Multifunctional Drug Treatment in Neurotrauma

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Summary: Although the concepts of secondary injury and neuroprotection after neurotrauma are experimentally well supported, clinical trials of neuroprotective agents in traumatic brain injury or spinal cord injury have been disappointing. Most strategies to date have used drugs directed toward a single pathophysiological mechanism that contributes to early necrotic cell death. Given these failures, recent research has increasingly focused on multifunctional (i.e., multipotential, pluripotential) agents that target multiple injury mechanisms, particularly those that occur later after the insult. Here we review two such approaches that show particular promise in experimental neurotrauma: cell cycle inhibitors and small cyclized peptides. Both show extended therapeutic windows for treatment and appear to share at least one important target. **Key Words:** Neurotrauma, neuroprotection, treatment, cell cycle inhibitors, small cyclized peptides.

INTRODUCTION

Trauma to the CNS causes both direct tissue damage and more delayed biochemical changes that lead to cell loss (secondary injury), demyelination, and related functional deficits.[1] Initiation of such biochemical cascades occurs from minutes to weeks after the insult. Numerous factors associated with delayed tissue loss have been identified from experimental studies of traumatic brain injury (TBI) and spinal cord injury (SCI); these include products of lipid degradation, disrupted ionic homeostasis, altered neurotransmitter release and receptor function, and inflammatory and immune changes.[1–3] Together, these biochemical and associated metabolic effects result in loss of neuronal and oligodendroglial cells, reactive astrogliosis, and proliferation/activation of microglia.[4,5]

Most neuroprotective strategies have been directed at individual components of this delayed reactive cascade, such as reducing free radical-induced actions, excitotoxicity, or inflammation. Whereas many such strategies have proven effective in experimental animal models of TBI or SCI, they have shown little or no neuroprotective actions in humans.[2] However, the majority of clinical neuroprotective approaches to date have been directed at reducing neuronal necrosis, which is a relatively early event that is largely completed within 6 to 8 h.[6] Yet only a relative minority of patients with neurotrauma can have treatment initiated within this time period. In addition, most therapies have aimed at modifying single components of the complex secondary injury cascade, even though it is recognized that many autodestructive biochemical changes occur in parallel. Use of multiple drug treatments, each directed to a different secondary injury component, has rarely been attempted (even experimentally) in neurotrauma,[7] although multifactorial combination drug approaches have long been standard therapy for certain infectious diseases and cancers. However, even if such combination treatments showed promise in animal models, the methodological difficulties and costs associated with such multi-drug comparison studies in treating clinical neurotrauma would likely prove prohibitive.

An alternative approach would be to identify single agents that can modify diverse secondary injury cascades. A number of such multifunctional or multipotential treatments has been proposed and successfully tested in experimental neurotrauma models. These have included naturally occurring substances, such as thyrotropin-releasing hormone (TRH), progesterone, heat shock protein, neurotrophic factors, and erythropoietin; drugs developed for other disorders such as statins or antibiotics; and agents developed through rational drug design.[2]

We have developed two multifunctional treatment approaches that have proved to be remarkably effective for the treatment of TBI and/or SCI. One was developed

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through a rational drug design program and was based on the tripeptide hormone TRH. The other has adapted drugs used extensively in experimental oncology with targets based on data developed from extensive genomics profiling in experimental TBI and SCI.

TRH AND NOVEL TRH ANALOGUES

In the early 1980s, we demonstrated that TRH, when used at higher than physiological concentrations, markedly improved outcome after experimental SCI, with a therapeutic window of at least 24 h.[8,9] TRH inhibits multiple secondary injury factors or processes, including declines of blood flow and bioenergetics, lipid degradation products such as peptidyl leukotriene and platelet activating factor, ionic dyshomeostasis (Na+, K+, Ca++, Mg++), endogenous opioids, and excitotoxins.[10–12] Subsequently, we found that TRH analogues that modified either the N-terminal or the middle amino acid of the tri-peptide hormone pyroglutamyl-histidyl-prolineamide were even more effective than TRH, with longer biological half-lives and fewer undesirable physiological actions. Such analogues proved highly effective in improving functional recovery and reducing lesion volume after experimental SCI or TBI.[13–17] The neuroprotective actions of TRH and TRH analogues in experimental neurotrauma have subsequently been confirmed

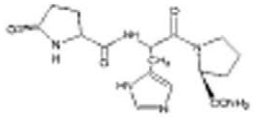
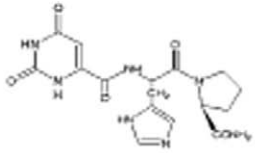
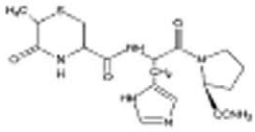
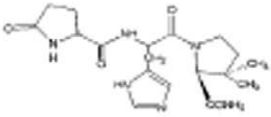
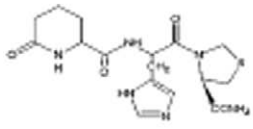
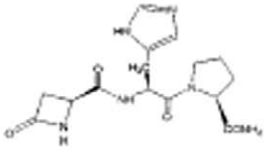
Drug	Structure	Endocrine	Analeptic	Autonomic	Neuroprotection
TRH		+	+	+	+
CG3509		+	+	+	+
CG3703		+	+	+	+
RX77368		+	+	+	—
MK-771		+	+	+	—
YM-14673		+	+	+	+

FIG. 1. Comparison of thyrotropin-releasing hormone (TRH) and various substituted TRH analogues that retain the endocrine, autonomic, and analeptic actions of TRH. Modifications of the N-terminus retain neuroprotective activity, whereas modifications of the C-terminus do not. +, positive effect; −, no effect. Reprinted with permission from Faden AI, et al. Ann NY Acad Sci 2005;1053:472-481.

by many laboratories.[18–22] Moreover, a small clinical trial of TRH suggested protective effects after SCI.[23]

TRH is metabolized through two major pathways: endopeptidase cleavage of pyroglutamyl to produce cyclohistidyly-proline diketopiperazine (CHP) or deamidation yielding the free acid form of TRH.[24,25] Various TRH analogues have been developed that modify one of its amino acids (FIG. 1).[26] Pyroglutamyl substitutions limit endopeptidase-mediated metabolism, resulting in compounds that have far longer biological half-lives than TRH (6–8 h vs 5 min); some of these are also more potent than TRH in terms of CNS activity. For example, YM-14673 is longer acting than TRH (8–36 times) and much more potent (10–100 times).[15] However, N-terminal substitutions retain the other physiological actions of TRH (i.e., endocrine, autonomic, and analeptic). We have also evaluated modifications of the histidyl residue (i.e., imidazole substitution); certain substitutions reduced the cardiovascular and/or endocrine activity while maintaining the neuroprotective actions of TRH (FIG. 2).[27] Critically, modification of the C-terminus results in compounds devoid of neuroprotective activity, although they retain endocrine, autonomic, and analeptic activity similar to TRH.

