

# Functional and cytoarchitectural spinal cord protection by ATL-146e after ischemia/reperfusion is mediated by adenosine receptor agonism

T. Brett Reece, MD,<sup>a</sup> Irving L. Kron, MD,<sup>a</sup> David O. Okonkwo, MD,<sup>b</sup> Jeffrey J. Laurent, MD,<sup>b</sup> Carlos Tache-Leon, MD,<sup>a</sup> Thomas S. Maxey, MD,<sup>a</sup> Peter I. Ellman, MD,<sup>a</sup> Joel Linden, PhD,<sup>c</sup> Curtis G. Tribble, MD,<sup>a</sup> and John A. Kern, MD,<sup>a</sup> Charlottesville, Va

**Background:** ATL-146e protects the spinal cord from ischemia/reperfusion injury, presumably via adenosine A<sub>2A</sub> receptor activation, but this relationship remains unproven. We hypothesized that spinal cord functional and cytoarchitectural preservation from ATL-146e would be lost with simultaneous administration of the specific adenosine A<sub>2A</sub> antagonist ZM241385 (ZM), thus proving that adenosine A<sub>2A</sub> receptor activation is responsible for the protective effects of this compound.

**Methods:** New Zealand White rabbits underwent 45 minutes of infrarenal aortic cross-clamping. Groups (n = 10) included sham, ischemia, ischemia plus ATL-146e (ATL-146E), ischemia plus ZM, or ischemia with both compounds (agonist-antagonist). Tarlov scores were recorded every 12 hours. After 48 hours, the spinal cord was fixed for histology and microtubule-associated protein 2 immunohistochemistry.

**Results:** Tarlov scores at 48 hours were significantly better in the sham and ATL-146E groups (5.0 and 3.9, respectively) compared with the other three groups (all  $\leq 1.3$ ;  $P < .001$ ). On hematoxylin and eosin, neuronal viability was higher in the sham, ATL-146E, and agonist-antagonist groups compared with the control and ZM groups ( $P < .05$ ). Microtubule-associated protein 2 expression was preserved in the sham and ATL-146E groups but was lost in the ATL + ZM, ZM241385, and control groups.

**Conclusions:** ATL-146e preserves the spinal cord in terms of both cytoarchitecture and function after reperfusion of the ischemic spinal cord, but this preservation is not completely blocked by competitive adenosine A<sub>2A</sub> receptor antagonism. Although ATL-146e does seem to partially function through activation of the adenosine A<sub>2A</sub> receptor, the neuroprotective mechanism may not be limited to this particular receptor. (J Vasc Surg 2006;44:392-7.)

**Clinical Relevance:** Paraplegia remains a real and devastating complication of vascular procedures that involve the thoracic aorta. Pharmacologic intervention has not yet had the proven success of other surgical adjuncts in limiting this complication, but treatment with adenosine analogues at reperfusion has shown promise in animal models. This study examined the specificity of the neuroprotective qualities of an adenosine receptor agonist in the setting of spinal cord ischemia/reperfusion injury with the ultimate goal of preventing neurologic complications in this specific subset of vascular patients.

Paraplegia is a significant and devastating complication for patients undergoing aortic aneurysm repair. This is especially true in higher-risk procedures of the thoracic aorta and in patients with previous abdominal aortic surgery. Paraplegia rates can range from 4% to 21%, depending on the location and extent of the aortic pathology.<sup>1-4</sup> Pharmacologic protection of the spinal cord during aortic surgery has not enjoyed the success that cerebrospinal fluid

drainage, local hypothermia, and distal perfusion have more recently demonstrated.<sup>5-7</sup> However, research has demonstrated promising success with some medications in multiple animal models of spinal cord ischemia/reperfusion.<sup>8</sup>

