

## A comparison of adenosine A<sub>2A</sub> agonism and methylprednisolone in attenuating neuronal damage and improving functional outcome after experimental traumatic spinal cord injury in rabbits

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**Object.** Steroid agents remain the lone pharmacological treatment in widespread use for acute spinal cord injury (SCI), although their utility remains in dispute in the neurotrauma literature. Adenosine A<sub>2A</sub> receptor activation with ATL-146e, a selective A<sub>2A</sub> agonist, has shown potential benefit in treating SCI; however, it has not been compared with the gold standard, methylprednisolone. The authors of this study evaluated ATL-146e and methylprednisolone for their ability to preserve neuronal viability and motor function in experimental SCI.

**Methods.** New Zealand White rabbits sustained SCI or sham injury via the Allen weight-drop technique. Ten minutes postinjury, animals received ATL-146e (ATL group, 0.06 µg/kg/min intravenously for 3 hours), methylprednisolone (steroid group, 30 mg/kg intravenously), or saline (trauma control group). Hindlimb motor function was recorded every 12 hours using the Tarlov motor grading scale (0, paralysis–5, normal hop). At 48 hours, fixed spinal cord tissue was evaluated for neuronal viability.

Hindlimb motor function in animals treated with ATL-146e was equivalent to that of sham-injured animals and was significantly better than that of trauma control animals at all time points and that of steroid-treated animals at 12 hours ( $p = 0.05$ ). Motor function in steroid-treated animals was worse than in those given ATL-146e and better than that of trauma control animals at later time points, but was not statistically significant (both  $p > 0.05$ ). Neuronal viability (measured in neurons/hpf) was significantly higher in both treatment groups compared with the trauma control group ( $12.1 \pm 1.4$  neurons/hpf for the ATL and  $13.3 \pm 1.4$  neurons/hpf for the steroid group compared with  $7.5 \pm 1.5$  neurons/hpf for the trauma control group; both  $p < 0.04$ ). Neuronal viability did not differ among ATL-146e-treated, steroid-treated, and sham-injured groups.

**Conclusions.** The use of ATL-146e is at least as effective as methylprednisolone in preserving function and is equivalent to methylprednisolone in preserving the structure of spinal cord tissue after blunt SCI. Adenosine A<sub>2A</sub> receptor activation may be an effective treatment for acute SCI while avoiding the adverse effects of steroid agents.

**KEY WORDS** • spinal cord injury • trauma • adenosine • A<sub>2A</sub> receptor • methylprednisolone • rabbit

**M**ORTALITY rates from SCI have improved significantly over the last three decades, mainly as a result of improvement in the treatment given to trauma patients in emergency medical systems.<sup>1,13,27</sup> Long-term morbidity rates and treatment outcomes for patients with SCIs, however, have not substantially improved. Although a recently published high-profile case has raised new hopes for rehabilitative options,<sup>31</sup> outcomes from SCIs, particularly those involving cord contusion and/or incomplete injury, will be most strongly affected by the identification

of effective interventions that improve cord functioning in the acute setting. It is thus disappointing that methylprednisolone remains the gold standard of treatment (and in many respects the lone treatment option), even though steroid agents were first used 20 years ago and their utility remains in dispute in the neurotrauma literature.<sup>4,16,24</sup>

The synthetic adenosine analog ATL-146e has both anti-inflammatory and neuroprotective effects.<sup>10,11</sup> It acts specifically as an agonist of A<sub>2A</sub> receptors, with minimal effect on other adenosine receptor subtypes.<sup>40</sup> In our laboratory, we demonstrated significant improvement in motor function and histopathological outcome in a dose-dependent fashion in an experimental model of spinal cord ischemia-reperfusion injury.<sup>10</sup> We expanded this work into a pi-

*Abbreviations used in this paper:* MAP = microtubule-associated protein; SCI = spinal cord injury; TBS = Tris-buffered saline; TNF = tumor necrosis factor.

