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Short communication

# Protective effects of 5-iodo-6-amino-1,2-benzopyrone, an inhibitor of poly(ADP-ribose) synthetase against peroxynitrite-induced glial damage and stroke development

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**Abstract**

Peroxynitrite triggers DNA single-strand breakage, which activates the nuclear enzyme poly(ADP-ribose) synthetase (PARS). Activation of PARS depletes its substrate, $NAD^+$, slowing the rate of glycolysis, electron transport, and ATP formation, resulting in cell necrosis. Here, we demonstrate that inhibition of PARS with the novel, potent PARS inhibitor 5-iodo-6-amino-1,2-benzopyrone ($INH_2BP$) protects against peroxynitrite-induced cell death (as measured by measurement of mitochondrial respiration and release of lactate dehydrogenase) in C6 glioma cells in vitro, and in a murine stroke model in vivo. Inhibition of PARS with $INH_2BP$ may represent a novel approach for the experimental therapy of stroke. 

*Keywords:* Nitric oxide (NO); Superoxide; Glia; Inflammation; Reperfusion

## 1. Introduction

Peroxynitrite and hydroxyl radical can induce DNA single-strand breakage, which is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS). Rapid activation of PARS depletes the intracellular concentration of its substrate, $NAD^+$, slowing the rate of glycolysis, electron transport and ATP formation. This process, as demonstrated in neurons, as well as in other cell types, including macrophages, smooth muscle cells, endothelial cells, epithelial cells, and pancreatic islet cells, can result in cell dysfunction and death. A major trigger of DNA single-strand breakage during reperfusion injury and various other pathophysiological conditions is peroxynitrite, a cytotoxic oxidant formed by the reaction of nitric oxide (NO) and superoxide (Zhang et al., 1994; Szabó et al., 1996, 1997b; Endres et al., 1997; Eliasson et al., 1997).

Although the studies using PARS knockout animals established that PARS is a major novel target for the experimental therapy of neuroinjury, data with potentially clinically applicable PARS inhibitors in stroke are lacking. The novel, potent PARS inhibitor 5-iodo-6-amino-1,2-benzopyrone ($INH_2BP$) was originally developed for antiviral and anticancer indications (Cole et al., 1991; Bauer et al., 1995, 1996). However, recent studies demonstrate that $INH_2BP$ inhibits peroxynitrite-induced injury in endothelial cells in vitro, and protects in murine models of local and systemic inflammation in vivo (Szabó et al., 1997a,c, 1998; Szabó, 1998). In the present study, we investigated whether $INH_2BP$ affects the course of peroxynitrite-induced cell death (as measured by measurement of mitochondrial respiration and release of lactate dehydrogenase) in C6 glioma cells in vitro and in a murine stroke model in vivo.

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## 2. Materials and methods

### 2.1. In vitro studies

#### 2.1.1. Cell culture

The rat astrocytoma cell line C6 (American Type Culture Collection, Rockville, MD, USA) was cultured in Ham's F12 medium supplemented with 15% horse serum, 2.5% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were cultured until 80% confluence. Cells were pretreated for 10 min with $INH_2BP$ (1–100 μM) and stimulated with peroxynitrite for 20 min (PARS assay) or 1 h (MTT and LDH assay) at 37°C. Peroxynitrite was synthesized by Dr. H. Ischiropoulos (University of Pennsylvania, Philadelphia, PA, USA), as previously described (Beckman et al., 1994). For treating the cells, authentic peroxynitrite was diluted in phosphate-buffered saline (PBS) pH 8.3 and was added to the cells in 1/10 of the volume of the cell suspension. Control samples were treated with PBS pH 8.3 only. Similar to previous studies (Szabó et al., 1996), this intervention did not affect the final pH of the culture medium, because of the small volume added, and because of the slight pH difference. The effect of decomposed peroxynitrite (kept in PBS pH 7.2 at 37°C for 2 h) was also tested in all assays and was found to have no effect on any of the parameters measured.

#### 2.1.2. Measurement of PARS activation

The incorporation of tritiated $NAD^+$ into nuclear proteins was measured as previously described (Szabó et al., 1996).

#### 2.1.3. Measurement of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction

The reduction of MTT, an indicator of cellular mitochondrial respiration, was measured by its conversion to formazan as previously described (Szabó et al., 1996).