Based on these observations, we developed dual-substituted TRH analogues (i.e., modifications at both the N-terminal and histidyl moieties). Such compounds (53a, 57a) have limited endocrine, autonomic, and analeptic effects while preserving or enhancing the neuroprotective actions (FIG. 2).[16,26] Compound 53a is at least two orders of magnitude more hydrophobic than either TRH or YM-14673, based on their partition coefficients between n-octanol and water (logP); thus it should have enhanced cellular permeability to the CNS.[16]

CYCLIC DIPEPTIDES

TRH is metabolized to a cyclic dipeptide (CHP), which, like other diketopiperazines, retains considerable physiological activity.[28] We have developed a series of diketopiperazines structurally related to CHP (FIG. 3).[29] One of these (35b) has been extensively examined using *in vitro* and *in vivo* model systems.[29–32] In neuronal cell culture models, 35b provides neuroprotection in necrotic

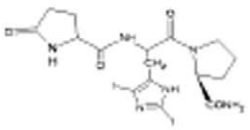
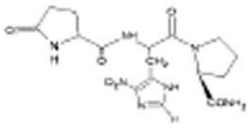
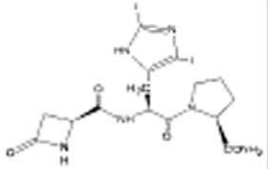
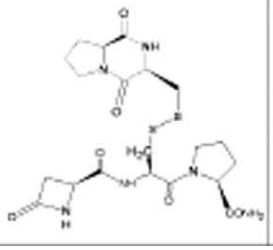
Drug	Structure	Endocrine	Analeptic	Autonomic	Neuroprotection
2,4-diiodo (Im)-TRH		—	NT	—	+
4(5)-NO2 (Im)-TRH		—	NT	+	+
53a		±	—	—	+
57a		—	—	—	+

FIG. 2. Comparison of the physiological actions of novel thyrotropin-releasing hormone (TRH) analogues that modify the imidazole structure. These analogues retain neuroprotective activity, but show reduced or absent endocrine, analeptic, and/or autonomic actions. +, positive effect; −, no effect; ±, partial effect; NT, not tested. Reprinted with permission from Faden AI, et al. Ann NY Acad Sci 2005;1053:472-481.

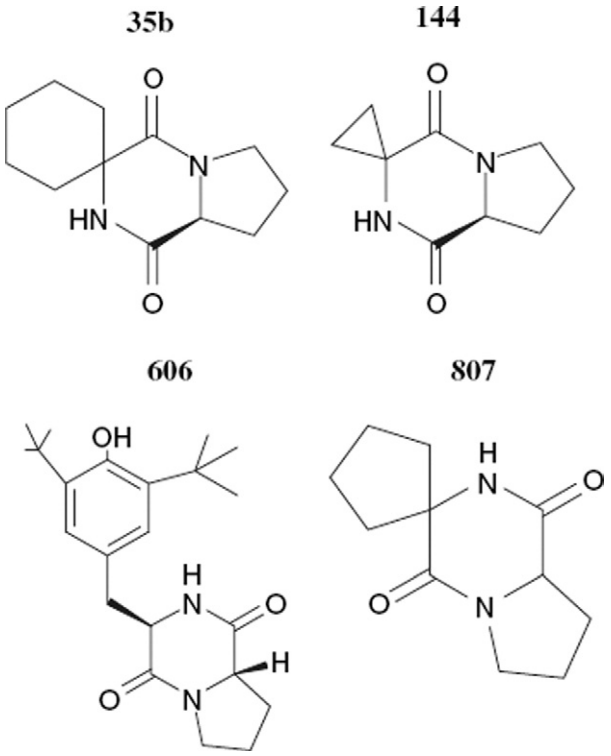


FIG. 3. Chemical structures of the cyclic dipeptides 35b, 144, 606, and 807. Reprinted from Faden et al. Neuropharmacology 2005;49:410-424.

cell death models (i.e, maitotoxin, glutamate, mechanical injury), as well as in apoptotic cell death models (i.e., staurosporine, beta amyloid) (FIG. 4).[31] Given intravenously, 35b reduced lesion volume by nearly 70%. It also improved functional (i.e., cognitive and motor) outcomes after either fluid percussion-induced traumatic brain injury (FPI) in rats or controlled cortical impact (CCI) injury in mice.[30,31] Treatment also significantly reduced apoptotic cell death in rat hippocampus after FPI. The therapeutic window for the drug is at least 8 h, and it shows a relatively flat dose response for neuroprotection between 0.1 and 10 mg/kg. Optimal doses are between 1 and 3 mg/kg, with repeated dosing over time showing no added benefit as compared to single bolus dose treatment. 35b is currently being developed by RemeGenix, Inc., for clinical trials in head injury.

Using the NIMH Psychoactive Drug Screening Program, 35b does not have significant binding affinities for any of 50 classical receptors, channels and transporters tested.[31] It also does not bind to either high- or low-affinity TRH receptors. To better address potential mechanisms, we performed temporal profiling using Affymetrix microarrays. Treatment with 35b after FPI upregulated various endogenous neuroprotective factors (BDNF, HSP 70, HIF1, mGluR7) and downregulated a number of recognized secondary injury factors (i.e., cyclins, calpains, cathepsin).[29,32] These findings were confirmed by PCR and protein measurements. Particularly noteworthy were the effects of treatment on cell cycle proteins, whose upregulation is associated with neuronal apoptosis, astrogliosis, and microglial activation after TBI or SCI.[33,34] Administration of 35b suppressed expression of the major upstream cell cycle proteins including cyclin D1, the retinoblastoma protein Rb, and E2F5.[29]

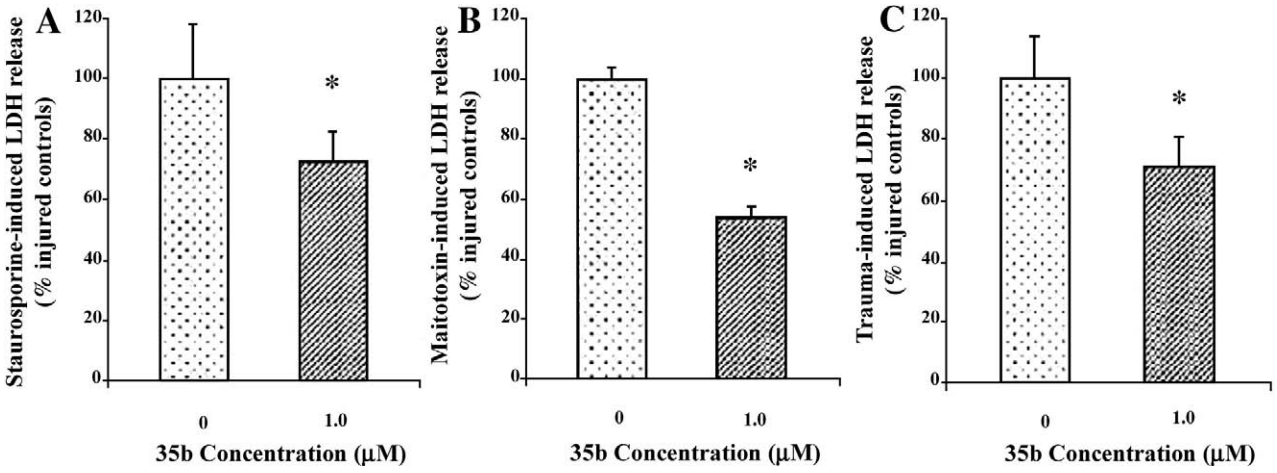


FIG. 4. Effect of 35b on lactate dehydrogenase (LDH) release in three different *in vitro* models of cell death: (A) apoptotic death induced by staurosporine; (B) necrotic death induced by maitotoxin; (C) traumatic death induced by a punch model that produces both initial physical disruption and later secondary death. Bars represent means and standard deviations for LDH released 16 to 18 h after injury in untreated injured cultures (dots) or injured cultures treated with 35b (diagonal lines) (n = 25 to 30 wells per condition). In (A), cultures were incubated with 0.3-μmol/L staurosporine for 16 to 18 h in the presence or absence of 35b. In (B), cultures were incubated with 0.1-nmol/L maitotoxin for 1 h in the presence or absence of 35b. Cultures were then washed and incubated for 16 to 18 h with or without 35b, as appropriate. In (C), 35b or media vehicle was present for 30 min before injury. Cells were then injured with a mechanical punch that delivered 28 parallel, uniformly distributed cuts to the surface of the cell layer. Thirty min after injury, cultures were washed again and incubated with 35b or vehicle, as appropriate, for 16 to 18 h. Reprinted from Faden et al. J Cereb Blood Flow Metab 2003;23: 342-354.

