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Research Report

# Aldehyde load in ischemia–reperfusion brain injury: Neuroprotection by neutralization of reactive aldehydes with phenelzine

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ABSTRACT

In ongoing studies of the neuroprotective properties of monoamine oxidase inhibitors, we found that phenelzine provided robust neuroprotection in the gerbil model of transient forebrain ischemia, with drug administration delayed up to 3 h post reperfusion. Since ischemia–reperfusion brain injury is associated with large increases in the concentrations of reactive aldehydes in the penumbra area, we investigated if the hydrazine function of phenelzine was capable of sequestering reactive aldehydes. Both aminoaldehydes and acrolein are generated from the metabolism of polyamines to putrescine by polyamine oxidase. These toxic aldehydes in turn compromise mitochondrial and lysosomal integrity and initiate apoptosis and necrosis. Previous studies have demonstrated that pharmacological neutralization of reactive aldehydes via the formation of thioacetal derivatives results in significant neuroprotection in ischemia–reperfusion injury, in both focal and global ischemia models. In our studies of acrolein and 3-aminopropanal toxicity, using an immortalized retinal cell line, we found that aldehyde sequestration with phenelzine was neuroprotective. The neuroprotection observed with phenelzine is in agreement with previous studies of aldehyde sequestering agents in the treatment of ischemia–reperfusion brain injury and supports the concept that "aldehyde load" is a major factor in the delayed cell losses of the ischemic penumbra.



## 1. Introduction

While it is not possible to protect the cells in an ischemic insult, the ischemic penumbra which surrounds this initial insult undergoes delayed cell death (Fisher, 2004). The concept of the ischemic penumbra suggests that there is a window of opportunity to provide pharmacological intervention and halt the delayed neuronal cell death. In this regard, the hallmark feature of cellular damage in the penumbra is the efflux of mitochondrial cytochrome *c* into the cytoplasm of neurons and the subsequent activation of caspases (Han et al., 2003; Zhao et al., 2005). This efflux of cytochrome *c* peaks at 4 to 6 h post-insult and appears to constitute the therapeutic window of opportunity for pharmacological agents to

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intervene (Green and Ashwood, 2005). There has been a vast array of mediators proposed to be involved in the delayed cell death of neurons in the ischemic penumbra. In studies of mediators involved in the premitochondrial phase of cell death in the penumbra, a number of investigators have demonstrated that increases in putrescine correlate with the ultimate volume of cell death in the penumbra (Baskaya et al., 1997; Paschen, 1992). While putrescine is not neurotoxic, aminoaldehydes and acrolein, generated by polyamine oxidase during the oxidation of spermine and spermidine to putrescine, are very toxic (Seiler, 2000). In this regard, polyamine oxidase (Liu et al., 2005) and the reactive aminoaldehyde 3-aminopropanal (Ivanova et al., 1998) are dramatically elevated in the penumbra with ischemia–reperfusion injury. Furthermore, inhibition of polyamine oxidase (Dogan et al., 1999a) or sequestration of reactive aldehydes via formation of thioesters with *N*-(2-mercaptopropionyl)glycine (Huang and Huang, 1990; Ivanova et al., 2002) provides significant neuroprotection in the penumbra. Increased 3-aminopropanal (3-AP) levels have also been shown to precede delayed neuronal cell death in the trimethyltin tin model of hippocampal CA3 neurodegeneration (Wood et al., 2006b). Studies *in vitro* and *in vivo* have also defined key roles for reactive aldehydes in apoptotic and necrotic mechanisms leading to both neuronal and glial cell death (Kruman et al., 1997; Ivanova et al., 1998; Ong et al., 2000; McCracken et al., 2000). In apoptotic mechanisms, acrolein demonstrates direct mitochondrial toxicity (Pocernich and Butterfield, 2003) while aminoaldehydes like 3-AP act at the premitochondrial phase of apoptosis, via lysosomal leakage or lysis (Li et al., 2003; Yu et al., 2003; Yu et al., 2004).

There are a number of pharmacological approaches that can reduce aldehyde load, but only a few have been investigated in stroke models. However, in all cases, these agents have been shown to be effective in a therapeutic window of treatment of at least 3 h post-insult in preclinical models of ischemia–reperfusion brain injury. These include aminoquanidine which can directly sequester aldehydes and which also can inhibit arginase thus decreasing the supply of ornithine for polyamine synthesis (Ivanova et al., 1998; Hipkiss, 2001); the mercapto agent *N*-(2-mercaptopropionyl)glycine (Huang and Huang, 1990; Ivanova et al., 2002) which sequesters aldehydes; and spin traps like phenylbutylnitrone which can be metabolized to *N*-t-butyl hydroxylamine thereby sequestering aldehydes (Atamna et al., 2001; Hipkiss, 2001).