ATL-146e is among the pharmacologic interventions demonstrating promise. ATL-146e presumably works through adenosine A<sub>2A</sub> receptor activation on several different cell types, most notably smooth muscle cells and neutrophils.<sup>9,10</sup> Although the mechanism of this drug's function has been proven in other organ models of ischemia/reperfusion, the neuroprotective effect during spinal cord ischemia and reperfusion has not been fully elucidated. The mechanism can possibly be proven by blocking adenosine A<sub>2A</sub> receptor activation with an antagonist specific to the adenosine A<sub>2A</sub> receptor. ZM241385 (ZM) has been used in drug development to exclusively block the effects of A<sub>2A</sub> activation.<sup>11-14</sup> ZM is a competitive antagonist that requires a higher molar concentration to block the agonist's effects. If ATL-146e does indeed work through

From the Departments of Surgery<sup>a</sup> and Neurosciences<sup>b</sup> and the Cardiovascular Research Center,<sup>c</sup> University of Virginia.

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Reprint requests: T. Brett Reece, MD, University of Virginia Health System, Department of Surgery, PO Box 801359, MR4 Building, Room 3116, Charlottesville, VA 22908 (e-mail: tbr5q@virginia.edu)

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activation of the adenosine  $A_{2A}$  receptor, then the protective effects of the drug would be lost when combined with a higher dose of ZM.

Furthermore, previous studies have shown highly variable results in terms of spinal cord cytoarchitectural changes after spinal cord ischemia/reperfusion in animal models. The problem remains that many studies are restricted to short reperfusion periods for debilitated animals, but the neuronal degeneration and the ability to identify this degeneration may progress for 7 days or longer. Presently, our institutional vivarium policy limits our reperfusion times to 48 hours for animals with complete hind-limb paralysis and an inability to void, so better markers of early cytoarchitectural changes must be identified. Neuronal viability alone, in our experience, is probably too limited in this time frame. Microtubule-associated protein (MAP)-2 has been shown in neuronal ischemia to be a very sensitive early marker of ischemic insult.<sup>15</sup> The addition of MAP-2 expression may be a superior marker of cellular damage compared with our previously described neuronal viability alone.

The aim of this study was to establish that the effects of ATL-146e are specific to adenosine  $A_{2A}$  activation. We hypothesized that protection of the ischemic spinal cord with ATL-146e requires adenosine  $A_{2A}$  activation and that the addition of MAP-2 better evaluates early ischemia/reperfusion spinal cord injury.

## METHODS

**Procedures.** All protocols were reviewed and approved by the Animal Care and Use Committee of the University of Virginia. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals*, as described by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996).

The study used mature New Zealand White rabbits in a model of normothermic spinal cord ischemia/reperfusion. After induction and endotracheal intubation, they were ventilated with a volume ventilator at a tidal volume of 20 mL and a rate of 25 breaths/min. Throughout the procedure, they received vaporized halothane, which was titrated to appropriate sedation. Arterial catheters were placed in the ears for blood pressure monitoring, and the marginal ear vein was accessed for delivery of both normal saline and any intravenous therapy.

The animals were divided into five groups: sham, ischemic controls, ATL-146E treatment, ZM treatment, and ATL + ZM. Sham animals ( $n = 8$ ) underwent laparotomy with an open abdomen for 45 minutes before closure. Ischemia-reperfusion animals (IR;  $n = 10$ ) underwent laparotomy with 45 minutes of ischemia by cross-clamping the infrarenal aorta and inferior vena cava in addition to cross-clamping both vessels at the iliac bifurcation to ensure no backflow perfusion. The final three groups underwent procedures identical to the control except that ATL-146e-treated animals (ATL-146E;  $n = 10$ ) received  $0.06 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  of ATL-146e for 3 hours beginning 10