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lot study in a rabbit model of blunt SCI and, again, administration of ATL-146e resulted in reductions in the extent of paralysis.<sup>12</sup> In addition, we demonstrated that improvements in functional outcome using ATL-146e after blunt SCI were sustained to 7 days postinjury.<sup>39</sup>

In the current study, we compare directly the early effects of A<sub>2A</sub> agonism and methylprednisolone in the treatment of traumatic SCI with both histopathological and functional/neurobehavioral end points.

### Materials and Methods

#### Induction of SCI

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

Thirty-three adult New Zealand White rabbits (weighing 3–3.5 kg) were anesthetized with ketamine and xylazine and subjected to blunt spinal cord trauma in a manner consistent with that previously described.<sup>12</sup> After induction of anesthesia, animals were endotracheally intubated and anesthesia was maintained with halothane, which was titrated to effect. An arterial catheter was placed in the ear for blood pressure monitoring, and the marginal ear vein was accessed for delivery of both normal saline and any intravenous therapies. The animals were then placed prone on a heating blanket.

Animals were randomly assigned to one of four groups: sham injury, trauma control, ATL-146e (ATL) treatment, and methylprednisolone (steroid) treatment. A midthoracic posterior laminectomy was performed, and an intact dura mater was exposed in all animals. The incisions were closed in the three sham-injured animals; these animals underwent no further intervention. Spinal cord-injured animals (10 per group) underwent identical exposure. Once the dura was exposed, a 10-g weight was dropped from 6 cm onto a brass impounder sitting on the dura, consistent with a modified Allen weight-drop technique. This 60 g/cm of injurious force produced a consistent, reproducible SCI.

#### Drug Administration and Physiological Monitoring

Animals in the trauma control group received an infusion of saline postinjury; those in the steroid-treated group received 30 mg/kg of methylprednisolone over 30 minutes beginning 10 minutes after the injury via a syringe pump. For the ATL-treated group, 8 µl of stock ATL-146e solution (5 g/L in dimethyl sulfoxide) was diluted in 4 ml of normal saline carrier. Animals in the ATL group received ATL-146e intravenously at a rate of 0.06 µg/kg/min infused over 3 hours via the ear vein.

An intravenous infusion of ATL-146e was chosen as the administration regimen because of its short plasma half-life, assuming that steady-state plasma levels are achieved within 10 minutes. The optimal duration and concentration of the ATL-146e was determined by dose-response curves generated in earlier experiments in which spinal cord ischemia infusion reperfusion injury were examined.<sup>9</sup> The duration of the ATL-146e infusion produces a therapeutic drug concentration similar to that of a single 30-mg/kg intravenous dose of methylprednisolone.<sup>6,7</sup>

All anterior spinal elements remained intact to preserve structural stability and to prevent further injury. The investigator administering the injury was blinded to the treatment-group assignment of the animal, with the exception of the sham-injured group.

Arterial blood gases were recorded every 30 minutes throughout the surgical procedure. The mean arterial pressure, PaCO<sub>2</sub>, PaO<sub>2</sub>, arterial pH, pulse rate, and rectal (core body) temperature were recorded at 30-minute intervals for each animal.

The animals were weaned off anesthesia until they were able to lift their heads. They were kept alive for 48 hours and were given Baytril and Buprenex every 12 hours. All animals that were unable to sit up independently received a bolus of intravenous fluids twice per day plus hand-fed nutritional supplements (Nutri-Cal; EVSCO Pharmaceuticals, Buena, NJ).

#### Hindlimb Function

Hindlimb motor function was graded at 12, 24, 36, and 48 hours

using a modified Tarlov motor grading scale. The Tarlov grading system is an established, reliable scale for evaluating hindlimb motor function. The Tarlov scale scores are based on an ability to move, sit, and hop (0, atony; 1, slight movement; 2, sits with assistance; 3, sits alone; 4, weak hop; 5, normal hop). Tarlov grading was performed by a member of the vivarium veterinary staff blinded to the experimental group and with no tie to the project. The median Tarlov grades for each treatment group were computed and compared statistically using Kruskal–Wallis nonparametric tests. Differences between individual treatment groups were further analyzed using the Mann–Whitney U-test and chi-square analysis.