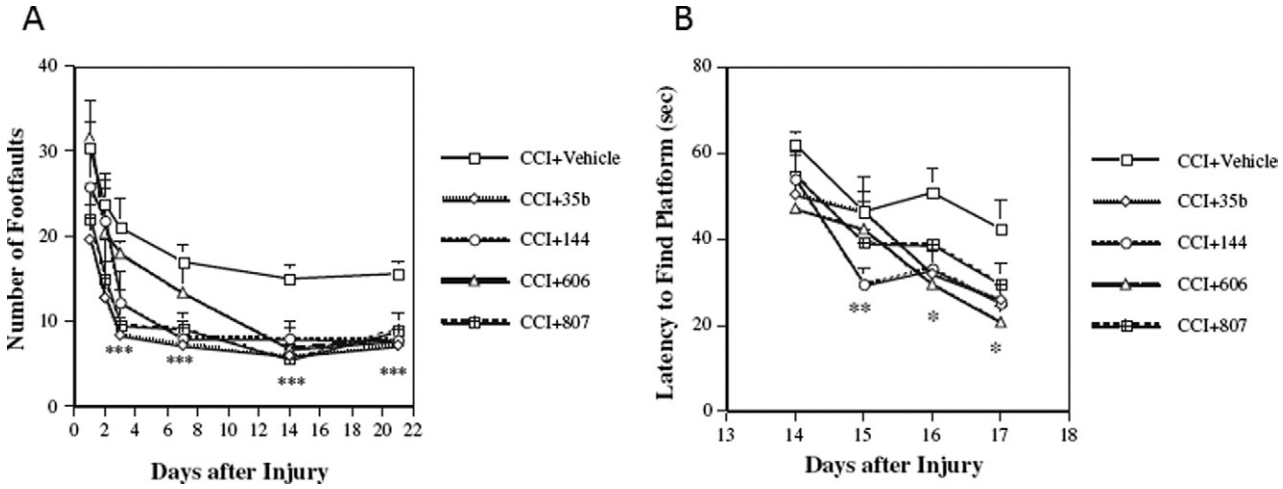
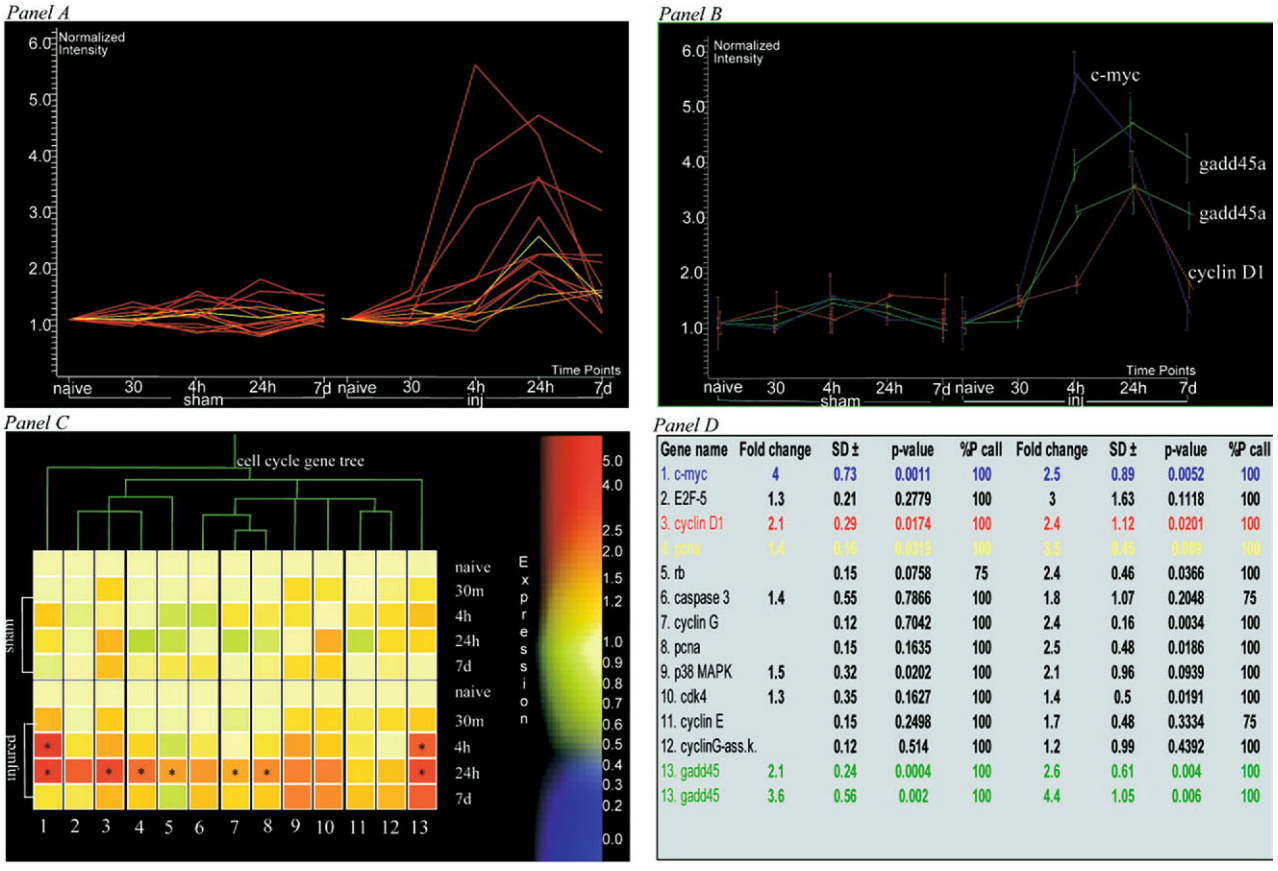


FIG. 5. A: Comparison of effects of diketopiperazines 35b, 144, 606, and 807 on motor function measured by beam walk test at 1, 2, 3, 7, 14, and 21 days post-trauma. All diketopiperazines significantly ($p < 0.001$) improved motor function at 14 and 21 days after injury, compared with vehicle-treated injured animals. 35b, 144, and 807 also showed significant ($p < 0.001$) protection at 3 and 7 days. $^{***}p < 0.001$ versus vehicle-treated injured (controlled cortical injury [CCI] + vehicle) animals. B: Water maze cognitive score after CCI in mice measured at 14, 15, 16, and 17 days post-injury. Diketopiperazines 35b, 144, 606 and 807 significantly ($p < 0.05$) improved cognitive function at 16 and 17 days after injury compared with vehicle-treated injured mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle-treated injured (CCI + vehicle) animals. Reprinted from Faden et al. Neuropharmacology 2005;49:410-424.