#### 2.1.4. Measurement of lactate dehydrogenase (LDH) release

Cell membrane injury was quantitatively assessed by the measurement of LDH release by damaged cells (Pflueger et al., 1990). Supernatant (50 μl) was transferred to flat-bottomed microtitre plates and 50 μl of freshly prepared lactic acid dehydrogenase substrate mixture (54 mM l(+)lactate; 0.66 mM 2 *p*-iodophenyl-3 *p*-nitrophenyl tetrazolium chloride; 0.28 mM phenazine methosulphate; and 1.3 mM nicotinamide dinucleotide in 0.2 M Tris buffer, pH 8.2) was added to each well. Following a 5-min incubation in the dark at room temperature, the amount of LDH released was determined by measuring the $OD_{490}$ with reference to the $OD_{630}$. The percentage cytotoxicity was calculated by comparison to the maximum release of LDH achieved by the addition of 100 μl 0.08% Triton X-100.

### 2.2. In vivo studies

#### 2.2.1. Ischemia model

The in vivo experiments were performed in strict accordance with the guidelines of the National Institutes of Health and the Division of Animal Care, Massachusetts General Hospital. Adult male 129/SV mice (18–20 g, Taconic farms, Germantown, NY, USA) were anesthetized for induction with 1.5% halothane and maintained in 1.0% halothane in 70% $N_2O$ and 30% $O_2$ using a Fluotec 3 vaporizer (Colonial Medical, Amherst, NH, USA). Ischemia was induced with a 8.0 nylon monofilament coated with a silicone resin/hardener mixture (Xantopren and Elastomer Activator, Bayer Dental, Osaka, Japan) as described previously (Hara et al., 1996). The filament was introduced into the left internal carotid artery up to the anterior cerebral artery. By so doing, the middle cerebral artery and anterior choroidal arteries were occluded. For filament withdrawal, after 2 h of occlusion, the animals were briefly reanesthetized. Core temperature was maintained at $36.5 \pm 1$°C with a thermostat (FHC, Brunswick, ME, USA) and a heating lamp (Skytron, Daiichi Shomei, Tokyo, Japan) until 1 h after reperfusion and during the treatment and monitoring period. Animals were killed after 22 or 70 h of reperfusion.

#### 2.2.2. Treatment protocol

$INH_2BP$ (10 mg/kg or 30 mg/kg in 5% dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA)) in PBS was administered in a total volume of 0.3 ml intraperitoneally (i.p.) 2 h before induction of ischemia. Control animals were injected i.p. with a corresponding volume of 5% DMSO in PBS. For 70-h reperfusion studies, animals were injected twice, 2 h before middle cerebral artery occlusion and again directly after reperfusion (30 mg/kg $INH_2BP$ or vehicle).

#### 2.2.3. Infarct measurement

For infarct measurements, animals were decapitated and the brains divided into five coronal 2-mm sections using a mouse brain matrix (RBM-2000C, Activational Systems, MI, USA) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) (22-h reperfusion experiments). After 24 h, TTC-staining is inappropriate for the assessment of infarct size, because of white-blood-cell infiltration into ischemic tissue. Therefore, for the 70-h reperfusion experiments infarcts were evaluated on hematoxylin and eosin (H and E)-stained cryostat sections (20 μm). After killing the animals under anesthesia, brains were snap-frozen in 2-methylbutane and then subjected to cryostat sectioning. Infarction volume was quantitated in TTC-stained sections or H and E-stained cryostat sections with

an image analysis system (M4, St. Catherines, Ontario, Canada) and calculated by summing the volumes of each section determined directly (Huang et al., 1994) or indirectly using the following formula: contralateral hemisphere ($mm^3$) − undamaged ipsilateral hemisphere ($mm^3$) (Swanson et al., 1990). Differences between 'direct' and 'indirect' volumes are likely to be accounted for by brain swelling.

*2.2.4. Assessment of neurological deficits*

Mice were tested for neurological deficit and scored as described by Bedersen et al. (1986), with the following minor modifications (Hara et al., 1996): (0) no observable neurological deficit (normal), (1) failure to extent right forepaw (mild), (2) circling to the contralateral side (moderate), and (3) loss of walking or righting reflex (severe). The rater was naive to the treatment protocol and to the groups' identity. Assessments were made 30 min after onset of ischemia and 24 h after reperfusion.