#### Qualitative Histopathological Analysis

At 48 hours postinjury, all animals were killed and perfusion fixed with 1 L 10% zinc formalin. Injured segments of spinal cords were harvested and embedded in paraffin. Five-micron sections were cut through the injury site and every fifth section was affixed to glass slides, deparaffinized in xylene, and rehydrated in serial dilutions of ethanol. Serial sections were then processed for immunohistochemical visualization of MAP-2, followed by a Luxol fast blue counterstain. In preparation for immunohistochemical analysis, sections were processed using a temperature-controlled microwave for antigen retrieval. Endogenous peroxidase was blocked by incubation in a solution of 1.65% H<sub>2</sub>O<sub>2</sub> in 0.025% Triton X in TBS. Sections were then incubated overnight in mouse anti-MAP-2 antibody (clone MT-01; Abcam Ltd., Cambridge, United Kingdom) at a dilution of 1:100 in TBS with 1% bovine serum albumin. The following day, sections were rinsed in TBS and Triton X and then incubated for 2 hours in biotinylated anti-mouse immunoglobulin G (dilution 1:200; Vector Laboratories, Burlingame, CA) in TBS with 1% bovine serum albumin. After incubation in an avidin-biotin-peroxidase complex (ABC standard “Elite” kit; Vector Laboratories) at a dilution of 1:100, sections were processed for visualization of the immunohistochemical complex using 0.05% diaminobenzidine (DAB; Sigma–Aldrich Chemical Co., St. Louis, MO) and 0.01% H<sub>2</sub>O<sub>2</sub>. Reacted sections were coverslipped for brightfield light microscopic examination.

#### Quantitative Histopathological Analysis

Serial sections of injured segments of spinal cords were prepared as described earlier and stained with H & E. Digital images of serial sections of ventral horn gray matter were obtained and coded. The number of intact, viable ventral horn motor neurons per high power field were counted in a blinded fashion by a single observer (A.S.H.) using standard criteria. Neurons were considered viable if they preserved their nucleolus, did not retract from the surrounding tissue, and showed no signs of axonal degeneration. The mean number of viable neurons per high power field for each treatment group was computed and compared statistically using analysis of variance and the Bonferroni multiple comparison post hoc test.

## Results

#### Physiological Monitoring

Blood gas analysis of PaO<sub>2</sub>, PaCO<sub>2</sub>, and pH in each experimental group confirmed physiological balance throughout the experiments. Furthermore, monitoring of mean arterial blood pressure and core body temperature revealed no differences among experimental groups (data not shown).

#### Hindlimb Motor Function

Tarlov scores at each observed time point for each experimental group are illustrated in Fig. 1. All animals subjected to sham injury had normal hindlimb function (Tarlov Score 5) throughout the observation period after surgery. Hindlimb motor function in sham-injured animals was significantly better than in the trauma control group