Gene name	Fold change	SD ±	p-value	%P call	Fold change	SD ±	p-value	%P call
1. c-myc	4	0.73	0.0011	100	2.5	0.89	0.0052	100
2. E2F-5	1.3	0.21	0.2779	100	3	1.63	0.1118	100
3. cyclin D1	2.1	0.29	0.0174	100	2.4	1.12	0.0201	100
4. pcna	1.4	0.15	0.0219	100	3.5	0.45	0.008	100
5. rb		0.15	0.0758	75	2.4	0.46	0.0366	100
6. caspase 3	1.4	0.55	0.7866	100	1.8	1.07	0.2048	75
7. cyclin G		0.12	0.7042	100	2.4	0.16	0.0034	100
8. pcna		0.15	0.1635	100	2.5	0.48	0.0186	100
9. p38 MAPK	1.5	0.32	0.0202	100	2.1	0.96	0.0939	100
10. cdk4	1.3	0.35	0.1627	100	1.4	0.5	0.0191	100
11. cyclin E		0.15	0.2498	100	1.7	0.48	0.3334	75
12. cyclinG-ass.k.		0.12	0.514	100	1.2	0.99	0.4392	100
13. gadd45	2.1	0.24	0.0004	100	2.6	0.61	0.004	100
13. gadd45	3.6	0.56	0.002	100	4.4	1.05	0.006	100

FIG. 6. Functional clustering of cell cycle genes shows high expression 4 to 24 h after injury. Functional clustering of genes based on involvement in cell cycle progression and apoptosis. Genes of this functional cluster also belong to smaller temporal clusters (gadd45a showed temporal clustering with c-myc [R2 = 0.99]), whereas PCNA, cyclin D1, cyclin G, Rb, and E2F5 belonged to the same temporal cluster [R2 = 0.99]). Data for all cluster members are shown in panels (A), (C), and (D), whereas data with standard deviations for multiple animals are shown in (B). B: Shows a self-organizing map graph subcluster applied to the cell cycle gene cluster shown in (A). Those genes showing significant p values (> 0.05) and fold changes (twofold) between sham and injured time points are indicated with an asterisk in (C). Reprinted from Di Giovanni et al. Ann Neurol 2003;53(4):454-468.

Various other cyclic dipeptides reduce lesion volume and improve behavioral outcome after CCI in mice with effects that are similar to those of 35b (FIG. 5).[29] In compound 606 the histidine moiety was replaced by 3,5-di-*tert*-butyltyrosine (DBT), a phenolic amino acid that can trap reactive oxygen species. In contrast to 35b, 606 blocked free radical-mediated cell death in neuronal cultures induced by FeSO4. Compound 144 also showed substantial neuroprotective actions *in vitro* and was highly protective in both the FPI and CCI models (FIG. 5).[29]

CELL CYCLE GENE AND PROTEIN EXPRESSION AFTER TBI OR SCI

Progression through the cell cycle is carefully regulated through the interplay of a number of cell cycle related proteins, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. Early events include the synthesis of cyclin D, which binds to CDK4 and CDK6; in the nucleus, CDK4/6 phosphorylate the retinoblastoma protein (Rb), leading to release of E2F transcription factors and transition to G1.[35,36] Apoptosis and cell cycle pathways share several common regulatory elements, including the retinoblastoma protein (Rb), E2F, and p53.

From extensive temporal profiling studies of gene expression changes after rodent SCI,[37,38] we identified a cluster of cell cycle genes that were coordinately regulated with the oncogene *c-myc,* which has been linked to neuronal cell death.[37,39] These gene expression changes, detected using Affymetrix chips (FIG. 6), were confirmed using RT-PCR, Western blots and immunocytochemistry.[34,37,38] Importantly, upregulated cell cycle proteins include key upstream regulatory elements that lead to G1 transition, including cyclin D1, Rb, and E2F5. Injury is also associated with downregulation of endogenous cell cycle inhibitors such as p27.

It is known that upregulation of cell cycle proteins in post-mitotic cells, such as neurons or oligodendroglial, results in caspase-mediated cell death.[40] Consistent with this view, we found that increased cell cycle expression