*2.3. Data analysis*

Data are presented as mean ± S.E.M. Comparisons were made by two-tailed Student's *t*-test. For neurological

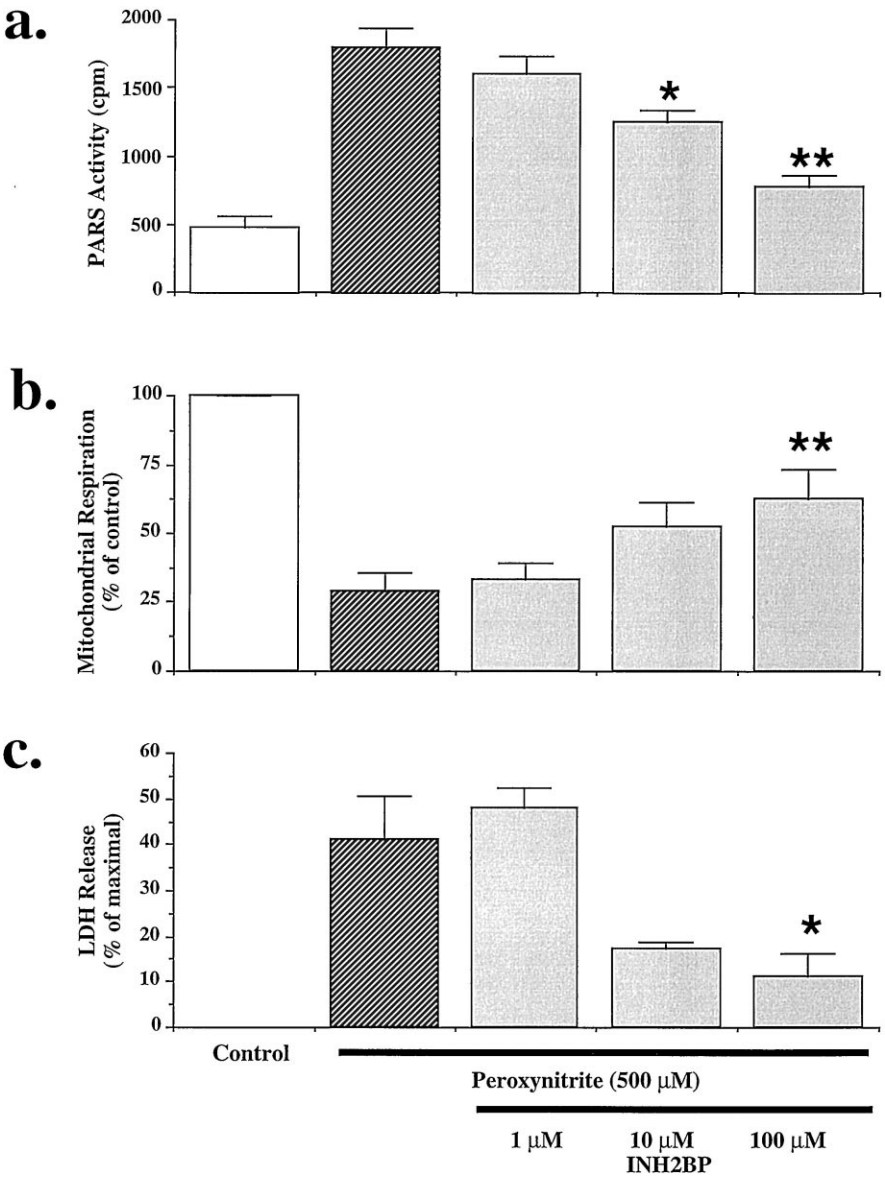


Fig. 1. $INH_2BP$ (1–100 μM) prevents PARS activation (A), the decrease in mitochondrial respiration, (B) and LDH release (C) in peroxynitrite (500 μM)-stimulated C6 cells. PARS activation is expressed as cpm per 1 million cells (A). Mitochondrial respiration in untreated cells corresponded to 0.061 ± 0.008 μg formazan produced per well per minute (B). LDH release is expressed as percentage of total cell lysis (elicited by the addition of the detergent Triton X-100; see Section 2) (C). $*\ P < 0.05$ or $**\ P < 0.01$ indicate significant effect of $INH_2BP$ compared to peroxynitrite alone; $n = 6–12$ wells per group.

