Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury

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Spinal cord injury (SCI) causes delayed secondary biochemical alterations that lead to tissue loss and associated neurological dysfunction. Up-regulation of cell cycle proteins occurs in both neurons and glia after SCI and may contribute to these changes. The present study examined the role of cell cycle activation on secondary injury after severe SCI in rat. SCI caused cell cycle protein up-regulation associated with neuronal and oligodendroglial apoptosis, glial scar formation and microglial activation. Treatment with the cell cycle inhibitor flavopiridol reduced cell cycle protein induction and significantly improved functional recovery versus vehicle-treated controls at 21 and 28 days post-injury. Treatment also significantly reduced lesion volume, as measured by MRI and histology, decreased astrocytic reactivity, attenuated neuronal and oligodendroglial apoptosis and reduced the production of factors associated with microglial activation. Thus, flavopiridol treatment improves outcome after SCI by inhibiting cell cycle pathways, resulting in beneficial multifactorial actions on neurons and glia.

Keywords: apoptosis; astrocyte; inflammation; microglia; neuron

Abbreviations: CDKIs = cyclin-dependent kinase inhibitors; CDKs = cyclin-activated kinases; MRI = magnetic resonance imaging; PRb = phosphorylated retinoblastoma protein; SCI = spinal cord injury

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Introduction

Spinal cord injury (SCI) causes neuronal cell death, astrocyte proliferation/scar formation and inflammation associated with microglial activation (Tator, 1996; Dumont et al., 2001). Up-regulation of cell cycle proteins occurs after central nervous system (CNS) trauma (Di Giovanni et al., 2003), and is associated with apoptotic cell death of post-mitotic cells, such as neurons, and proliferation of astrocytes and microglia (Becker and Bonni, 2004). Cell cycle proteins are normally down-regulated in post-mitotic neurons (Okano et al., 1993) and re-entry into the cell cycle can cause apoptosis in such cells (Nguyen et al., 2002; Becker and Bonni, 2004). Up-regulation of cell cycle proteins is correlated with neuronal apoptosis after experimental SCI (Di Giovanni et al., 2003) and brain injury (Natale et al., 2003). Cyclins (Sherr, 1993), cyclin-activated kinases (CDKs) (Nishitani and Lygerou, 2002) and cyclin-dependent kinase inhibitors (CDKIs) (Benton and Whittemore, 2003) control progression through the cell cycle (Boonstra, 2003). In response to mitogenic stimulation, CDK4 and CDK6 are activated and translocated to the nucleus, which prompts the entrance into G1 phase (Sherr, 1995). In the nucleus, CDK4/6 phosphorylates the retinoblastoma protein (Rb), which results in the dissociation of phosphorylated Rb (pRb) from the Rb/E2F complex and the activation of the E2F transcription factor (Kitagawa et al., 1996; Sears and Nevins, 2002). E2F appears to induce apoptosis by increasing expression of key components of the caspase pathway (Nahle et al., 2002) and by inducing p53 and p73 (Sears and Nevins, 2002; Greene et al., 2004). p53 and p73 lead to activation of pro-apoptotic Bcl-2 family members, resulting in release of mitochondrial pro-apoptotic proteins such as cytochrome c, a critical component of the intrinsic caspase pathway (Nahle et al., 2002; Nguyen et al., 2003). Traumatic brain injury also results in cell cycle protein expression in astrocytes and microglia (Natale et al., 2003). The former is associated with glial scarring (Hoke and Silver, 1994). The latter participate in phagocytosis, proliferation and production of pro-inflammatory molecules such as interleukin (IL) 1β, IL6, inducible nitric oxide synthase, and reactive oxygen species (Raivich et al., 1999; Lynch et al., 2004), among others.
Administration of cell cycle inhibitors, such as flavopiridol, roscovitine or olomoucine, can provide neuroprotection in various *in vitro* models, such as etoposide, kainic acid, or colchicine induced injury (Jorda et al., 2003; Cernak et al., 2005; Di Giovanni et al., 2005). Flavopiridol also blocks astrocyte and microglial proliferation *in vitro* (Cernak et al., 2005; Di Giovanni et al., 2005). The present study examined the effects of continuous intrathecal flavopiridol administration on behavioural recovery and lesion size from severe acute experimental spinal cord contusion injury. Outcomes also included evaluation of cell cycle activation, neuronal and oligodendroglial cell death, activation and proliferation of astrocytes and microglia, as well as release of microglial associated inflammatory factors.