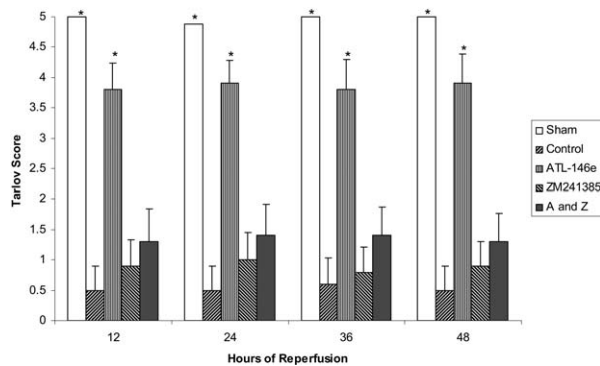
minutes before reperfusion, antagonist animals (ZM;  $n = 10$ ) received 10 times the molar equivalent ( $0.42 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) of ZM for 3 hours, and the combined-therapy group (agonist-antagonist;  $n = 10$ ) received both of these drugs at the previous dose and rate, simultaneously. Both drugs were given by using a Harvard microinfusion pump (Harvard Apparatus, Holliston, Mass).

The animals recovered until they were able to independently lift their heads. They survived for 48 hours under close supervision. All animals that were unable to sit up independently received a bolus of intravenous fluids twice per day and were hand-fed. At 48 hours, all animals were again anesthetized and perfusion-fixed with formalin. Spinal cords were harvested and placed in formalin for histology and immunohistochemistry.

**Function.** The Tarlov scale was used to assess functional outcomes at 12, 24, 36, and 48 hours. The Tarlov scale grades the animals according to their ability to move, sit, and hop. A score of 0 indicates complete paraplegia with no movement in the hind limbs. A score of 1 indicates that the animal has some hind limb movement. A score of 2 means the animal can sit with assistance, whereas 3 means it can sit on its own. A score of 4 means the animal has a weak hop, and 5 means the animal has a normal hop. The reported scores were graded by a blinded observer with no tie to the project.

**Neuronal viability.** Fixed spinal cords were sectioned into 5- $\mu\text{m}$  sections. Eight to 12 sections of each spinal cord were stained with hematoxylin and eosin. These sections were graded by a blinded observer who counted the number of viable motor neurons in each high-power field. Neurons were considered nonviable on the basis of loss of nuclear structure, cellular retraction from the surrounding tissue, and loss of cellular architecture. Neuronal viability was based on the number of viable neurons per high-power field.

**MAP-2 immunohistochemistry.** Lumbar spinal cords were embedded in paraffin, and transverse sections were cut with a microtome. Ten-micrometer sections were mounted on slides and deparaffinized in xylenes and progressive alcohol rinses. In preparation for immunocytochemistry, sections were processed with a temperature-controlled microwave antigen-retrieval approach described previously in detail.<sup>16</sup> Endogenous peroxidase was blocked by incubation in a solution of 1.65% hydrogen peroxide in 0.025% Triton X in Tris-buffered saline (TBS). Sections were then incubated overnight in mouse anti-MAP-2 antibody (clone HM-2; Sigma-Aldrich Co, St Louis, Mo) at a dilution of 1:100 in TBS with 1% bovine serum albumin. The following day, sections were rinsed in TBS/Triton X and then incubated for 2 hours in biotinylated anti-mouse immunoglobulin G (1:200; Vector, Burlingame, Calif) in TBS with 1% bovine serum albumin. After incubation in an avidin-biotin-peroxidase complex (ABC standard Elite kit; Vector; dilution, 1:100), sections were processed for visualization of the immunohistochemical complex by using 0.05% diaminobenzidine (Sigma-Aldrich) and 0.01% hydrogen peroxide. Once the sections were stained, they were



**Fig 1.** Functional outcomes. Hind-limb function was significantly preserved in the sham and ATL-146E groups compared with the other three groups at every time point ( $*P < .01$  vs control, ZM, and agonist-antagonist, A and Z).

randomly assigned names, and by using digital imaging, the percentage of gray matter staining for MAP-2 was obtained. Thus, MAP-2 is expressed as a percentage of staining per section of gray matter.

