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# Inhibition of adenosine kinase attenuates inflammation and neurotoxicity in traumatic optic neuropathy

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ABSTRACT

Traumatic optic neuropathy (TON) is associated with apoptosis of retinal ganglion cells. Local productions of reactive oxygen species and inflammatory mediators from activated microglial cells have been hypothesized to underlie apoptotic processes. We previously demonstrated that the anti-inflammatory effect of adenosine, through $A_{2A}$ receptor activation had profound protective influence against retinal injury in traumatic optic neuropathy. This protective effect is limited due to rapid cellular re-uptake of adenosine by equilibrative nucleotside transporter-1 (ENT1) or break down by adenosine kinase (AK), the key enzyme in adenosine clearance pathway. Further, the use of adenosine receptors agonists are limited by systemic side effects. Therefore, we seek to investigate the potential role of amplifying the endogenous ambient level of adenosine by pharmacological inhibition of AK. We tested our hypothesis by comparing TON-induced retinal injury in mice with and without ABT-702 treatment, a selective AK inhibitor (AKI). The retinal-protective effect of ABT-702 was demonstrated by significant reduction of Iba-1, ENT1, TNF-α, IL-6, and iNOS/nNOS protein or mRNA expression in TON as revealed by western blot and real time PCR. TON-induced superoxide anion generation and nitrotyrosine expression were reduced in ABT-702 treated mice retinal sections as determined by immunoflourescence. In addition, ABT-702 attenuated p-ERK1/2 and p-P38 activation in LPS induced activated mouse microglia cells. The results of the present investigation suggested that ABT-702 had a protective role against marked TON-induced retinal inflammation and damage by augmenting the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine.



## 1. Introduction

Traumatic optic neuropathy (TON) is partial or complete loss of function of optic nerve (ON) due to either a direct injury or indirectly after head trauma sequelae, such as edema, hemorrhage, and concussion (Steinsapir and Goldberg, 2011). Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute TON. Optic nerve injury mediated loss of retinal ganglion cells (RGCs) through apoptosis has been hypothesized due to several underlying common mechanisms, including lack of neurotrophin support, increased extracellular glutamate levels, damage from free radicals, and disruption of cellular homeostasis (Pang et al., 2010). All these mechanisms cause activation of microglial cells and inflammatory responses such as release of free radicals, cytokines, and prostaglandins and complement molecules (Lucas et al., 2006). Therefore, counteracting inflammation may possess neuroprotective effect in TON.

Adenosine is a ubiquitous homeostatic purine nucleoside that accumulates extracellularly in response to metabolic stresses such as hypoxia and inflammation. Activation of either G protein-coupled adenosine receptors (ARs; A1R, A2AR, A2BR, and A3R) by extracellular adenosine can modulate cell signaling. However, A2A receptor activation significantly modulates neuronal integrity and neuroprotection by adenosine receptor modulation has been demonstrated in several model systems (Lusardi, 2009). In accordance, we have demonstrated that $A_{2A}$AR signaling had a protective effect in traumatic optic neuropathy by attenuating microglia induced inflammatory response (Ahmad et al., 2013).

ARs agonists have limited therapeutic use due to systemic side effects (Fredholm et al., 2005). However, a promising alternative might

*Abbreviations:* TON, traumatic optic neuropathy; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; MAP kinase, mitogen-activated protein kinase; ERK, P38, extracellular signal-regulated kinase; AR, adenosine receptor; AKI, adenosine kinase inhibitor; ABT-702, 2-p-[2-Carboxyethyl]phenethylamino-5′-N-ethylcarboxamidoadenosine; LPS, lipopolysaccharides.

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be the augmentation of the adenosine levels by targeting enzymes or nucleoside transporters that regulate the extracellular levels of adenosine (Shen et al., 2012). Metabolic clearance of adenosine occurs through key enzyme adenosine kinase (ADK) and evidence shows that the inhibition of this enzyme increases extracellular adenosine levels in cell and tissues (Boison and Shen, 2010). Indeed, the inhibition of ADK has been proven to possess potential therapeutic usefulness in a wide range of neurological disorders (Boison, 2008). In this context, we previously reported that pharmacologic inhibition of ADK augments adenosine and exerts activity in retina of diabetic mice (Elsherbiny et al., 2013). Here, we seek to investigate the retinal protective role of ABT-702, a selective adenosine kinase inhibitor against marked TON-induced retinal inflammation and damage.