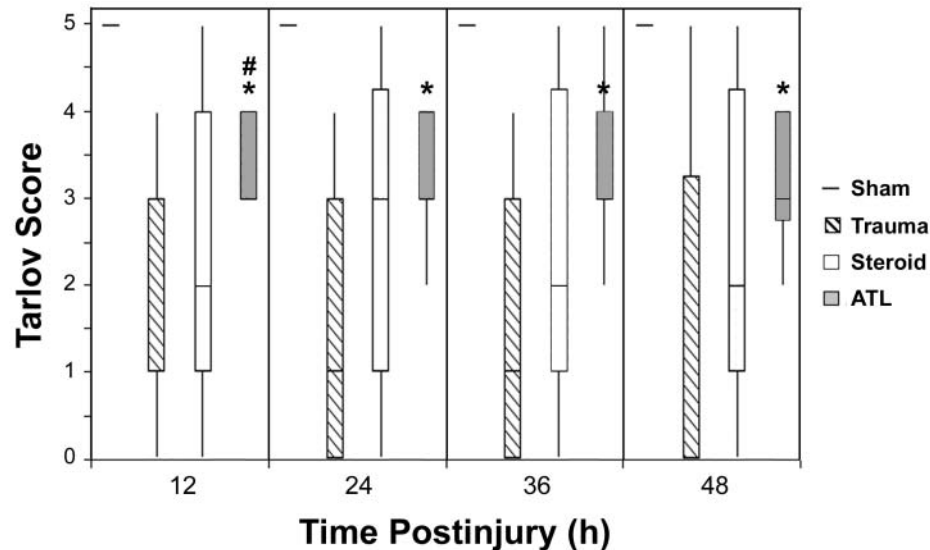


FIG. 1. Box plot illustrating a comparison of hindlimb motor function (using the Tarlov motor score) during the first 48 hours postinjury. Boxes represent interquartile range; whiskers represent all values within  $1.5 \times$  interquartile range. All animals subjected to sham injury had normal hindlimb function (Tarlov Score 5) throughout the observation period following surgery. Hindlimb motor function was equivalent in sham-injured and in ATL-146e-treated animals. Tarlov scores for sham-injured animals were significantly higher than were scores for trauma controls and steroid-treated animals at all time points. All ATL-146e-treated animals fared significantly better than did trauma control ones at all time points, and they fared significantly better than steroid-treated animals at 12 hours. A trend toward improved hindlimb function, but not statistical significance, was noted in ATL-treated animals compared with steroid-treated animals at 24, 36, and 48 hours. Tarlov scores trended worse in trauma control compared with steroid-treated animals, but this difference failed to reach statistical significance. Thus, at 48 hours postinjury, ATL-146e-treated animals had hindlimb motor function preservation equivalent to sham-injured animals, whereas motor function in steroid-treated animals was significantly worse than it was in their sham-injured counterparts. Asterisk denotes statistical significance compared with trauma control group; number sign represents statistical significance compared with steroid group.

and the steroid-treated group at all time points. The following Tarlov scores are reported as medians with the interquartile ranges displayed parenthetically. At 12 hours, the sham-injury, trauma control, and steroid groups scored 5, 1 (1–3), and 2 (1–4), respectively ( $p = 0.001$ ); at 24 hours they scored 5, 1 (0–3), and 3 (1–4), respectively ( $p = 0.004$ ); at 36 hours they scored 5, 1 (0–3), and 2 (1–4), respectively ( $p = 0.005$ ); and at 48 hours they scored 5, 0 (0–3), and 2 (1–4), respectively ( $p = 0.01$ ).

No statistically significant differences were noted, however, between results for sham-injured and ATL-146e-treated animals. At all time periods, the Tarlov scores were 5 for the sham-injured animals. Grades for the ATL animals were 4 (3–4) at 12 hours, 3 (3–4) at 24 hours, 3 (3–4) at 36 hours, and 3 (3–4) at 48 hours ( $p > 0.05$  for all).

Animals treated with ATL-146e fared significantly better than trauma control animals did at all time points (12 hours,  $p = 0.001$ ; 24 hours,  $p = 0.007$ ; 36 hours,  $p = 0.007$ ; 48 hours,  $p = 0.035$ ) and fared significantly better than the steroid-treated animals did at 12 hours ( $p = 0.05$ ). A trend toward improved hindlimb function, but not statistical significance, was noted in ATL-treated animals compared with steroid-treated animals at 24, 36, and 48 hours.

In summary, at 48 hours postinjury, ATL-146e-treated animals showed hindlimb function preservation equivalent to that of sham-injured animals, and motor function in steroid-treated animals was worse than in the ATL-146e group and better than in the trauma control group, but not significantly.

#### Qualitative Histopathological Analysis

Light microscopic examination of spinal cord tissue stained with MAP-2 and Luxol fast blue revealed distinct patterns within each treatment group. Spinal cord tissue from sham-injured animals had normal cytoarchitecture (Fig. 2A). The morphological characteristics of steroid-treated and ATL-146e-treated animals were remarkably similar, displaying small degrees of contusional change in the gray matter but overall excellent preservation of cytoarchitecture (Fig. 2C and D). Examination of injured spinal cord segments from trauma control animals, however, revealed marked contusional change in the gray matter, partial loss of gray–white differentiation, and loss of alpha motor neurons in the ventral horn (Fig. 2B).