Materials and Methods

**Spinal cord injury**

Contusion SCI was performed in adult male Sprague Dawley rats as previously described (Yakovlev and Faden, 1994). Briefly, rats (275–325 g) were anaesthetized with sodium pentobarbital (67 mg/kg, I.P.) and injury was induced using a 10 g weight dropped from 50 mm (25 mm for pilot studies) onto an impounder positioned on the exposed spinal cord at vertebral level T9 to produce a severe injury (25 mm drop resulted in a moderate injury). Sham-injured animals received a laminectomy without weight drop. All experiments complied fully with the principles set forth in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council [DHEW pub. No. (NIH) 85-23, 2985], and were approved by the Georgetown University IACUC.

**Flavopiridol administration**

Thirty minutes after contusion injury, rats received an intrathecal injection 2 segments below the injury site (spinal T11) using a 10 µl Hamilton syringe with a 32 G bevelled needle. Five microlitres of flavopiridol (250 µM in vehicle, representing a total of 1.25 pmol, Aventis) or vehicle (1% DMSO in 0.9% saline) was injected into the intrathecal space. A polyethylene catheter (P-100, 1.52 outside diameter) attached to an Alzet mini-osmotic pump (Alzet, Cupertino, CA; Model 2001) was inserted into the intrathecal space immediately following the injection and advanced toward the lesion site, resting 1–2 mm below the lesion site, as described previously (Iannotti et al., 2004). The mini-osmotic pump was loaded with either flavopiridol (100 µM in 1% DMSO in saline) or vehicle (1% DMSO in 0.9% saline) and administered 1 µl of drug (representing 0.1 pmol/day) or vehicle per hour for 7 days.

**Functional assessment**

The Basso–Beattie–Bresnahan (BBB) scale, toe spread, righting reflex, withdrawal reflex (to extension) and contact placing test were used to assess neurological function, as previously detailed (Kerasidis et al., 1987; Faden and Halt, 1992; Basso et al., 1996). A second method of assessing function, the combined behavioural score (CBS; Kerasidis et al., 1987), was modified by removing tasks that animals were unable to perform, such as inclined plane task, and utilized as described. All functional scores were obtained at Days 1, 7, 14, 21 and 28 by two individuals blinded to treatment. The CBS is presented as a percentage of sham performance on the different tasks.

**MRI analysis**

At 28 days post-injury, rats underwent magnetic resonance imaging (MRI) using a 7 Tesla 20-cm bore MRI (Bruker Biospin Billerica, MA). Rats in the pilot study underwent a 3D imaging protocol (Pirko et al., 2005), in which rats received an injection of gadolinium (Omniscan, Amersham Health, Waukesha, WI; 1.7 mmol/kg, 1 ml I.V.). Parameters for the 3D TI weighted RARE sequence were field of view 90 mm × 35 mm × 35 mm; TR = 200 ms, TE = 5.9 ms, MTX = 256 × 256 × 256 and Rare Factor = 4. For the subsequent study, severely injured rats underwent a 2D T2 weighted imaging protocol, in which the rats (n = 10/group) received no gadolinium contrast agent and the field of view was 90 × 90 mm. The TR = 3640 ms, TE = 121 ms, MTX = 256 × 256. Hyperintense areas on MRI images were assessed using Image J analysis software.

**Histology**

The day after T2 weighted MRI imaging for the 28 day study (n = 10/group), or 24 h, 3 days or 7 days (n = 3/group) after injury for the short-term studies, rats were anaesthetized (100 mg/kg sodium pentobarbital, I.P.) and intracardially perfused with 100 ml of 0.9% saline followed by 300 ml of 10% buffered formalin. A 1-cm section of the spinal cord centred at the lesion epicentre, T-9, was dissected, post-fixed in 10% buffered formalin overnight and cryoprotected in 30% sucrose for 48 h.

Lesion volume was assessed based on the Cavaliere method of stereology, as described previously (Iannotti et al., 2004), using every fifth spinal cord section from 5 mm rostral to 5 mm caudal the lesion site, which were stained with cresyl violet and photographed (AxioPlan Zeiss Microscopy system; Carl Zeiss, Inc., Thornwood, NY).