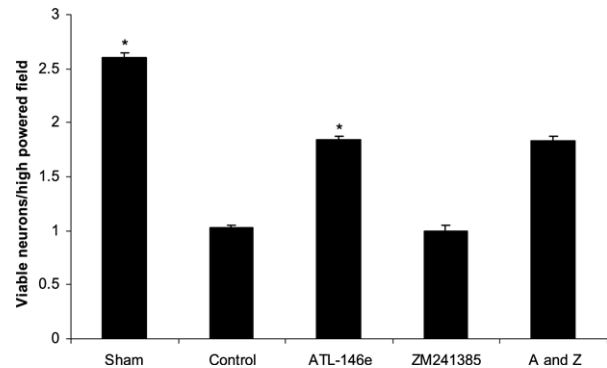
**Statistics.** The five groups were analyzed by using analysis of variance with a Bonferroni multiple comparison test to determine significant differences. Assumptions were tested, and nonparametric test results verified the results of the analysis of variance. The statistics for this study were performed by an independent statistician.

## RESULTS

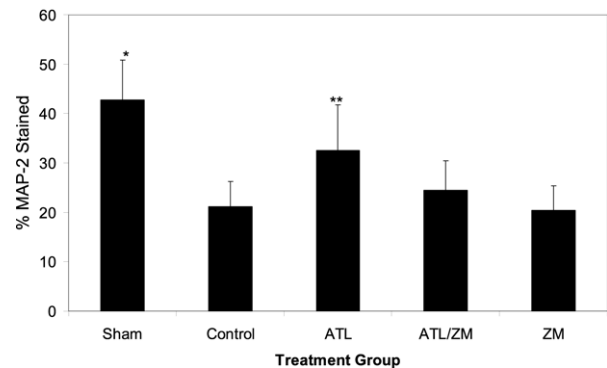
**Functional outcomes.** Tarlov scores at 12 hours were as follows: sham,  $5 \pm 0$ ; IR,  $0.5 \pm 0.40$ ; ATL-146E,  $3.8 \pm 0.44$ ; ZM,  $0.9 \pm 0.43$ ; and agonist-antagonist,  $1.3 \pm 0.54$ . Tarlov scores at 24 hours were as follows: sham,  $4.9 \pm 0.13$ ; IR,  $0.5 \pm 0.40$ ; ATL-146E,  $3.9 \pm 0.38$ ; ZM,  $1.0 \pm 0.45$ ; and agonist-antagonist,  $1.4 \pm 0.52$ . Tarlov scores at 36 hours were as follows: sham,  $5 \pm 0$ ; IR,  $0.6 \pm 0.43$ ; ATL-146E,  $3.8 \pm 0.49$ ; ZM,  $0.9 \pm 0.42$ ; and agonist-antagonist,  $1.4 \pm 0.48$ . Tarlov scores at 48 hours were as follows: sham,  $5 \pm 0$ ; IR,  $0.5 \pm 0.40$ ; ATL-146E,  $3.9 \pm 0.48$ ; ZM,  $0.9 \pm 0.41$ ; and agonist-antagonist,  $1.3 \pm 0.47$ . At all time points, both the sham and ATL-146E groups had significantly better function compared with the IR, ZM, and agonist-antagonist groups (each  $P < .001$ ). These findings are depicted graphically in Fig 1.

**Neuronal viability.** Sham animals had the highest neuronal viability, with  $2.6 \pm 0.09$  per high-power field. ATL-146E-treated animals had the next highest neuronal viability, with  $1.84 \pm 0.04$  per high-power field, closely followed by the agonist-antagonist group, with  $1.833 \pm 0.05$  per high-power field. The worst two neuronal viabilities were found in ZM ( $1.00 \pm 0.05$ ) and control ( $1.025 \pm 0.03$ ) animals. Sham, ATL-146E, and agonist-antagonist animals were all significantly better than both ZM and IR animals ( $P < .01$ ). Neuronal viability outcomes are presented graphically in Fig 2.

**MAP-2 immunohistochemistry.** MAP-2 expression was significantly preserved in sham animals compared with



**Fig 2.** Neuronal viability. Neuronal viability by hematoxylin and eosin demonstrated significant preservation of neuronal cytoarchitecture per high-power field in the sham group and both groups receiving ATL-146E ( $*P < .01$  vs IR and ZM).