#### Quantitative Histopathological Analysis

The number of intact, viable ventral horn motor neurons per high power field were counted in a blinded fashion and compared statistically among treatment groups. Neuronal viability was significantly higher in the sham-injured control group and in both treatment groups compared with the trauma control group. Values of neuronal viability for each group reported as neurons/hpf  $\pm$  standard error of the mean are as follows: sham group,  $12.4 \pm 0.3$ ; ATL group,  $12.1 \pm 1.4$ ; and steroid group,  $13.3 \pm 1.4$  compared with the trauma control group,  $7.5 \pm 1.5$ ;  $p < 0.04$ . Neuronal viability did not differ among ATL-treated, steroid-treated, and sham-injured animals. Results are depicted in Fig. 3.

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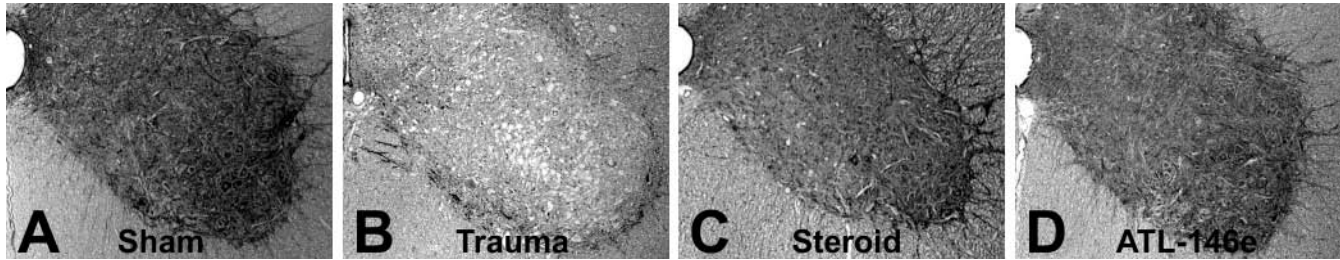


FIG. 2. Photomicrographs of ventral horn gray matter obtained in each treatment group. A: Spinal cord tissue from sham-injured animals had normal cytoarchitecture. B: Spinal cord tissue from trauma control animals displayed distinct patterns of central contusional change, gray matter vacuolization, partial loss of gray–white differentiation, and loss of ventral horn motor neurons. Spinal cord tissue from steroid-treated (C) and ATL-146e-treated (D) animals showed similar patterns of preservation of overall cytoarchitecture with only minimal contusional change, minimal loss of gray–white differentiation, and the presence of numerous pyramidal ventral horn motor nuclei with prominent nucleoli. Anti-MAP-2 and Luxol fast blue, original magnification  $\times 25$ .

### Discussion

Steroid treatment for blunt SCI remains controversial. The use of methylprednisolone is based principally on a series of studies in which improved neurological outcomes occurred if steroids were initiated within 8 hours of injury in the setting of blunt (nonmissile) SCI, with the caveat that patients receiving steroids had a trend toward higher rates of pneumonia and pulmonary embolus.<sup>3–5</sup> Subsequent studies as well as reviews of methods and data from the National Acute Spinal Cord Injury Study have failed to support the efficacy of methylprednisolone and have increased the controversy regarding its use in treating SCI.<sup>17,23,24</sup>

The proposed mechanism of action of steroids in blunt SCI has been attributed to effects on local blood flow, inhibition of immunological injury, and/or reduction of free radical-mediated lipid peroxidation and neuronal damage.<sup>19–21,32</sup> Adenosine and adenosine analogs have been shown to inhibit the inflammatory response, improve cerebral blood flow, and limit neuronal damage;<sup>2,12,26,50–52</sup> however, the use of systemic adenosine causes hypotension as well as cardiac and hemodynamic compromise that negatively counter possible benefits of adenosine therapy.<sup>28,36</sup>