## 2. Materials and methods

### 2.1. Experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Georgia Regents University, Augusta, GA, USA guidelines. Eightto tenweek-old male wild-type (WT) mice (16 mice in each group) in C57BL/6 background were used for experiments. Mice were anesthetized according to standard protocol and limbal conjunctival peritomy was performed on one eye of each mouse. Forceps dissection under the conjunctiva posteriorly allowed access to the optic nerve, upon which pressure was placed 1 mm posterior to the globe until pupillary dilation was noted (approximately 10 s). Blood vessel close to optic nerve was carefully avoided in TON surgery. Mock-operated contra lateral eye served as the control. After one week, all mice were sacrificed. Eyes were enucleated and sectioned for histological analysis. Retinas were harvested for Western or Real Time PCR analysis. In pharmacologic studies, age-, weight- and sex-matched C57BL/6 mice were rendered optic nerve crush and then injected i.p. with vehicle (DMSO), or ABT-702 (AKI, Adenosine Kinase Inhibitor) (1.5 mg/kg bwt, i.p.) every other day for the duration of the study (n = 4–6/group).

### 2.2. Western blot analysis

Protein expression was measured by western blotting. In brief, washed cultured cells or retinal tissues were lysed in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.25% deoxycholate, supplemented with 40 mmol/L NaF, 2 mmol/L $Na_3VO_4$, 0.5 mmol/L phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 × *g* at 4 °C for 30 min. Protein was determined by Bradford method (Bio-Rad, Hercules, CA). 50–100 μg was boiled in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β-actin (Sigma), Iba1 (Wako, Japan), ADK, iNOS, nNOS, nitrotyrosine and ENT1 from Santa cruz Biotechnology Inc., CA, and phospho-ERK and ERK (Cell Signaling Technology, Beverly, MA) were detected with a horseradish peroxidase-conjugated antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

### 2.3. Immunohistochemical analysis

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (10 μm) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum (NGS) and then incubated overnight at 4 °C with primary antibodies: Rabbit anti-nitrotyrosine, rabbit anti-Iba-1 (Wako Pure Chemical, Wako, TX), or mouse anti-pERK1/2 antibody (Cell signaling technology, USA). Thereafter, sections were briefly washed with 1X PBS-T (0.1%) or 0.3% Triton X-100 and incubated with appropriate secondary antibodies (Invitrogen). Slides were examined under the fluorescence microscope (Carl Zeiss). Specificity of the reaction was confirmed by omitting the primary antibody. Data (10 fields/retina, *n* = 4–6 in each group) were analyzed using fluorescence microscopy and Ultra-View morphometric software or Image J software (NIH) to quantify the intensity of immunostaining.

### 2.4. Real-time PCR (isolation of RNA, synthesis of cDNA)

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad in a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). 50 ng of cDNA was amplified in each real-time PCR using Bio-Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers (Table 1). An average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization.

### 2.5. Analysis of dihydroethidium (DHE) fluorescence for the detection of superoxide

The detection of superoxide anion in the mouse eye sections was performed as described previously (Ahmad et al., 2013). In brief, mouse eyes were frozen in OCT and stored at −80 °C until use. Enzymatically intact eye sections were thawed in room temperature, rehydrated with PBS, incubated with dihydroethidium (DHE; 10 μMol/L in PBS) for 30 min at 37 °C in a humidified chamber protected from light. After incubation, sections were washed with PBS. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and emits red fluorescence. For the detection of ethidium, samples were examined with a fluorescence microscope (Axioskop 2 plus with AxioCam; Carl Zeiss, Germany; Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence was quantified using Image J software (NIH).

### 2.6. Mouse microglia cell culture, drugs treatment

The mouse micrgolial cell line EOC-20 was obtained from the American Type Culture Collection (ATCC CRL-2469, Manassas, VA, USA). Cells were maintained at 37 °C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 4 mM L-glutamine, and 20% conditioned medium from bone-marrow-derived Ladmac cells (ATCC CRL-2420) as a source of colony stimulating

**Table 1**
The primer sets used for the detection of mouse genes by quantitative real-time PCR analysis.

| Gene | Primer sequence (5′-3′) | Accession number |
|---|---|---|
| TNF-α | CCCTCACACTCAGATCATCTTCT<br>GTCACGACGTGGGCTACAG | NM_013693.2 |
| ENT1 | CAAGTATTTCACAAACCGCCTGGAC<br>GAAACGAGTTGAGGCAGGTGAAGAC | Am J Physiol Heart Circ Physiol 299:H847–H856, 2010 |
| Iba-1 | GTCCTTGAAGCGAATGCTGG<br>CATTCTCAAGATGGCAGATC | NM_019467 |
| iNOS | ACA TCG ACC CGT CCA CAG TAT<br>CAG AGG GGT AGG CTT GTC TC | Primer Bank ID 6754872 |
| IL-6 | TAGTCCTTCCTACCCCAATTTCC<br>TTGGTCCTTAGCCACTCCTTC | NM_031168.1 |
| GAPDH | CAT GGC CTC CAA GGA GTAAGAGAG<br>GGA GAT GCT CAG TGT TGG | M32599 |
| 18S | AGT GCG GGT CAT AAG CTT GC<br>GGG CCT CAC TAA ACC ATC CA | NR_003278 |