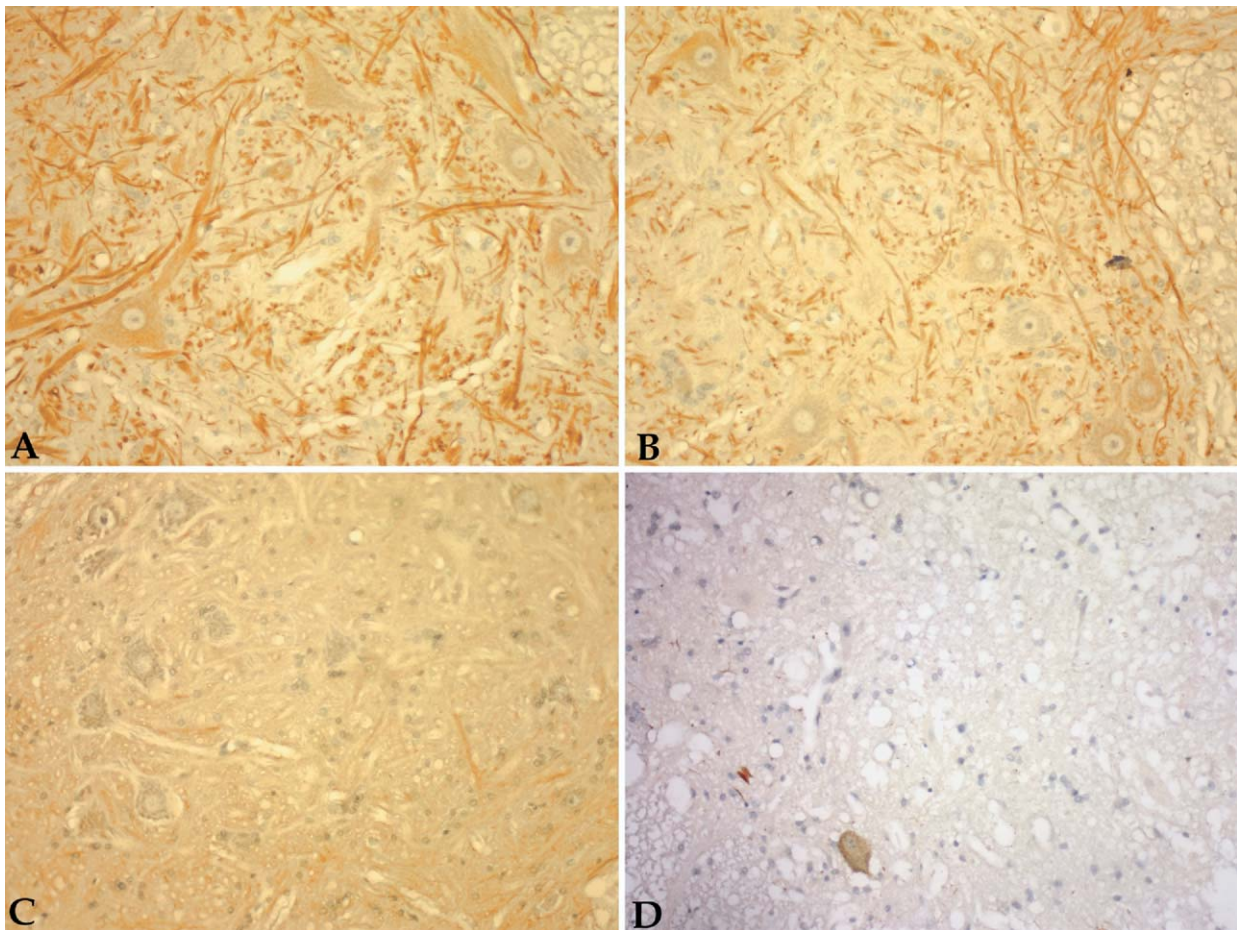


**Fig 3.** Microtubule-associated protein (MAP)-2. These photomicrographs depict the MAP-2 expression of spinal cord sections after ischemia/reperfusion. (A) and (B) depict the sham and ATL-146E groups, respectively, with preservation of the filamentous structures within the gray matter. (C) depicts the combined-therapy group, with some background staining but with complete loss of the neural filaments. Finally, (D) depicts the control and ZM groups, with complete loss of even background staining for MAP-2.

all other groups (all  $P < .01$ ). MAP-2 expression in ATL-146E animals was significantly better than in the ZM and IR groups, with a trend toward better preservation than in the agonist-antagonist group ( $P = .1$ ). MAP-2 expression was statistically similar among agonist-antagonist, ZM, and IR animals. Representative sections are shown in Fig 3, and MAP-2 expression is quantified in Fig 4.