Four specific adenosine receptors have been defined that may allow isolation of desired antiinflammatory effects from detrimental hemodynamic effects. One, the adenosine A<sub>2A</sub> receptor, is expressed on inflammatory cells, including neutrophils, mast cells, macrophages, eosinophils, platelets, and T cells,<sup>8</sup> and activation of the A<sub>2A</sub> receptor has been shown to be inhibitory on these cells.<sup>29,46</sup>

We have developed several compounds that act specifically on the adenosine A<sub>2A</sub> receptor. One of them, ATL-146e, has been shown to reduce inflammatory response by impairing oxidative burst in neutrophils.<sup>47</sup> Presumably through inflammatory inhibition, ATL-146e has been shown to improve outcomes in models of heart, lung, and renal ischemia–reperfusion injury.<sup>18,37,41</sup> Moreover, in an *in vivo* model of spinal cord ischemia–reperfusion injury, we demonstrated that ATL-146e given at the time of reperfusion preserved motor function while attenuating levels of several markers of inflammation, including intercellular adhesion molecule and systemic TNF.<sup>11</sup> Despite presumed immune modulation, results of toxicology studies have revealed that using ATL-146e A<sub>2A</sub> receptor activation does

not cause immune suppression or increased infection rates.<sup>29</sup>

We now understand that many pathophysiological features of spinal cord trauma are modulated by actions of the adenosine A<sub>2A</sub> receptor. Specifically, A<sub>2A</sub> receptor agonism can suppress both the inflammatory response and the induction of a specific pathway of apoptosis known to have deleterious effects in central nervous system injury.<sup>14,45</sup> Spinal cord injury induces a characteristic inflammatory response that can result in the apoptotic death of neurons and white matter damage after injury. Of particular importance is the markedly increased production and release of TNF and increased expression of TNF receptors in response to trauma.<sup>38,54,58</sup> Tumor necrosis factor causes white matter damage and also induces apoptosis via signaling through a family of “death receptors” linked to caspase activation. Activation of TNF receptors results in the activation of caspase-8, which directly activates the extrinsic pathway and indirectly activates the intrinsic pathway of apoptosis by cleavage and activation of the proapoptotic protein Bid.<sup>55,56</sup> It is important to note that apoptotic pathways of injury have been confirmed in human SCI as well.<sup>14</sup>

Previous studies have revealed that adenosine A<sub>2A</sub> receptor activation may affect each of these cascades of TNF-induced apoptosis by suppressing leukocyte recruitment and by reducing cytokine release, most notably that of TNF by monocytes.<sup>30,46</sup> Furthermore, immunological injury leads to a characteristic cavitation of white matter, with subsequent wallerian degeneration and scarring.<sup>42</sup> Suppression of inflammation mediated by A<sub>2A</sub> receptor agonism may also limit this process of white matter injury; A<sub>2A</sub> agonism also reduces both the Bad/Bcl-mediated extrinsic pathway of apoptosis and the caspase-8–induced, Bid-mediated intrinsic pathway of apoptosis,<sup>25,30,59</sup> both of which are active in SCI.

Two additional effects of A<sub>2A</sub> agonism may protect neurons in the setting of spinal cord trauma. Evidence of disruption of blood flow within the spinal cord develops early after the initial mechanical injury.<sup>33,43,48,49</sup> Disruption in blood flow results in local infarction due to hypoxia and ischemia.<sup>43</sup> This process is particularly damaging to gray matter because of its high metabolic requirement. The local microvasculature has A<sub>2A</sub> receptors, activation of which causes vasodilation.<sup>22,34,35,44</sup> Consequently, A<sub>2A</sub> ago-

