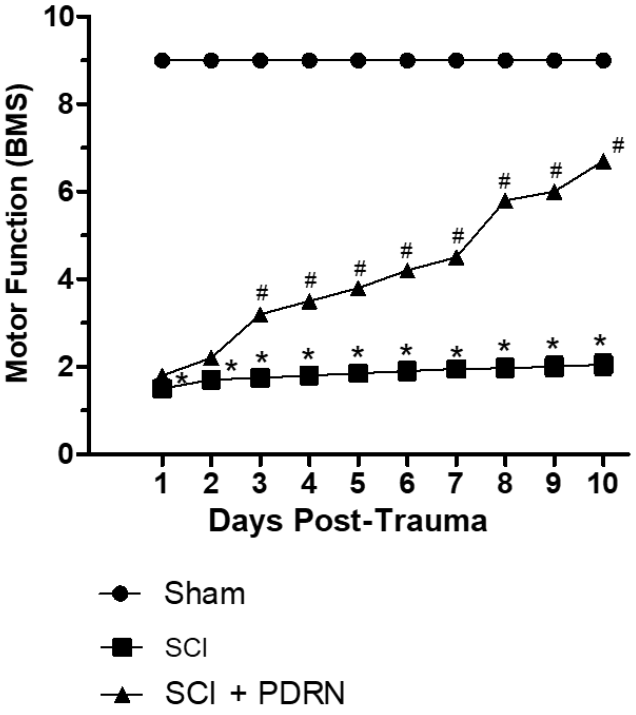


Figure 2

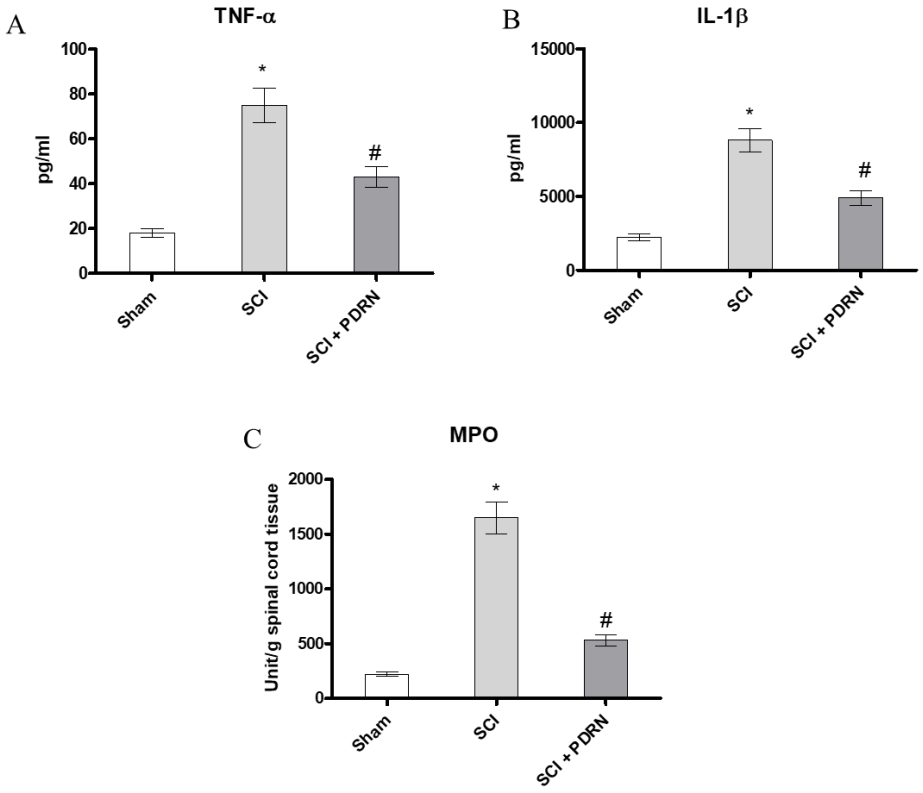

A
TNF-α
pg/ml
100
80
60
40
20
0
*
#
Sham
SCI
SCI + PDRN
B
IL-1β
pg/ml
15000
10000
5000
0
*
#
Sham
SCI
SCI + PDRN
C
MPO
Unit/g spinal cord tissue
2000
1500
1000
500
0
*
#
Sham
SCI
SCI + PDRN