## DISCUSSION

Transient occlusion of the thoracoabdominal aorta during vascular surgery carries a significant risk for postprocedural neurologic deficit. Recent studies in thoracoabdominal aneurysms suggest that this risk can be minimized with several modalities of spinal cord preservation, including cerebrospinal fluid drainage via the lumbar drain, regional hypothermia, and left heart bypass with distal perfu-



**Fig 4.** Microtubule-associated protein (*MAP*)-2. MAP-2 immunohistochemistry is depicted as the percentage of gray matter stained. \* $P < .01$  vs all other groups; \*\* $P < .01$  vs ZM and ischemia/reperfusion.

sion.<sup>5-7</sup> Although these techniques have improved neurologic outcomes in many patients, the extra procedure adds risks of additional complications, and they are not uniformly successful. A pharmacologic means of protecting the spinal cord from ischemia/reperfusion injury may obviate the need for more invasive strategies.

We have previously shown functional preservation of the spinal cord after ischemic injury with the use of hypothermic adenosine given in a retrograde venous fashion during ischemia.<sup>17-21</sup> The instability of adenosine at body temperature and the interruption of arterial flow to the ischemic tissue necessitated retrograde delivery during the ischemic period. These studies demonstrated significant functional improvement of the spinal cord with this retrograde delivery of cold adenosine during ischemia. However, the significant effort required for spinal cord protection via this route limits its potential clinical utility.

Recent studies have indicated that adenosine's effects are mediated through at least four defined receptors.<sup>9</sup> One such receptor, the adenosine  $A_{2A}$  receptor, seems to be responsible for many of the anti-inflammatory properties of adenosine. The adenosine  $A_{2A}$  receptor is predominantly

expressed on inflammatory cells and vascular smooth muscle. Both of these cell types are thought to play roles in the pathogenesis of injury after the reperfusion of ischemic tissues. Activation of the adenosine  $A_{2A}$  receptor can impair the inflammatory process and, at higher doses, lead to vasodilatation.

ATL-146e is a selective  $A_{2A}$ -receptor agonist with nominal cross-reactivity with  $A_3$  receptors.<sup>22</sup> ATL-146e has been shown in previous studies to preserve spinal cord function in models of both ischemic and traumatic spinal cord injury.<sup>23-26</sup> Although activation of the adenosine  $A_{2A}$  receptor was thought to be responsible for these protective actions, to our knowledge no study to date has addressed this mechanistic presumption.

In this study, we administered a competitive adenosine  $A_{2A}$  antagonist, ZM, to test whether competitive antagonism of the adenosine  $A_{2A}$  receptor blocks the neuroprotective effect of ATL-146e. A ZM dose of 10 times the concentration of ATL-146e was used to maximize receptor saturation by the antagonist. The neurobehavioral results of this study demonstrate that ATL-146e-mediated preservation of spinal cord function is lost with competitive

receptor blockade by ZM. Additionally, ZM alone did not result in further deterioration of function compared with control, thus implying that endogenous adenosine A<sub>2A</sub> receptor activation plays little or no role in innate functional protection from this mode of ischemic injury.

In terms of cytoarchitecture, our study presents some confounding results. Gray matter MAP-2 staining, indicating ischemic cytoskeletal injury, was significantly diminished in the IR control group and the ZM group compared with the ATL-146e and sham groups. MAP-2 staining in the agonist-antagonist group tended to be worse than the MAP-2 staining in the ATL-146e group, but with only a trend statistically ( $P = .1$ ).