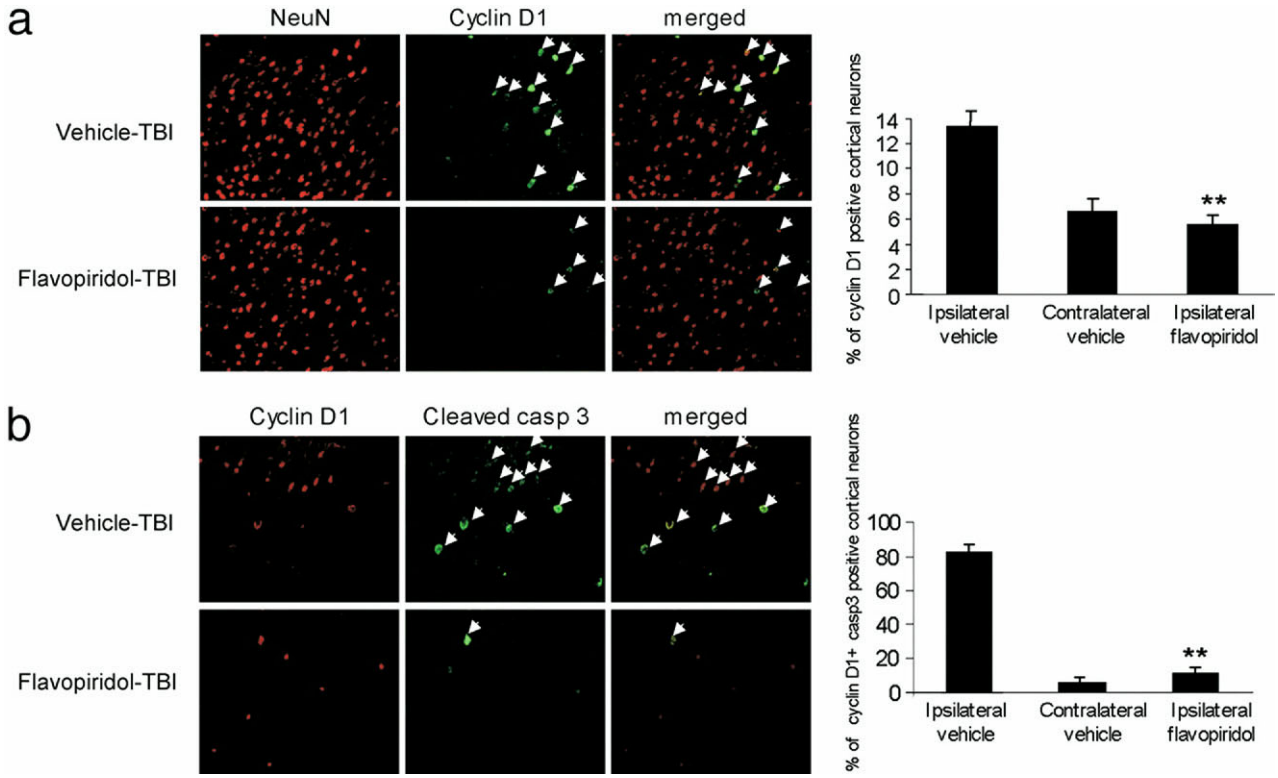


FIG. 7. Immunofluorescence shows the expression of cyclin D1 and active caspase 3 in cortical neurons after traumatic brain injury (TBI), and effects of flavopiridol treatment. a: Double immunofluorescence in coronal sections of the brain cortex around the injury site shows staining for cyclin D1 in vehicle, and flavopiridol-treated animal after TBI and for the neuronal marker NeuN. Cyclin D1 expression is induced in cortical neurons after TBI and strongly reduced after flavopiridol compared with vehicle (arrows). (Original magnification, ×125.) Bar graphs show quantitation of cortical neurons expressing cyclin D1 in ipsilateral and contralateral cortex in vehicle-treated rats compared with flavopiridol (ipsilateral) (**, $p < 0.01$). b: Double immunofluorescence in coronal sections of the brain cortex around the injury site shows staining for cyclin D1 in vehicle, and flavopiridol-treated animal after TBI, and for active caspase 3. Flavopiridol strongly reduces cyclin D1-positive and active caspase 3-positive neurons (arrows). (Original magnification, ×125.) Bar graphs show quantitation of cortical neurons co-expressing cyclin D1 and cleaved caspase 3 in ipsilateral and contralateral cortex in vehicle-treated rats compared with flavopiridol (ipsilateral) (**, $P < 0.01$). Reprinted from Di Giovanni S, et al. Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc Natl Acad Sci U S A 2005;102:8333-8338.

in neurons was associated with active caspase 3 expression and/or TUNEL positive staining after TBI [33,40] or SCI[37] (FIG. 7). Upregulation of cell cycle proteins is also readily observed in primary neuronal culture models after stimulation of caspase-dependent apoptosis with ceramide,[41] β-amyloid,[42] KCl withdrawal,[43] or DNA damage.[44–46] In addition, kainic acid-induced excitotoxicity of cerebellar granule cells is associated with increased

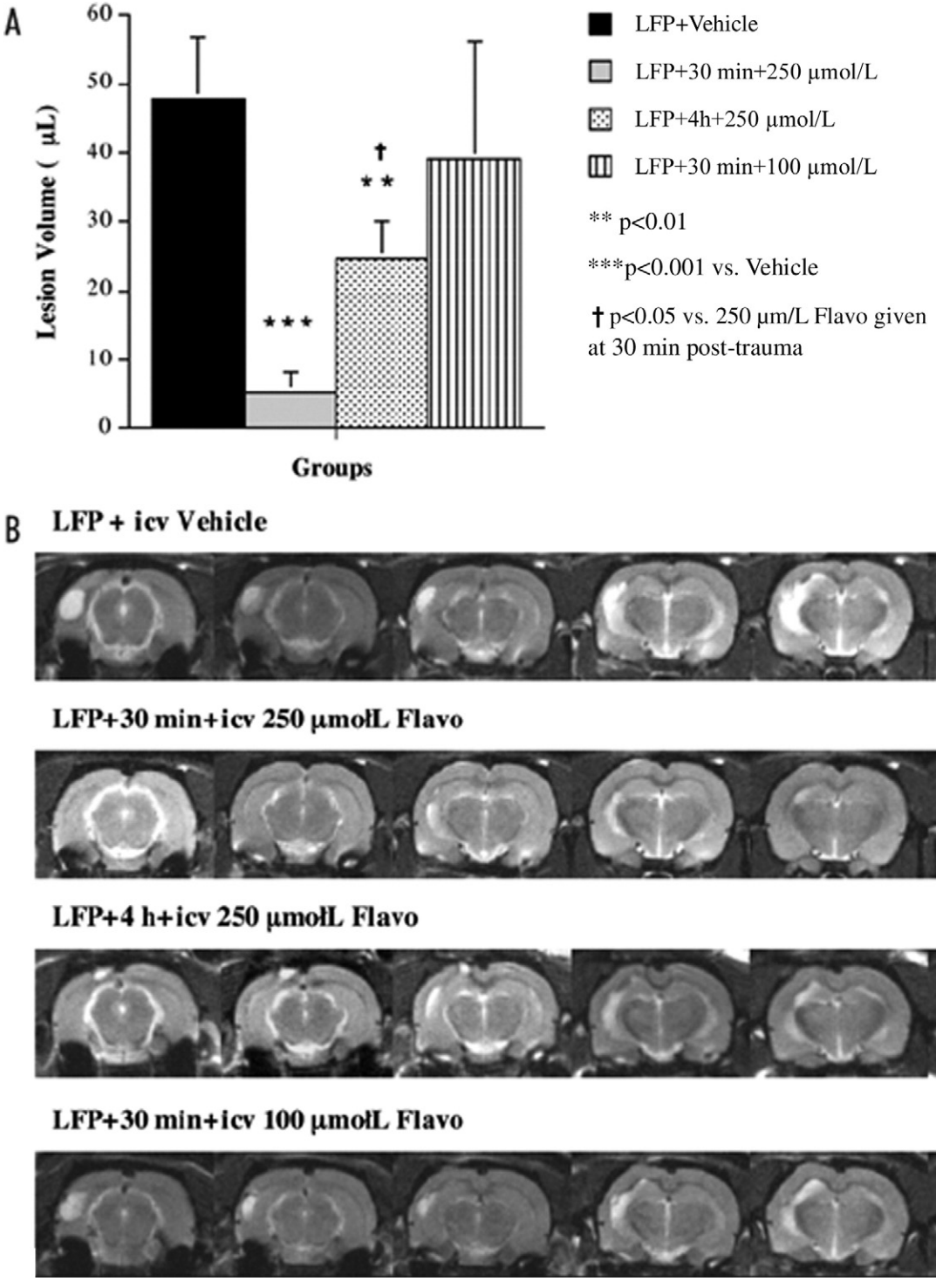


FIG. 8. Effect of flavopiridol on lateral fluid percussion (LFP)-induced lesion volume 21 days after injury in the rat. Data are from rats treated 30 min or 4 h after injury with flavopiridol (5 µL of 100 or 250 µmol/L solution for 5 min) or vehicle. A: Lesion volumes (n = 12; mean ± standard error of the mean). B: representative T2-weighted MRI of vehicle or flavopiridol-treated rats. Regions of marked hyperintensity are evident in the cortex and hippocampus. $^{**}p < 0.001$. $^{***}p < 0.0001$. Reprinted from Cernak I, et al. Role of the cell cycle in the pathobiology of central nervous system trauma. Cell Cycle 2005;4:1286-1293.

expression of cyclins D and E, PCNA and E2F1, as well as with increased expression of caspases 3 and 9.[47] Similarly, trophic withdrawal-induced cell death is associated with increased expression of both cyclins and cyclin-dependent kinases.[48]

SCI and TBI cause active astrogliosis that causes glial scar formation and proliferation/activation of microglia. After injury, cell cycle proteins are highly expressed in GFAP positive cells, as well as in activated microglia. For example, cyclin D1 expression is found to be increased in microglia after transient forebrain ischemia in the rat[49] and global ischemia in the gerbil.[50] Astrocyte proliferation was also associated with increased cell cycle proteins after ischemia.[50] After SCI, microglia and astrocytes demonstrate a marked reduction in p27, an endogenous cell cycle inhibitor.[51] Upregulation of cell cycle proteins is observed in primary cell culture models, including astroglial proliferation after exposure to serum[52] or microglial proliferation/activation after exposure to lipopolysaccharide (LPS).[53] After CNS injury, astrocytes undergo rapid proliferation and contribute to the formation of the glial scar.[54] This scar may provide a physical barrier to axonal growth,[55] as well as a "wall" to prevent migration of inflammatory cells into undamaged tissue.[56,57] Microglia, the primary immunological cell in the CNS, undergo rapid proliferation and transition from a resting, ramified phenotype to an ameboid phagocytic phenotype that is nearly indistinguishable from infiltrating macrophages.[58] Activated microglia produce pro-inflammatory molecules, such as interleukin (IL)-1β, IL6, inducible nitric oxide synthase,[59] complement components,[60] and reactive oxygen species,[61] which serve to modify both secondary injury and endogenous neuroprotective responses.