**Immunohistochemistry**

Standard fluorescent immunohistochemistry was performed on sections, obtained as described above. Immunohistochemistry was performed at 24 h, 72 h, 7 days or 28 days; time points were chosen to most closely match the reported peaks of caspase-3, cell cycle protein, or inflammatory-related gene and protein expression (Di Giovanni et al., 2003; Byrnes et al., 2006). Antibodies included ED1 (Serotec, Raleigh, NC), GFAP (DAKO, Carpinteria, CA), NeuN (Chemicon, Temecula, CA), MAP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved-Caspase 3 (Cell Signaling, Danvers, MA), APC (Calbiochem, San Diego, CA), Cyclin G1 (Santa Cruz Biotechnology), Cyclin D1 (Labvision, Fremont CA), p22phox (Santa Cruz Biotechnology) p-Pro (Abcam, Cambridge, MA), and osteopontin (Santa Cruz Biotechnology). Appropriate secondary antibodies linked to FITC or Cy3 fluorophores (Jackson Immunoresearch, West Grove, PA) were incubated with tissue sections for 1 h at room temperature. Slides were coverslipped using mounting media with DAPI or TO-PRO-3, which bind to and label DNA (Vector Labs, Burlingame, CA; Invitrogen, Carlsbad, CA). Negative controls were produced by incubation with non-immune sera rather than primary antibody.
To examine apoptotic DNA degradation using the TUNEL assay we employed the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (#S7110, Chemicon International). The TUNEL assay was performed between the wash step after primary antibody and the incubation step with the secondary antibodies, following the manufacturer’s instructions.

Immunofluorescence was detected using an AxioPlan Zeiss Microscopy system (Carl Zeiss, Inc., Thornwood, NY) or confocal microscopy. Confocal fluorescence microscopy imaging was performed using Zeiss LSM 510 Meta confocal laser scanning microscope. Visualization of the fluorophores was achieved using the 488 nm argon laser, a 543 nm helium/neon laser and a 633 nm helium/neon laser. The Multi Track protocol of LSM 510 META (all lines are scanned sequentially with each of the lasers) was used to prevent the ‘cross-talk’ associated with simultaneous excitation with all three lasers. The configuration parameters were as follows: (i) Filters: Ch3-BP 560-615, Ch2-BP 505-530, ChS1 659-702; (ii) Beam Splitters: MBS-HFT UV/488/543/633, DBS1-NFT 635 VIS, DBS2-NFT 545, DBS3-plate, FW1-none; (iii) scan zoom 1; (iv) frame size 1024 × 1024 pixels. Confocal gain remained consistent for analysis of all antibodies of interest.

Quantitation of immunolabelling

For ED1, GFAP and p22phox immunolabelling, the proportional area of tissue occupied by immunohistochemically stained cellular profiles within a defined target area (the lesion site and surrounding tissue) was measured using the Scion Image Analysis system using a method modified from that described by Popovich et al. (1997).

For quantitation of cleaved caspase-3 or cyclin G1 positive cells, immunolabelling was performed with an immunoenzymatic detection (Vector Laboratories ABC Kit, Vector Laboratories), rather than immunofluorescence, according to the manufacturer’s instructions. DAB and/or NovaRed were used as enzyme substrates, and tissue was counterstained with hematoxylin. Total immunolabelled cell counts were obtained using unbiased stereology and the Stereologer Program (Systems Planning and Analysis, Alexandria, VA). For unbiased stereology, it was essential that the entire 10 mm of tissue surrounding the lesion site, beginning at a random starting point (chosen using the random number generator on the Stereologer Software), was sampled using every 20th section, for a total of 12 sections (30 μm section thickness; average mounted thickness = 0.23 μm). The multi-level sampling design in the Stereologer software, based on the optical fractionator sampling method, was used to estimate immunolabelled cell numbers. Briefly, a 100 × 100 μm unbiased counting frame with a 40X objective was placed over digital images obtained from the Panasonic KR222 Color CCD camera attached to an Olympus BH-E microscope. Immunostained profiles that fell within the counting frame or were touching the inclusion lines were counted as the nuclei came into view while focusing through the z-axis. Settings for counting were: SSF = 0.05; ASF = 0.04; TSF = 0.429; sampling height = 15 μm, guard zone = 2 μm; average CE = 0.11.

Western blot

At 24 h post-injury, the 1 cm section of the spinal cord encompassing the lesion site was dissected from 4 rats/group. Tissue was homogenized in RIPA Buffer [1% Na deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 0.01 M Tris–HCl, pH 8.0, 0.14 M NaCl, 1 mM iodoacetamide, 1 mM AEBSF, 1 mM aprotinin], and centrifuged to isolate protein. Twenty-five micrograms of protein were run in an SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The blot was then probed with cyclin D1 (Santa Cruz, 1:1000) or pRb (Abcam, 1:1000). Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce, Rockford, IL). β-Actin was used as a control for gel loading and protein transfer. Scion Image Analysis (http://www.scioncorp.com/) was used to assess pixel density of resultant blots for comparison between groups. Data are presented as a ratio to β-actin.

Statistical analysis

Quantitative data are presented as mean ± SEM. Lesion volume and immunohistochemical data were obtained by an investigator blinded to treatment group. BBB scores were analysed with two-way ANOVA and repeated measures. All remaining data were analysed using Student’s t-test or one-way ANOVA, where appropriate. All statistical tests were performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc., San Diego, CA) and SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois). A P value < 0.05 was considered statistically significant.