With respect to the hematoxylin and eosin neuronal viability data, the sham, ATL-146e, and agonist-antagonist groups demonstrated preserved neuronal viability compared with the ischemia/reperfusion control and ZM groups. As with the MAP-2 data, coadministration of an antagonist with agonist failed to completely block the effect of ATL-146e on neuronal viability.

We suspect that the disconnect between the functional and the cytoarchitectural outcomes in the agonist-antagonist group may be due to three factors. First, the coadministration of agonists and antagonists causes a rightward shift of the dose-response curve of the agonist, thus limiting, but not eliminating, the effects of the agonist. Therefore, the combination of ATL-146e and the antagonist ZM may not result in complete blockade of ATL-146e effects. Second, although ATL-146e is a selective A<sub>2A</sub> receptor agonist, its effects are not limited to the adenosine A<sub>2A</sub> receptor only. Thus, competitive blockade of the adenosine A<sub>2A</sub> receptor would not completely block the actions of ATL-146e. ATL-146e is known to have limited activity at the adenosine A<sub>3</sub> receptor as well,<sup>22</sup> and the adenosine A<sub>3</sub> receptor has been shown to mediate ischemic preconditioning in isolated heart models of ischemia/reperfusion.<sup>26-28</sup> The low-level activation of the A<sub>3</sub> receptor could account for the incomplete blockade of the protective cytoarchitectural effects seen in our study with coadministration of an A<sub>2A</sub> competitive antagonist. The third factor affecting interpretation of our results is that, in our ischemia/reperfusion model, functional preservation occurs before cytoarchitectural changes. More specifically, in previous data, the neurobehavioral effects of ATL-146e were detected at earlier time points than protective effects on cytoarchitecture, which can take a week or longer to manifest.<sup>29</sup> On the basis of that study, we would expect the functional deficit to predict eventual cellular structure deterioration. Thus, in terms of this study, it may be that the process of cytoarchitectural degeneration was prolonged by administration of an antagonist and extended beyond the survival time used in the study design. Therefore, the divergence between functional and cytoarchitectural outcomes in this study may simply reflect a dampened neuronal response to ATL-146e in combination with ZM that still ultimately results in neuronal death.

This study raises intriguing questions about the potential contribution of A<sub>3</sub> receptor activation in adenosine-

mediated spinal cord protection. Although adenosine A<sub>2A</sub> receptor activation is the principal player in the attenuation of spinal cord ischemia/reperfusion injury, we cannot exclude a smaller contribution by adenosine A<sub>3</sub> receptor agonism. The study also builds on multiple previous studies to provide further evidence that systemic delivery of ATL-146e preserves spinal cord function and neuronal viability after ischemia/reperfusion injury. Clinical trials using this compound in this type of injury are imminent. To date, to our knowledge, no known adverse effects have been identified with the use of ATL-146e in animals or humans. Because ATL-146e is now in clinical trials as a cardiac imaging agent, it will soon be eligible for trials in other disease paradigms, including spinal cord injury and spinal cord ischemia/reperfusion.

This study supports our hypothesis that treatment of the ischemic spinal cord at reperfusion with ATL-146e can attenuate injury, with the activation of the adenosine A<sub>2A</sub> receptor playing a significant, but possibly not exclusive, role in its protective mechanism. In conclusion, ATL-146e treatment preserves spinal cord function and cytoarchitecture after spinal cord ischemia/reperfusion and may provide another option for neuroprotection in patients undergoing risky vascular procedures in the future.

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#### AUTHOR CONTRIBUTIONS

Conception and design: JAK, TBR, DOO, CGT, CT-L, ILK

Analysis and interpretation: TBR, DOO, JAK, VCGT, ILK, PIE

Data collection: TBR, CGT, CT-L, DOO, PIE, Writing the article: TBR, DOO, JAK

Critical revision of the article: TBR, DOO, JAK

Final approval of the article: TBR, DOO, JAK

Statistical analysis: Independent statistician

Obtained funding: JAK, JLL, ILK

Overall responsibility: TBR, DOO, JAK

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