deficits, the Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's test was used. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. $INH_2BP$ inhibits peroxynitrite-induced PARS activation and neuronal injury in glial cells in vitro

Pretreatment with $INH_2BP$ (1–100 μM) caused a dose-dependent inhibition of the peroxynitrite-induced activation of PARS in the C6 cells. The most potent effect of $INH_2BP$ was achieved with 100 μM concentration (Fig. 1). Peroxynitrite induced a decrease in MTT reduction and an increase in the LDH levels in the culture medium, indicative of suppressed mitochondrial respiration and disrupted cell membrane integrity, respectively (Fig. 1). $INH_2BP$ dose-dependently protected against peroxynitrite-induced injury (Fig. 1).

### 3.2. $INH_2BP$ reduces infarct size after transient middle cerebral artery occlusion

Pretreatment with 30 mg/kg i.p. ($n = 8$) 2 h before ischemia significantly reduced infarct size after 2 h of middle cerebral artery occlusion and 22 h reperfusion in 129/SV mice compared to controls ($n = 10$) (Fig. 2). With $INH_2BP$ at 10 mg/kg ($n = 11$), there was a trend toward smaller infarct sizes compared to controls (Fig. 2). All animals exhibited a neurological score of 2 or higher 30 min after the onset of ischemia. At 22 h of reperfusion, deficits were significantly ($P < 0.05$) improved in the 30 mg/kg group compared with controls ($1.8 \pm 0.1$ vs. $1.2 \pm 0.3$ vs. $1.0 \pm 0.2$ in vehicle-treated, 10 and 30 mg/kg $INH_2BP$-treated animals, respectively). To evaluate if the protective effects of $INH_2BP$-treatment sustain over time, 70-h reperfusion experiments were performed. After 70 h, treated animals ($2 \times 30$ mg/kg i.p., $n = 5$) had approximately 30% smaller infarcts compared to controls ($n = 8$) ($101 \pm 9$ $mm^3$ vs. $142 \pm 8$ $mm^3$, $P < 0.01$). The differ-