Figure 3

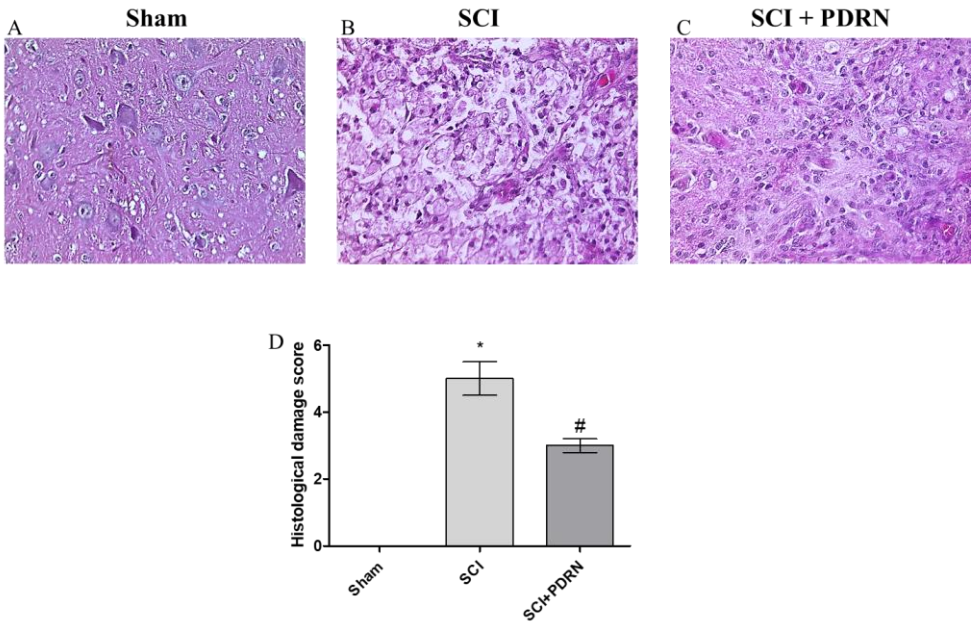

A
Sham
B
SCI
C
SCI + PDRN
D
Histological damage score
0
2
4
6
*
#
Sham
SCI
SCI+PDRN

Figure 4

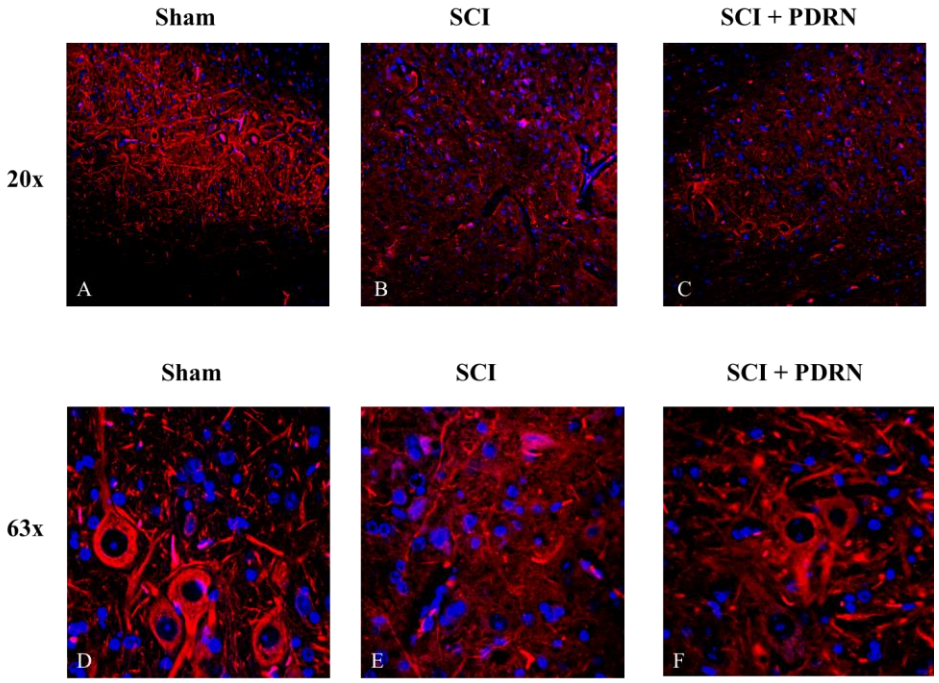


Figure 5

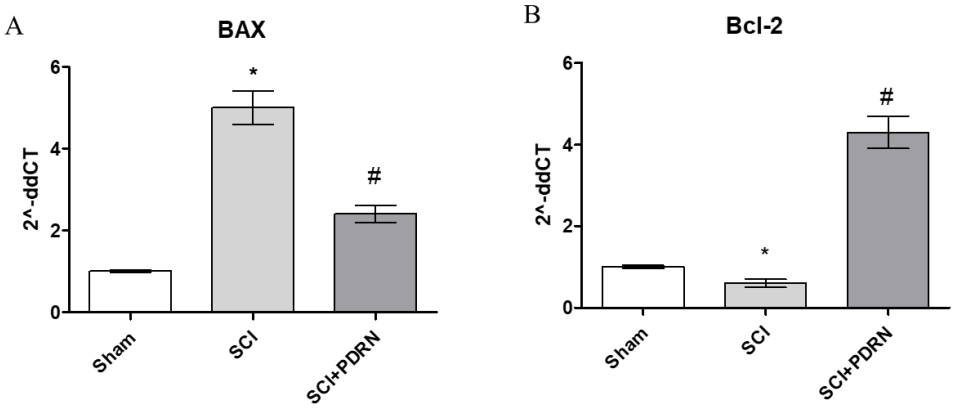

A
BAX
2^-ddCT
6
4
2
0
*
#
Sham
SCI
SCI+PDRN
B
Bcl-2
2^-ddCT
6
4
2
0
*
#
Sham
SCI
SCI+PDRN