Results

Flavopiridol administration attenuates lesion volume after SCI

Lesion volume assessment by MRI was performed with either T1 or T2 weighted imaging protocols. Gadolinium labelling and T1 hypointense areas, representing areas of demyelination or axon loss, has been proposed to correlate with the lesion volume (Miller et al., 1998). Gadolinium appearance within the spinal cord indicates blood-spinal cord barrier breakdown or ‘leakiness’ (Pirko et al., 2005). T2 weighted MRI hyperintense areas correspond to areas of edema, inflammation, demyelination, axon loss and astrogliosis (Hart et al., 1998).

Initial pilot studies, in which a single dose of flavopiridol was applied 30 min after injury, demonstrated a decrease in gadolinium uptake as measured by gadolinium contrast enhanced T1 MRI imaging after moderate spinal cord contusion injury (Fig. 1). The average lesion volume was 13.1 ± 0.9 mm³ after flavopiridol treatment, while volume after vehicle treatment was 19.6 ± 2.7 mm³. Modification of the original treatment protocol to include continuous infusion of flavopiridol demonstrated an even larger decrease in lesion volume, as measured by T2 weighted MRI images at 28 days post-injury after severe SCI (Fig. 1c and d). After vehicle administration, lesion volumes averaged 29.4 ± 7.8 mm³; administration of flavopiridol decreased lesion volume by 62% to 11.1 ± 0.9 mm³ (P < 0.05, one-tailed t-test, n = 7/group).
In order to confirm the MRI imaging analysis, perfused spinal cord tissue from the same animals was stained with cresyl violet; non-biased stereology was used to assess lesion volume for injured tissue with or without flavopiridol infusion for 7 days. The average lesion volume assessed for vehicle-treated spinal cord tissue was $39.7 \pm 6.9 \text{mm}^3$; flavopiridol treatment significantly reduced lesion volume ($P < 0.05$, unpaired two-tailed $t$-test, $n = 10$/group), with an average lesion volume of $21.3 \pm 3.2 \text{mm}^3$ (Fig. 2). This reduction occurred in both white and grey matter, with an overall decrease in cavity formation and tissue loss. Correlation analysis of lesion volumes between MRI and histological measurements revealed a statistically significant correlation ($P < 0.05$, Linear Regression, $n = 10$/group), with an $r^2$ value of 0.66 (Fig. 2).

MAP2 staining was also performed to analyse tissue viability after injury. MAP2 is a neuronal-specific protein whose expression is significantly decreased in degenerating neurons. MAP2 expression appeared diminished at 24 h after SCI; flavopiridol visibly attenuated this decrease (Fig. 2).

**Flavopiridol administration improves functional recovery after SCI**

Functional testing analyses were performed weekly following SCI. By 21 days post-injury, flavopiridol-treated rats had significantly greater ($P < 0.001$, Repeated Measures ANOVA followed by Tukey Post-test, $n = 10$/group) BBB scores ($8.2 \pm 0.6$) as compared to vehicle-treated animals ($6.3 \pm 0.5$; Fig. 3a). This improvement remained through the end of the study, at which point vehicle-treated rats had reached a BBB score of $6.4 \pm 0.6$, whereas flavopiridol-treated rats had reached an average score of $8.0 \pm 0.4$. Moreover, whereas only 20% of the vehicle-treated rats had reached a score of at least 8 by 28 days post-injury, 70% of the flavopiridol-treated rats had achieved 8 or higher by 14 days. This percentage increased to 80%
by 21 days post-injury, including 20% (or 2 rats) who had achieved scores of at least 10, indicating weight-supported plantar stepping. All of the flavopiridol-treated rats had achieved a score of at least 8 by 28 days post-injury.

A second functional test, a combined behavioural score (CBS) encompassing performance on tests including contact placing, hindpaw withdrawal in response to stretch, righting reflex, and toe spread, was also performed. Performance on this combined test was significantly different between the two groups by day 28 post-injury (Fig. 3b): vehicle-treated animals had achieved 29.1% ± 5 of the sham (non-injured) score, while flavopiridol treatment resulted in a significant \((P < 0.05, \text{two-way}
\)
ANOVA/Repeated Measures followed by Bonferroni Post-test, \( n = 10/\text{group} \) increase to 61.7\% ± 6.