Increases in cell cycle protein expression have also been reported in chronic neurodegenerative disorders. For example, both neurons and glia show increased PCNA and cyclin D expression in human Alzheimer's patients.[62,63] Furthermore, DNA replication has been identified in apoptotic neurons in human Alzheimer's patients.[64] In an animal model of Alzheimer's disease, genetic APP23 mice demonstrate an increase of cell cycle related proteins in astrocytes.[65]

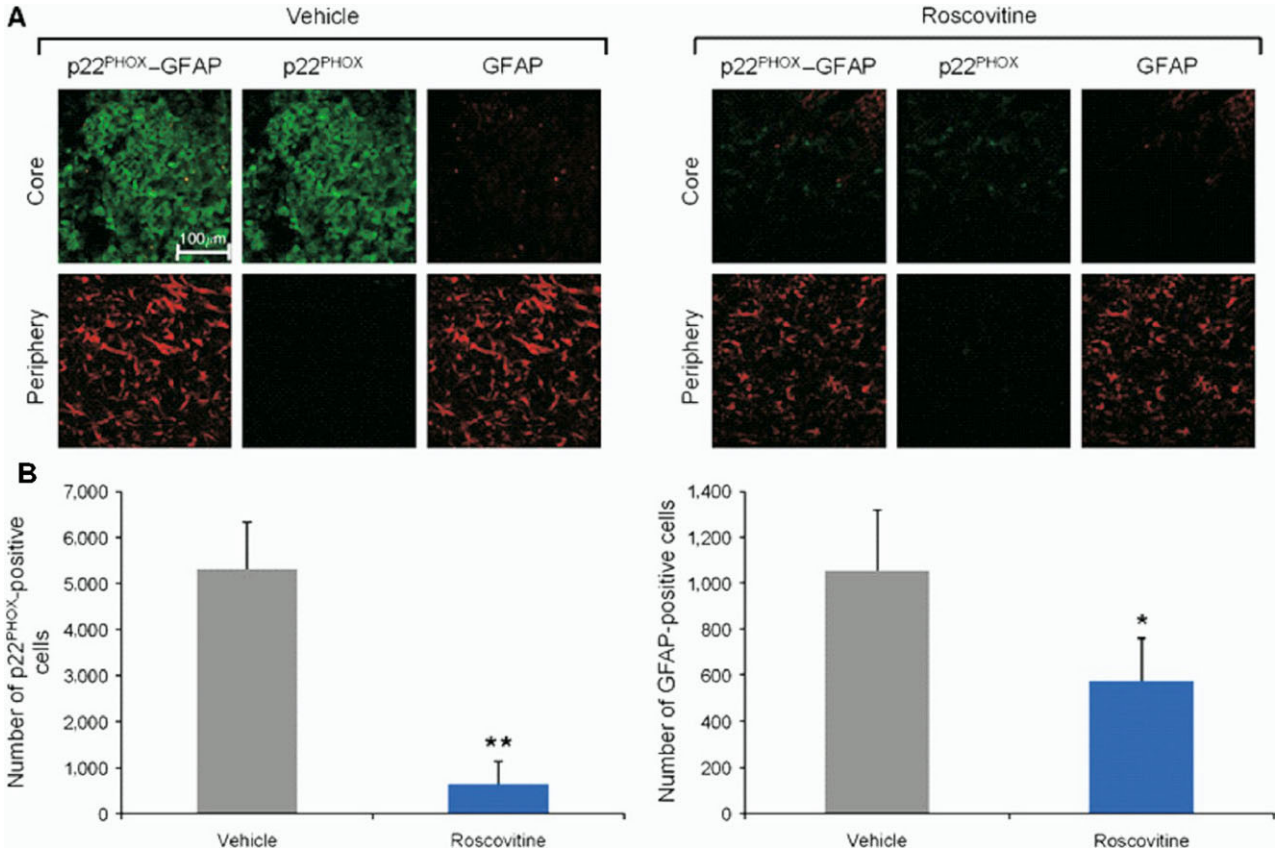


FIG. 9. Roscovitine treatment decreases activation of microglia and astroglia at 7 days after injury, as indicated by immunostaining for p22PHOX and GFAP. A: Representative composite confocal images after double-immunostaining for GFAP (red) and p22PHOX (green) of the core and periphery of the injury show that injury-dependent increase in the number of p22PHOX-positive microglia is concentrated in the core, whereas the increase in GFAP-positive astroglia occurs especially in the periphery of the lesion. Roscovitine attenuated both changes. There is no colocalization between markers for microglia and astroglia. Images showing the separate p22PHOX and GFAP channels are included. B: Cell counting indicates that roscovitine treatment results in a significant decrease in both p22PHOX (n = 3 sections, threshold = 50, unpaired *t*-test, $^{**}p = 0.0029$) and GFAP-positive cells (n = 3 sections, threshold = 150, unpaired *t*-test, $^{*}p = 0.0298$) per brain section. Reprinted from Hilton et al. J Cereb Blood Flow Metab 2008;(advance online publication).