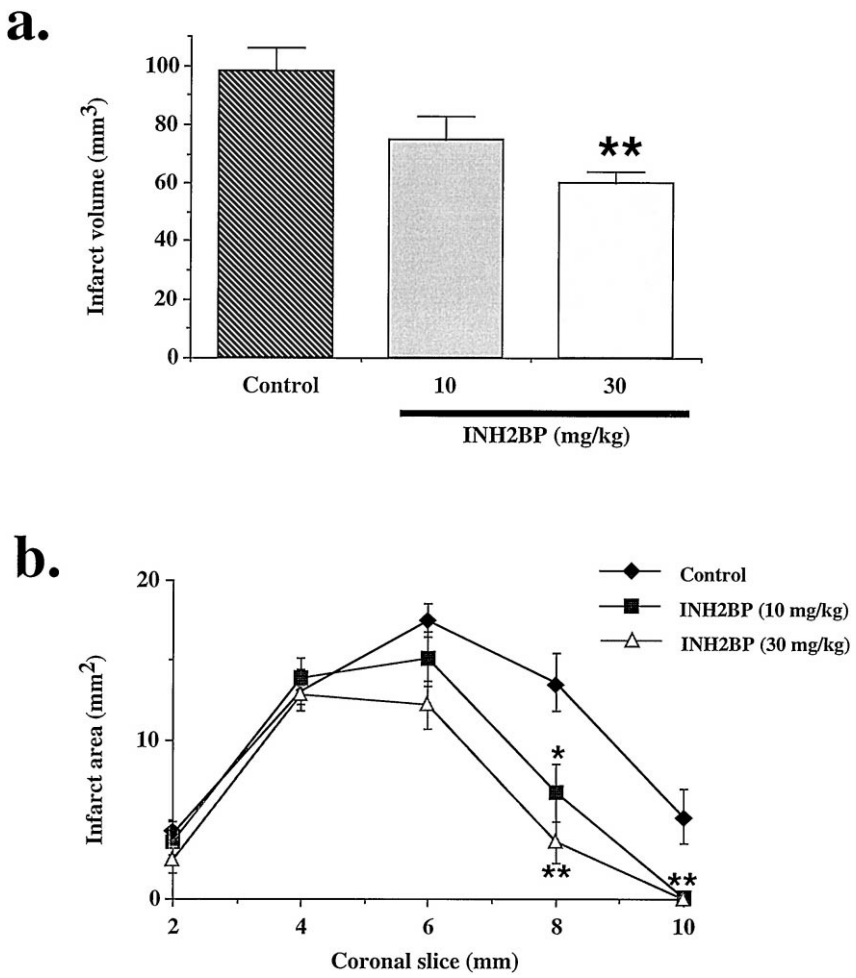


Fig. 2. Reduction in infarct size in $INH_2BP$-treated mice (30 mg/kg) compared to vehicle-injected controls 22 h after 2-h middle cerebral artery occlusion. Brain infarct volume (A) and brain infarct area (2-mm coronal sections) (B) in vehicle-injected mice (diamonds) and mice treated with 10 mg/kg (squares) or 30 mg/kg (triangles) $INH_2BP$. Drug was injected i.p. 2 h before onset of ischemia. $* P < 0.05$ or $** P < 0.01$ vs. vehicle; $n = 8$–11 per group.

ence was also significant ($P < 0.01$) when infarct size was evaluated with an indirect method that corrects for brain swelling ($70 \pm 3$ mm$^3$ vs. $102 \pm 5$ mm$^3$).

## 4. Discussion

The present study demonstrated that inhibition of PARS with the novel, potent inhibitor $INH_2BP$ provides a dose-dependent protection against glial cell injury in vitro and against stroke development in vivo. Along with a reduction in infarct size, neurological deficits were improved in treated animals indicating functional recovery after PARS inhibition.

Multiple studies demonstrate that a significant portion of the neuronal injury is related to overproduction of NO, due to the NMDA receptor activation, and subsequent activation of the neuronal NO synthase in the reperfused brain. NO, in turn, combines with superoxide, and the rapid formation of peroxynitrite induces rapid and pronounced oxidative and peroxidative injury (Szabó, 1996; Dalkara and Moskowitz, 1997; Samdani et al., 1997). Part of this oxidant injury in reperfusion is related to DNA single-strand breakage and activation of an energy-consuming cycle governed by the nuclear enzyme PARS. Recent studies using the prototypical PARS inhibitor 3-aminobenzamide (Endres et al., 1997) or genetically engineered animals lacking functional PARS gene (Endres et al., 1997; Eliasson et al., 1997) demonstrated the activation of PARS in neurons in the reperfusion phase of a murine model of stroke, and protection against the neuronal injury by inhibition of PARS. The activation of PARS in reperfusion is mainly related to activation of the neuronal isoform of NO synthase (Endres et al., 1998).

From these recent studies, PARS emerges as a major target for the development of novel pharmacological interventions for the treatment of stroke-related neuronal injury. However, only limited number of drug candidates are currently available for the safe and effective inhibition of PARS in vivo. $INH_2BP$ and related benzopyrone analogues were originally developed for antiviral and anticancer indications, considering the role of PARS in viral replication and malignant transformation (Cole et al., 1991; Bauer et al., 1995, 1996). In vivo studies demonstrated that $INH_2BP$ has good bioavailability and low toxicity (Bauer et al., 1995, 1996; Szabó et al., 1997c). So far, it has not been evaluated whether the protective effects of PARS inhibition or deletion after cerebral ischemia sustain over time, or, alternatively, whether infarct development is merely delayed (Eliasson et al., 1997; Endres et al., 1997). In this report we show that infarct sizes after $INH_2BP$ treatment are significantly reduced even 3 days after ischemia. The mechanism of action is directly related to inhibition of PARS, since $INH_2BP$ lacks oxidant and free radical scavenging properties (Szabó et al., 1998), but is a potent cellular inhibitor of PARS (Szabó et al., 1997a,c, 1998). On the basis of the present studies, which demonstrate an effective prevention of neural injury by $INH_2BP$ in vitro and in vivo, we conclude that the experimental therapy of stroke may represent a novel indication for the development of $INH_2BP$ and related PARS inhibitors.

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