**Flavopiridol administration decreases astrocyte reactivity and microglial-related factor expression**

Analysis of GFAP labelling—an indicator of astrocyte reactivity associated with glial scar formation—showed a clear reduction in proportional area of labelled tissue around the lesion epicentre as early as 3 days post-injury in flavopiridol-treated animals. GFAP staining remained low at 28 days post-injury in flavopiridol-treated tissue in comparison to vehicle-treated tissue (Fig. 4a and b). Quantitation demonstrated a remarkable and highly significant decrease in GFAP staining at both days 3 (\( P < 0.05 \); unpaired two-tailed \( t \)-test, \( n = 3/\text{group} \); Fig. 4d) and 28 (\( P < 0.05 \); unpaired two-tailed \( t \)-test, \( n = 10/\text{group} \); Fig. 4c) post-injury. Immunolabelling for certain factors released from activated microglia—including osteopontin and p22phox (Byrnes et al., 2006)—at 3 or 28 days post-injury demonstrated a reduction with flavopiridol treatment (Fig. 4d–h). Vehicle-treated tissue showed a high level of punctate labelling associated with large, round cells in the lesion epicentre for osteopontin (Fig. 4d). Little, if any, labelling for these factors was observed following flavopiridol treatment (Fig. 4e). Plasma membrane staining for p22phox was found in both vehicle- and flavopiridol-treated tissue (Fig. 4f and g), although there was quantitatively less staining in the flavopiridol-treated tissue at 28 days (\( P < 0.05 \); unpaired two-tailed \( t \)-test, \( n = 3/\text{group} \); Fig. 4h), suggesting an overall down-regulation in microglial activity.

Analysis of ED1, a marker of the presence of macrophages and activated microglia, using either immunohistochemistry or western blot, revealed no significant difference between vehicle- or flavopiridol-treated spinal cord tissue at either 3 or 28 days post-injury (data not shown). Overall, these data suggest that flavopiridol attenuates microglial activation, without affecting the invasion of these inflammatory cell types into the lesion site.

**Flavopiridol reduces cell cycle activation after injury**

Immunohistochemistry was performed to study the effect of SCI on the expression of cell cycle-related proteins. In sham-injured tissue, numerous NeuN positive cells were observed in both ventral and dorsal spinal horns, but few were co-labelled with either cyclin G1 or cyclin D1 (Fig. 5a, data not shown). However, following SCI, cyclin G1 expression was frequently observed in cells also labelled with NeuN at 24 h (Fig. 5a). Quantitation of cyclin G1 positive cells within the grey matter of the spinal cord in the 1 cm surrounding the lesion site demonstrated an average of 14,330 ± 2,470 cells.

Continuous infusion of flavopiridol attenuated the expression of cyclin G1, reducing the average number of cyclin G1 positive cells to 5,906 ± 1,306 (Fig. 5b). Few cells were found to be double-labelled with cyclin G1 and NeuN (Fig. 5a).

Cyclin D1 protein expression was also observed following SCI (Fig. 6). This protein expression was noted throughout the spinal cord at 72 h post-injury, most densely around the periphery of the spinal cord. This labelling was primarily found in astrocytes, which were also most dense at the spinal cord periphery, as demonstrated by GFAP and cyclin D1 double-labelling. Treatment with flavopiridol reduced the appearance of cyclin D1 expression at this time. Western blot analysis for cyclin D1 revealed that the reduction in cyclin D1 protein by flavopiridol was statistically significant (Fig. 6c).

Finally, activation of the cell cycle results in phosphorylation of Rb, ultimately resulting in activation...
Flavopiridol improves SCI recovery

Flavopiridol reduces apoptosis after SCI

To investigate flavopiridol’s effects on neuronal and oligodendroglial cell death after SCI, flavopiridol and vehicle-treated tissue were assessed at 24 or 72 h post-injury for TUNEL and cleaved-caspase-3 staining. Cell nuclei were indicated by chromatin staining with TO-PRO-3. TUNEL-labelling revealed positive cells in the vehicle-treated cord; in comparison, there was a decrease in the appearance of TUNEL-positive cells in the flavopiridol-treated cord (Fig. 8). In the vehicle-treated sample the TUNEL positive cells have nuclei manifesting highly condensed chromatin masses, a marker of Stage II caspase-dependent apoptosis (Yuste et al., 2005).

At 72 h after SCI, cleaved-caspase 3 was also increased in NeuN positive neurons, further indicating apoptosis (Fig. 9). This increase was attenuated by treatment with flavopiridol. Unbiased stereology was used to count the total number of caspase-3 positive cells within the spinal cord at 24 h after injury. In the 10 mm of tissue surrounding the lesion site, a total of 19 810 ± 2938 cleaved caspase-3 positive cells were found in the vehicle-treated spinal cord. Treatment with flavopiridol significantly reduced this number to 9199 ± 3623 (P < 0.05, unpaired two-tailed t-test, n = 3/group; Fig. 9b). A total of only 1338 ± 125 cleaved caspase-3 cells were found in sham-injured spinal cord. Quantitation of cells double-labelled with NeuN and caspase-3 using unbiased stereology revealed that many of these apoptotic cells were neurons, and the amount of neuronal apoptosis was significantly decreased with flavopiridol treatment (P < 0.05, unpaired two-tailed t-test, n = 3/group; Fig. 9c).