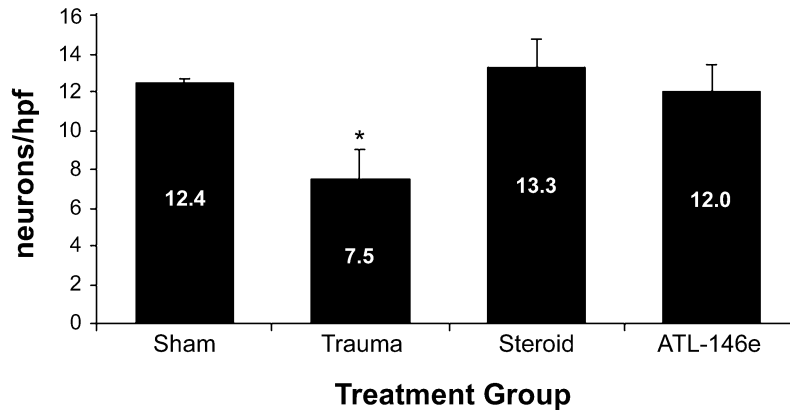


FIG. 3. Bar graph showing neuronal viability of spinal cord ventral horn motor neurons (viable neurons/hpf) among sham-injured, trauma control, steroid-treated, and ATL-146e-treated groups at 48 hours postinjury. Neuronal viability was significantly higher in the three other groups compared with the trauma control group (\* $p < 0.04$ , analysis of variance).

nism may increase blood flow and limit hypoxic–ischemic damage in SCI. (A similar effect caused by methylprednisolone was reported in the early experimental work with the compound.<sup>57</sup>) In addition,  $A_{2A}$  agonism has a direct effect of decreasing the metabolic rate and membrane currents in neurons,<sup>15,53</sup> further shielding neurons in the lesion cavity and possibly creating a milieu in which neurons can survive an otherwise lethal insult.

Given the parallels between secondary injury mechanisms in traumatic SCI and with that of spinal ischemia–reperfusion injury, the extension of ATL-146e therapy into blunt SCI was a natural progression. A pilot study was performed in our laboratory to evaluate the efficacy of using ATL-146e to treat blunt SCI in rabbits. In that study, adenosine  $A_{2A}$  receptor activation with ATL-146e after blunt spinal cord trauma led to significant improvements in hindlimb function at 12 hours postinjury with a trend toward preserving histological viability of motor neurons.<sup>12</sup> We later extended these findings to confirm that the use of ATL-146e results in sustained improvements in hindlimb function through 7 days postinjury.<sup>39</sup> Positive results notwithstanding, in those studies we did not compare ATL-146e use against the current gold standard of therapy, methylprednisolone.

In the current study, we confirmed and expanded our previous results demonstrating that ATL-146e administered early after blunt SCI significantly protects spinal cord function. Improvements in hindlimb motor function were preserved throughout the 48-hour postinjury observation period. Treatment with methylprednisolone, although trending toward improvement, did not produce statistically significant functional preservation compared with results for trauma control animals.

Furthermore, in terms of histopathological comparisons, ATL-146e treatment preserved neuronal viability at levels comparable to those measured for sham-injured animals and significantly better than those obtained in trauma control animals. The methylprednisolone treatment likewise preserved neuronal viability, but the implication of this observation remains unknown because the animals treated with methylprednisolone were not superior functionally to trauma control animals.

In our next generation of studies we will investigate whether functional improvements noted with ATL-146e are sustained at longer survival times. Such studies are hampered in a rabbit model by restrictions on our protocols (for example, rabbits unable to support their own weight at 48 hours postinjury must be killed for ethical reasons at our institution). As such, we have established a rodent model of weight-drop injury in our laboratory, and experiments involving ATL-146e are ongoing. In future studies we will also evaluate the therapeutic window and the utility of combination pharmacotherapy (methylprednisolone plus ATL-146e), as well as the effect of  $A_{2A}$  agonism using ATL-146e on markers of inflammatory and apoptotic injury.

### Conclusions

The adenosine  $A_{2A}$  receptor agonist ATL-146e is at least as effective as methylprednisolone in preserving function and is equivalent to methylprednisolone in preserving the structure of spinal cord tissue after blunt SCI. The ATL-146e may represent an effective treatment option for blunt SCI in the acute phase while bypassing adverse effects of steroid agents.

### Disclosure

Drs. Linden and Kron own equity in Adenosine Therapeutics, LLC, which owns the proprietary rights to ATL-146e.

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