Figure 6

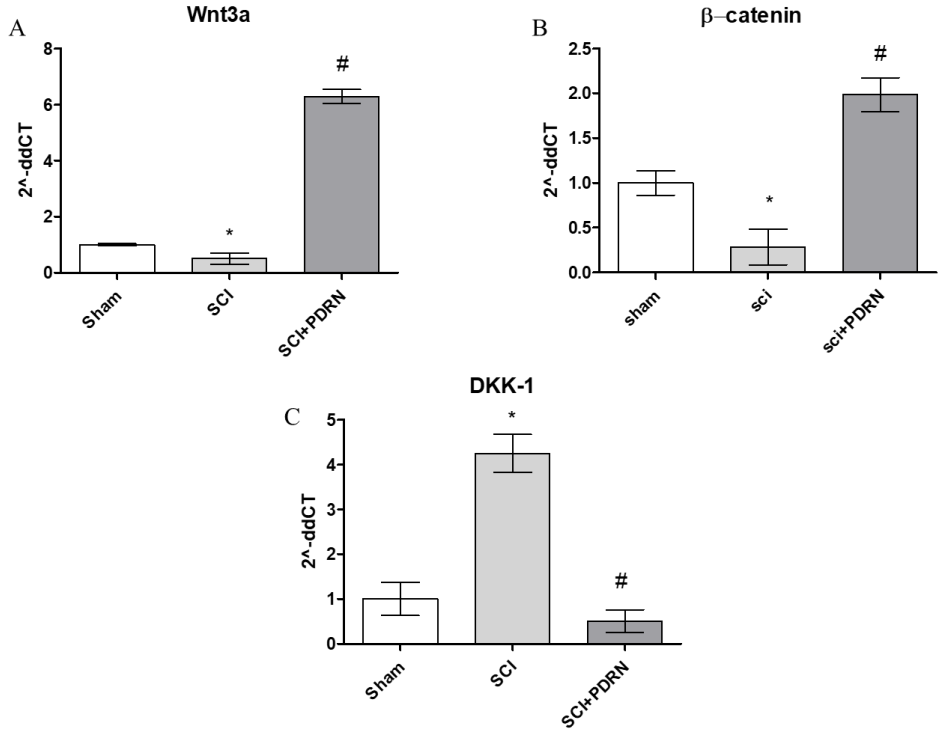
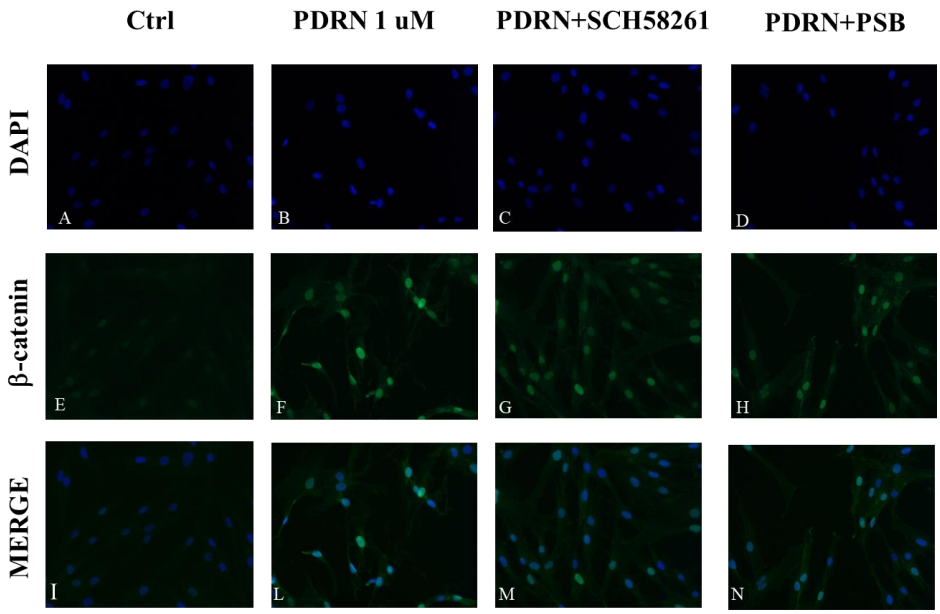

A
Wnt3a
2^-ddCT
8
6
4
2
0
*
#
Sham
SCI
SCI+PDRN
B
β–catenin
2^-ddCT
2.5
2.0
1.5
1.0
0.5
0.0
*
#
sham
sci
sci+PDRN
C
DKK-1
2^-ddCT
5
4
3
2
1
0
*
#
Sham
SCI
SCI+PDRN

Figure 7


Ctrl
PDRN 1 uM
PDRN+SCH58261
PDRN+PSB
DAPI
β-catenin
MERGE
A
B
C
D
E
F
G
H
I
L
M
N