Double-labelling also demonstrated the presence of oligodendrocytes that were positive for caspase-3 (Fig. 10). Double-labelling was found in vehicle treated tissue after SCI. Following treatment with flavopiridol, double-labelling was rarely seen, suggesting a decrease in oligodendroglial apoptosis.

Discussion

Flavopiridol is a semi-synthetic flavonoid derived from the bark of rohitukin, an indigenous plant from India that inhibits all CDKs examined to date with an IC_{50} ~ 100 nM (for CDK7 the IC_{50} ~ 300 nM) (Newcomb et al., 2004). It causes cell cycle arrest in G1 or at the G2/M transition (Swanton, 2004). Here we demonstrate that flavopiridol interferes in cell cycle progression and reduces phosphorylation of Rb, one of the key regulatory components of the cell cycle. This protein is normally phosphorylated during the cell cycle by cyclin D/CDK4 or CDK6 and cyclin E/CDK2 (Herwig and Strauss, 1997). Phosphorylation of Rb results in activation of the E2F transcription factor and, ultimately, progression of the cell to S phase of the cell cycle in mitotic cells and apoptosis in post-mitotic cells, such as neurons (Kitagawa et al., 1996; Sears and Nevins, 2002). We also demonstrate here that flavopiridol treatment reduces cyclin D1 and G1 expression after SCI, confirming the capacity of flavopiridol to inhibit cell cycle pathways in this model. However, increased cyclin D1 expression is not always associated with apoptosis; studies have shown that increased CDK4 activity may be the required event to induce neuronal death (Park et al., 2000), possibly explaining why we observed a lack of cyclin D1 expression in neurons but an increase in neuronal apoptosis after SCI. Increased CDK4 activity is accompanied by an increase in Rb phosphorylation, followed by caspase-3 cleavage and neuronal apoptosis (Park et al., 2000). Further, cyclin G1 expression has been associated with neuronal apoptosis after SCI (Di Giovanni et al., 2003). Flavopiridol has been extensively studied as an anti-neoplastic agent in clinical trials, which could serve to facilitate potential clinical development of this drug for SCI (Senderowicz, 1999, 2005).

Cell cycle proteins appear to play an important role in the response to CNS injury. These proteins are significantly increased after injury in neurons showing apoptosis and caspase activation (Di Giovanni et al., 2003; De Biase et al., 2005). Inhibition of cell cycle progression has been reported to provide neuroprotection in other models of CNS injury (Cernak et al., 2005; Di Giovanni et al., 2005; Tian et al., 2006), although the mechanisms involved have been only partially addressed. Recently, Tian et al. (2006) demonstrated that olomoucine, a CDK 5 inhibitor, improved function and reduced scar formation after spinal cord transection. However, they did not demonstrate treatment effects on cell cycle activation or effects on the post-traumatic inflammatory response; nor did they provide evidence for a mechanism of protective action. Moreover, olomoucine has a more limited profile of activities as compared to flavopiridol, and is a far less potent and effective neuroprotective agent than flavopiridol in a variety of in vitro models (Cernak et al., 2005; Di Giovanni et al., 2005). Using a more clinically relevant injury model we show that flavopiridol treatment decreases neuronal and oligodendroglial apoptosis, markedly reduces astrocytic reactivity and limits the release of microglial-activation related inflammatory factors. Additionally, the protective effects in a severe traumatic model are noteworthy, as such injury is much less responsive to neuroprotective treatment in animal or human SCI (Tator, 2002).

Pilot studies revealed that a single injection of the drug had only modest effects after SCI, resulting in decreased...
Fig. 4 Astrocyte and microglial marker immunohistochemistry after injury and treatment. Immunohistochemistry performed for astrocytes (a, b), osteopontin (e, f), and p22phox (g, h) at 3 and 28 days post-injury. Heavy astrocytic (GFAP) labelling was found surrounding the
lesion site (c) at 28 days after SCI in vehicle-treated tissue (shown as a mosaic image of the entire 10 mm cord section surrounding the lesion epicentre); a). However, little GFAP labelling was found in samples that had received flavopiridol continuous infusion (b). Quantitation of the proportional area of GFAP labelling in the spinal cord (through the 1cm surrounding the lesion site) showed a significant decrease in GFAP labelling at 28 (c) and 3 (d) days post-injury in flavopiridol treated tissue (P < 0.05; n = 3/group at 3 day; 10/group at 28 days). Immunolabelling for osteopontin and p22phox, factors expressed by microglia, was also decreased by flavopiridol treatment (f, h) in comparison to vehicle (e, g). All images are obtained from 1 mm rostral to the lesion epicentre, in the centre region of a transverse spinal cord section. Quantitation of p22phox labelling in the spinal cord (through the 1 cm surrounding the lesion site) showed a significant decrease in labelling at 28 days post-injury in flavopiridol-treated tissue (e; *P < 0.05). Bar = 1 mm (a, b); 50 μm (e, f); 100 μm (g, h).