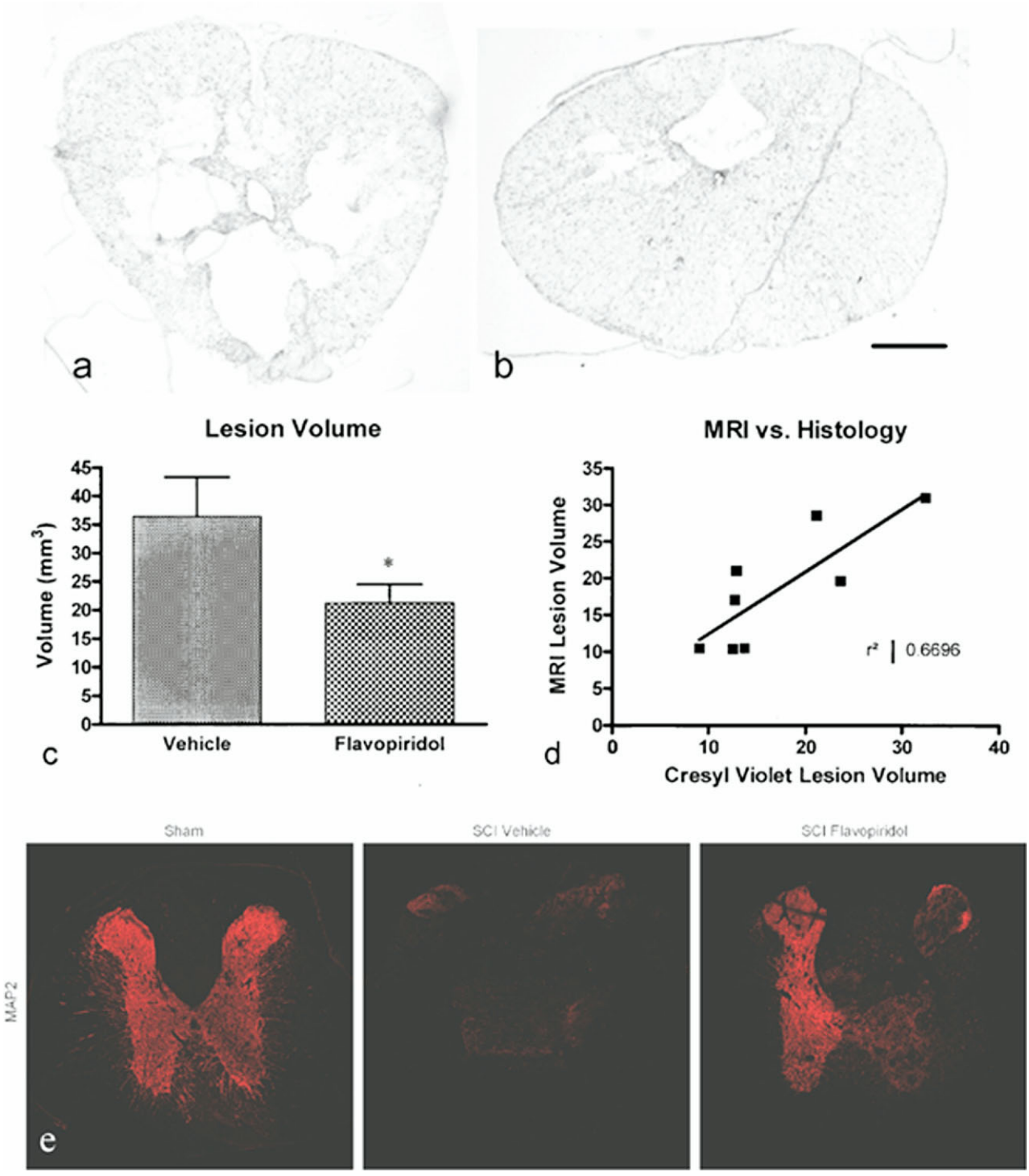
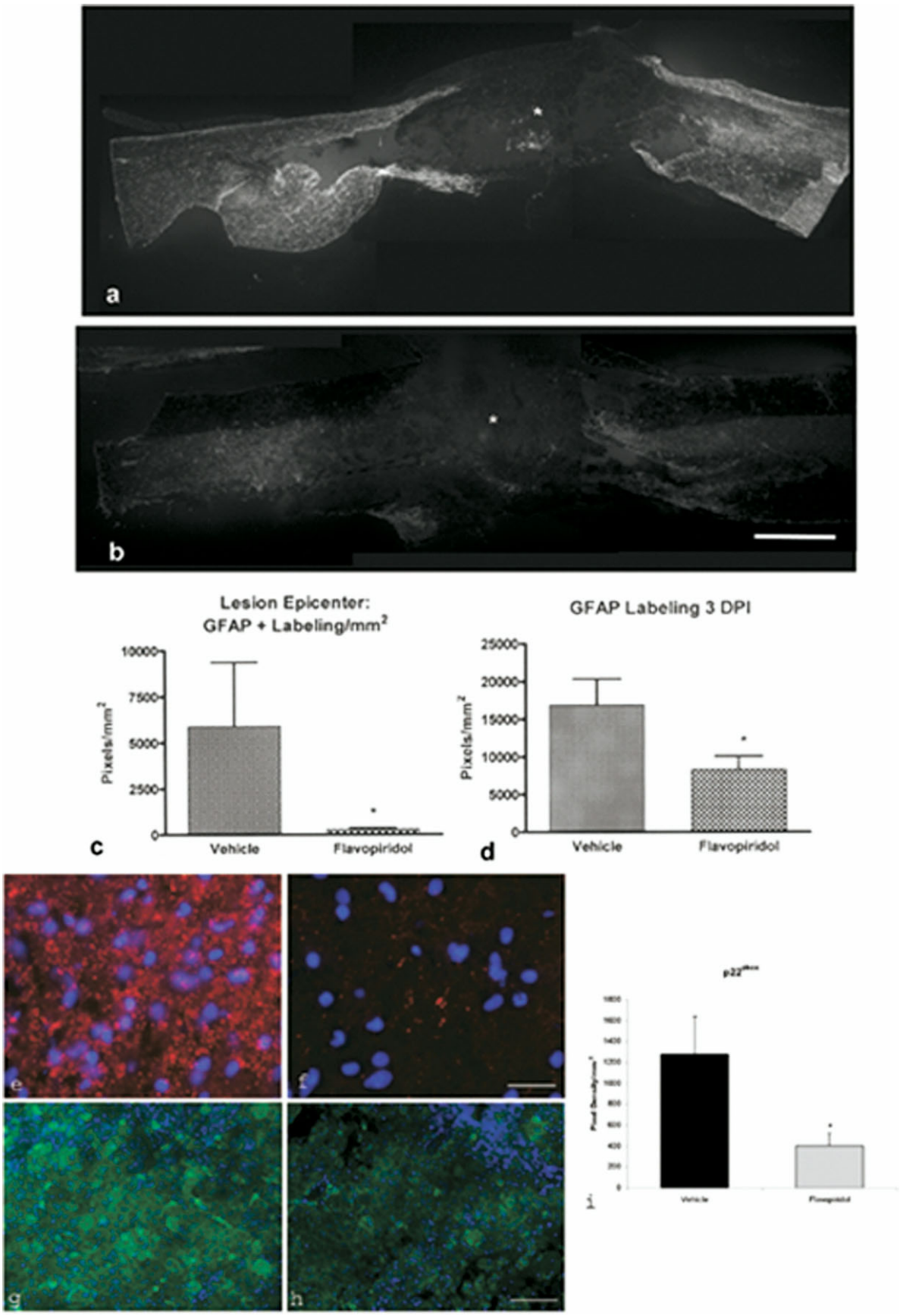


FIG. 10. Cresyl violet staining of tissue sections from flavopiridol- or vehicle-treated spinal cords. Images are taken from the center of the lesion site for both vehicle-treated (a) and flavopiridol-treated (b) samples, and demonstrate a clear decrease in tissue loss with flavopiridol treatment. Cavalieri volume estimation was performed to assess lesion volume (c). Analysis of correlation between MRI obtained lesion volume and histologically measured lesion volume shows a statistically significant correlation ($r2 = 0.6696$; $p < 0.05$; (d) wide-field high-resolution confocal images of a complete transversal section of the injured spinal cord, and (e) demonstrates that MAP2 expression is diminished throughout the cord at 24 h after spinal cord injury (SCI). This is attenuated by treatment with flavopiridol. Bar = 500 μm. Reprinted from Byrnes KR, et al. Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury. Brain 2007;130:2977-2992. By permission of Oxford University Press.


a
b
Lesion Epicenter:
GFAP + Labeling/mm²
Pixels/mm²
10000
7500
5000
2500
0
Vehicle
Flavopiridol
c
GFAP Labeling 3 DPI
Pixels/mm²
25000
20000
15000
10000
5000
0
Vehicle
Flavopiridol
d
e
f
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h

INHIBITION OF CELL CYCLE

Cell cycle inhibitors have been developed and extensively evaluated in experimental cancer models, and several have been tested in humans. The best characterized and studied among these are flavopiridol, a semi-synthetic flavonoid derived from rohitukin bark,[66] and the purine analogues roscovitine and olomoucine.[67] Flavopiridol blocks all the CDKs and also inhibits the transcription of cyclin D1.[68,69] In contrast, the purine analogues preferentially inhibit CDK2 and CDK5, although at higher concentrations these may inhibit other kinases.[70]