Fig. 5 Flavopiridol attenuates the increase in Cyclin G1 protein levels induced by spinal cord injury. Wide-field high-resolution confocal images using a 10× objective (first column, a) of a complete transveral section of the injured spinal cord demonstrates that cyclin G1 expression is increased in neurons (NeuN positive) at 24 h after SCI. This increase is attenuated by treatment with flavopiridol. The expression of cyclin G1 in non-injured (sham) spinal cord is modest. The absence of NeuN staining is an indicator of injury-induced neuronal death after injury; the reduction in size of NeuN-stained cells further indicates neuronal injury. Flavopiridol treatment attenuates this NeuN decreased signal. The arrows in column I indicate the general area same image from which a narrower field is presented are near-full resolution in columns 2–4, where arrows indicate neuronal cells with nuclear overexpression of cyclin G1 protein. Quantitation of cyclin G1 labelling with unbiased stereological methods indicates a significant reduction with flavopiridol treatment in comparison to vehicle (b).
volume of gadolinium entrance into the spinal cord lesion, suggesting an improvement in blood-spinal cord barrier integrity, (Fig. 1) but little functional improvement (data not shown). However, infusion of flavopiridol for 7 days post-injury, during which many secondary injury events are occurring, such as scar formation and inflammation, significantly reduced lesion volume and improved functional recovery (Figs 2 and 3). While it is difficult to identify true lesion volume in vivo without either experimental artifact from histology or underestimation from MRI, these two methods showed comparable changes (Fig. 3d). Therefore, the difference found between vehicle and flavopiridol-treated tissue is likely a reliable outcome.

The flavopiridol-treated group demonstrated a 2–3 point improvement in the BBB score compared to the vehicle-treated group, reflecting the ability of rats to place their

Fig. 6 Flavopiridol reduces Cyclin D1 protein levels induced by SCI. Wide-field high-resolution confocal images of a complete transversal section of the injured spinal cord demonstrates that cyclin D1 expression is found throughout the cord at 72 h after SCI, but is most dense at the periphery (a). This is also the area of the greatest GFAP (astrocyte) labelling. Inserts demonstrate double-labelling of GFAP and cyclin D1. Cyclin D1 and GFAP expression is attenuated by treatment with flavopiridol. The expression of cyclin D1 in non-injured (sham) spinal cord is modest. Western blot analysis of protein levels for cyclin D1 (shown are three bands/group) show that flavopiridol reduces the protein expression at 24 h (b), which could be quantified (c). Bars represent mean ± SEM; *P < 0.05.
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Fig. 7 Flavopiridol attenuates pRb immunolabelling induced by spinal cord injury. Confocal images taken using a 20× objective of the injured spinal cord demonstrate that pRb is expressed in neurons (NeuN positive) at 72 h after SCI (a). This expression is attenuated by treatment with flavopiridol. The presence of pRb in non-injured (sham) spinal cord is modest. The absence of NeuN staining is an indicator of injury-induced neuronal death after injury. The arrows indicate neuronal cells (NeuN positive) with high levels of pRb. pRb expression was quantified with western blot (b). Shown are three bands/group. Quantitation of pixel density per band indicates a significant ($P < 0.05$) reduction in pRb expression at 24 h post-injury with flavopiridol treatment (c). Bands represent mean ± SEM, *$P < 0.05$.
paws in plantar positions, or sweep hind limbs in walking motions. This 2–3 point improvement is both statistically significant and noteworthy, as experimental treatments usually fail to provide improvement following this level of injury (Tator, 2002). As the BBB was not originally designed to test locomotion of severely injured animals, additional objective tests of hind limb motion, based on the combined behavioural score (Kerasidis et al., 1987), including withdrawal, righting, and contact placing, were utilized. The original CBS scale is heavily influenced by the inclined plane test; due to the severity of the injury in the current study, rats were unable to perform this task and the scale was therefore re-configured without this test. With the combined behavioural score described in the methods sections, rats in the flavopiridol treatment group also demonstrated considerable and significantly improved performance.

Dividing cells, such as microglia and astrocytes, undergo cell cycle activation and proliferation after CNS injury (Kato et al., 2003) and are therefore a potential target of flavopiridol treatment. Astrocyte activation after SCI, as measured by GFAP staining at 28 days post-injury, was significantly inhibited by flavopiridol treatment. Although glial activation may serve either protective or destructive roles after SCI, the glial scar provides a physical barrier to regeneration and plasticity, and is a source of multiple inhibitory factors that may limit neuroplasticity (Davies et al., 1996; Silver and Miller, 2004). Thus, the ability of flavopiridol to limit scar formation may facilitate endogenous restorative potential.

Flavopiridol treatment also reduced markers of microglial-related inflammation, including osteopontin and p22phox—factors associated with microglial activity after SCI (Shaked et al., 2005; Byrnes et al., 2006)—in agreement with our previous in vitro findings (Cernak et al., 2005). There was, however, no significant effect of flavopiridol on ED1 labelling, a marker for activated microglia and macrophages, at either 7 or 28 days post-injury; this may reflect the fact that much of the ED1 labelling in the lesion epicentre after injury reflects

Fig. 8 Flavopiridol attenuates TUNEL staining after SCI. TUNEL staining of sham, vehicle-treated and flavopiridol-treated samples at 3 days post-SCI indicate TUNEL staining in vehicle-treated samples, which is attenuated with flavopiridol treatment (green). Nuclear staining (TO-PRO-3; red) demonstrates nuclear pyknosis (inserts), which was rarely, if ever, found in sham or flavopiridol-treated tissue. Images were obtained 3 mm caudal to the lesion epicentre, in the centre of a transverse spinal cord section. Bar = 50 μm.
Flavopiridol attenuates the increase in cleaved-caspase-3 (active) induced by SCI. Cleaved-caspase-3 is increased in neurons (NeuN positive) at 72 h after SCI (arrows, a). This increase is attenuated by treatment with flavopiridol. The presence of cleaved-caspase-3 in non-injured (sham) spinal cord is modest. The absence of NeuN staining is an indicator of injury-induced neuronal death after injury. As shown before, flavopiridol treatment attenuates this decreased NeuN signal. The diamond arrows indicate a non-neuronal cell with strong cleaved-caspase-3 signal. Flavopiridol treatment significantly reduced both overall cleaved caspase-3 staining (b) and neuronal apoptosis (c), as measured by unbiased stereological counting. Bars represent mean ± SEM; *P < 0.05.
Cell cycle activation has been associated with apoptotic cell death in non-proliferating cell types, such as neurons and oligodendrocytes (Di Giovanni et al., 2005), and both cell types undergo apoptotic cell death after spinal cord injury (Shuman et al., 1997; Grossman et al., 2001; Beattie et al., 2002a, b; McBride et al., 2003; Stirling et al., 2004; Knoblach et al., 2005). Flavopiridol can prevent apoptosis of neurons from various insults in vitro (Mirjany et al., 2002; Verdaguer et al., 2004). Consistent with such observations, we show a reduction in both TUNEL positive and caspase-3 positive cells at 24 hs, 72 h and 7 days after SCI in flavopiridol-treated versus vehicle-treated animals; double-labelling for neurons and oligodendrocytes indicates that both of these cell types undergo apoptosis. Quantitation revealed that neuronal apoptosis was significantly reduced by flavopiridol treatment and double-labelling of oligodendrocytes with cleaved-caspase 3 was rarely observed. In conclusion, the present studies show that flavopiridol decreases cell death, astrocytic reactivity and microglial activation after severe SCI, reducing secondary injury and improving recovery of function. Our data suggest that this effect reflects flavopiridol’s ability to inhibit cell cycle pathways. Therefore, cell cycle inhibitors may provide a unique multi-modal therapeutic approach for the treatment of SCI. The fact that flavopiridol was effective in a severe SCI model, which typically is far less responsive to neuroprotective strategies either in animal models or clinically, also suggests a potentially larger treatment population for this approach as compared to such clinical treatments as methylprednisolone or naloxone—both of which have questionably modest protective effects after incomplete SCI in humans (Fehlings, 2001; Hurlbert, 2006; Sayer et al., 2006; Tator, 2006).

Moreover, if subsequent studies in SCI confirm such beneficial effects after late systemic administration, as we have shown in traumatic brain injury (Cernak et al., 2005), treatment with cell cycle inhibitors may provide another advantage to current clinical options, as neither methylprednisolone nor naloxone proved effective when administered more than 8 h after injury (Bracken and Holford, 1993).

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References


Flavopiridol improves SCI recovery