Each of these agents shows neuroprotection *in vitro*, such as against etoposide-induced neuronal apoptosis[33] or apoptosis of cerebellar granule cells after KCl withdrawal.[43] Moreover, olomoucine inhibits hypoxia-induced neuronal cell death in culture,[71] whereas flavopiridol inhibits kainite-mediated or colchicines-mediated apoptotic cell death.[72,73] A recent study in our laboratory determined that inhibition of multiple cyclin-dependent kinases reduces etoposide-induced neuronal apoptosis, including CDK1 and CDK4.[74]

Cell cycle inhibitors show inhibitory effects on the proliferation and activation of mitotic cells, such as microglia and astrocytes *in vitro.* For example, stimulation of microglia with LPS induces proliferation. Pre-treatment of microglia with cell cycle inhibitors, such as flavopiridol or roscovitine, for 1 h prior to the addition of LPS results in a significant suppression of microglial proliferation[33] and nitric oxide production.[74] Importantly, roscovitine treatment of microglial cells stimulated with LPS reduced microglial-induced neurotoxicity.[74] Similarly, proliferation of astrocytes induced by the addition of 10% serum was completely inhibited by flavopiridol.[33]

In vivo, cell cycle inhibition using pharmacological approaches has shown neuroprotective effects. For example, early treatment with flavopiridol, administered centrally, showed remarkable neuroprotection after FPI in rats.[40] Lesion volume was reduced by approximately 70% and chronic behavioral recovery (motor and cognitive) was indistinguishable from sham-injured controls. Caspase-mediated neuronal cell death after TBI was nearly completely attenuated. In addition to neuroprotection, significant effects on mitotic cells were also observed. GFAP expression and markers of microglial activation were markedly reduced. These changes were associated with near complete suppression of cell cycle proteins in neurons, astroglia, and microglia, respectively.[40] Delayed administration of flavopiridol was similarly found to have neuroprotective effects. In a follow-up study, flavopiridol was administered centrally at 30 min or 4 h after FPI, or systemically (intraperitoneally) at 24 h after FPI;[33] each of these treatments resulted in markedly reduced lesion volumes that were approximately 90%, 50%, and 60%, respectively (FIG. 8).

The more specific cell cycle inhibitor roscovitine, which does not have potentially confusing effects on gene transcription, has similar actions after FPI. Administration of roscovitine 30 min after FPI resulted in highly significant reductions in lesion volume and improved behavioral outcome (motor and cognitive). This cell cycle inhibitor also reduced astrogliosis and produced a marked inhibition of microglial activation-related inflammation[74] (FIG. 9).

Research in SCI confirms the strong beneficial effects of treatment with cell cycle inhibitors. We have shown that flavopiridol treatment, centrally administered by mini-osmotic pump beginning 30 min post-trauma and continuing over 7 days, significantly improved motor recovery and reduced lesion volume at 28 days.[34] Treatment-reduced cell cycle protein induction in neurons and astrocytes; this reduction was associated with decreased cleaved caspase-3 labeling in neurons and oligodendrocytes, as well as reduction in glial scar. Neuronal loss, measured by MAP-2 staining, was alleviated by flavopiridol treatment, and tissue loss was significantly reduced overall. Further treatment with the cell-cycle inhibitor markedly limited microglial activation and associated inflammatory factors (FIGS. 10 and 11). The cell cycle inhibitor olomoucine has also been shown to decrease lesion volume and improve function after SCI.[75] Reductions in microglial-related inflammation[76] and astrocytic scar[75] were found with olomoucine treatment, supporting the beneficial effect of cell-cycle inhibition after SCI. Preliminary work using cyclin D1

FIG. 11. 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) labeling was found surrounding the lesion site (*) at 28 days after SCI in vehicle-treated tissue (shown as a mosaic image of the entire 10-mm cord section surrounding the lesion epicenter); (a) however, little GFAP labeling was found in samples that had received flavopiridol continuous infusion (b). Quantitation of the proportional area of GFAP labeling in the spinal cord (through the 1 cm surrounding the lesion site) showed a significant decrease in GFAP labeling at 28 (c) and 3 (d) days post-injury in flavopiridol-treated tissue ($p < 0.05$; n = 3/group at 3rd day; 10/group at 28 days). Immunolabeling 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 epicenter, in the center region of a transverse spinal cord section. Quantitation of p22phox labeling in the spinal cord (through the 1 cm surrounding the lesion site) showed a significant decrease in labeling at 28 days post-injury in flavopiridol-treated tissue (i; $*p < 0.05$). Bar = 1 mm (a, b); 50 μm (e, f); 100 μm (g, h). Reprinted from Byrnes KR, et al. Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury. Brain 2007;130:2977-2992. By permission of Oxford University Press.

knockout mice subjected to SCI has shown decreased lesion volumes in knockouts as compared to wild type controls, consistent with the pharmacological inhibition studies.[34]

Research in experimental cerebral ischemia is highly consistent with the TBI and SCI studies. Dominant negative CDK4/5 animals show reduced neuronal cell death after focal or global brain ischemia,[77] as does treatment with CDK inhibitors.[78,79] Moreover, after focal cerebral ischemia, cyclin D1 knockouts or animals treated with olomoucine show reduced astrocyte proliferation. Excitotoxic cell death after kainic acid administration is attenuated by treatment with anti-sense oligonucleotides directed against CDK4 or cyclin D1.

SUMMARY

Activation of cell cycle proteins in the CNS causes proliferation of mitotic cells, such as astroglia or microglia, but induces apoptosis in post-mitotic cells, such as neurons or oligodendroglia. Acute injuries to the CNS, including TBI and SCI, cause upregulation of many cell-cycle proteins in both mitotic and post-mitotic cells. These changes cause neuronal and oligodendroglial cell death, astroglial scar formation and proliferation/activation of microglia, with the release of associated inflammatory factors. Treatment with cell-cycle inhibitors results in striking neuroprotection, likely related to its multifunctional actions on these diverse cell types. Because cell-cycle proteins have such diverse effects, even selective inhibitors of these pathways may serve as multifunctional neuroprotective agents.

Another approach to multi-potential drug treatment of CNS injury is to use compounds that modulate different signal transduction pathways that are involved in secondary injury. TRH is a naturally occurring brain hormone, which when used at higher than physiological levels as a drug can inhibit many factors and mechanisms implicated in delayed cell death. Thus, TRH and TRH analogues can improve blood flow and bio-energetic state; limit loss of ionic homeostasis; reduce lipid degradation; and inhibit the actions of endogenous opioids, leukotrienes, platelet-activating factor, and possibly glutamate.[29,32] The neuroprotective effects do not appear to be mediated by TRH receptors, as they occur at supraphysiological doses and can be dissociated from the other physiological effects of TRH (i.e., endocrine, analeptic, autonomic).

Diketopiperazines that are structurally related to a metabolic product of TRH have marked neuroprotective activity, but do not act on either high- or low-affinity TRH receptors. They also have diverse multifunctional neuroprotective actions. As with cell-cycle inhibitors, the prototype compound 35b inhibits the activation of many cell-cycle proteins after injury. But they also reduce other known secondary injury factors, including calpains and cathepsins, while upregulating several well-established endogenous neuroprotective factors including brain derived neurotrophic factor, heat shock protein 70, and hypoxia-inducible factor 1. Each of the latter factors has considerable protective activity in animal models. These findings underscore the attractiveness of multifunctional drug approaches for the treatment of neurotrauma and other neurodegenerative disorders.

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