Monoamine oxidase inhibitors are also compounds that have demonstrated significant neuroprotective actions (reviewed in Sowa et al., 2004). In this regard, a number of biogenic amines, in addition to polyamines, are possible precursors of reactive aldehydes (Toninello et al., 2004). Inhibition of amine oxidases therefore might underlie some of the neuroprotective properties of monoamine oxidase inhibitors (MAOI). However, in our studies of phenelzine, we found that this MAOI provided dramatic neuroprotection in the ischemic gerbil model and we undertook studies to understand these neuroprotective actions. In this regard, prior studies of aldehyde-mediated hepatotoxicity had demonstrated that hydralazine forms a hydrazone with reactive aldehydes and neutralizes their cytotoxicity (Kaminskas et al., 2004). We therefore undertook experiments to determine if the hydrazine function of phenelzine could also neutralize reactive aldehydes and block their cytotoxicity.

## 2. Results

### 2.1. *Ischemic gerbil*

In our studies of the neuroprotective actions of phenelzine, we found that subcutaneous administration once daily for 7 days, starting 3 h post reperfusion, provided significant neuroprotection of hippocampal CA1 neurons in the gerbil model of transient global ischemia–reperfusion injury (Fig. 1). This level of protection is comparable to that of *N*-(2-mercaptopropionyl) glycine (MPG) in the rat MCAO model (Ivanova et al., 2002).

### 2.2. *Hydrazone formation*

Previous studies have demonstrated by mass spectrometry that MPG forms thioesters with reactive aminoaldehydes at pH 7.4, thereby inactivating them (Ivanova et al., 2002). Similarly, we demonstrated that phenelzine readily formed a hydrazone derivative with 3-AP at pH 7.2 (Fig. 2) and at pH 4.5 (lysosomal pH). The hydrazone (M.Wt.=191) was derivatized with tBDMS (+114; M.Wt.=305; Wood et al., 2006a). GC–MS analysis revealed a strong [$MH^+$] ion of 306 with a retention time of 5.25 min. Using [$^2H_4$]phenelzine, the [$MH^+$] ion of 310 also had a retention time of 5.25 min verifying the hydrazone derivative.

### 2.3. *Neuroprotection of retinal ganglion cells*

As reported previously (Wood et al., 2006b), 400 μM 3-AP elicited a robust LDH release without cellular detachment (Fig. 3). Our previous observations that serum proteins in the cell culture media avidly sequester much of the 400 μM 3-AP (i.e. the $TD_{50}$ for 3-AP shifts over 6-fold to the left in low serum medium; Wood et al., 2006b), the free concentration of 3-AP in our assay would be less than 70 μM during the critical premitochondrial phase (up to 4 h post administration) of cell death when 3-AP enters the lysosomes and initiates lysosomal leakage (Yu et al., 2003). In the case of acrolein and $H_2O_2$, which demonstrate direct mitochondrial toxicity, the concentration response curves for LDH release demonstrated greater potency and efficacy for these cellular stressors.

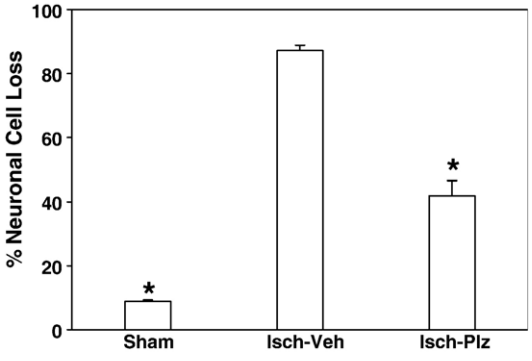


**Fig. 1 – Protection of hippocampal CA1 neurons in the ischemic gerbil model. Global ischemia was for 5 min, and phenelzine (15 mg/kg, sc; Isch–Plz) was administered 3 h post reperfusion and every 24 h thereafter for 7 days. Neuronal cell counts were made on day 8. *$p<0.05$ compared to values in ischemia–vehicle (Isch–Veh) gerbils.**

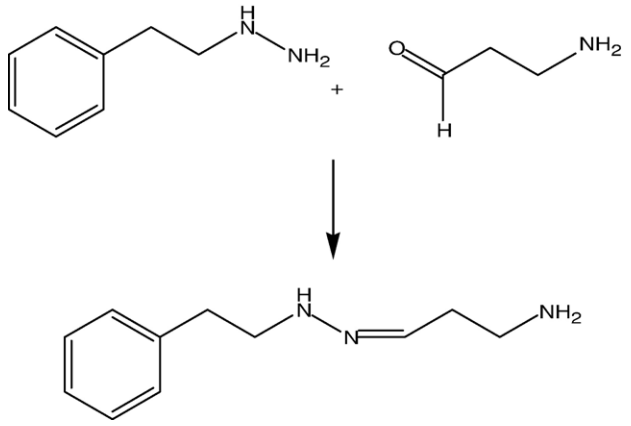

**Fig. 2 – Formation of the hydrazone after incubation of phenelzine and 3-AP in PBS for 2 h.**

Using this paradigm, we first demonstrated that MPG provided neuroprotection against 3-AP with a therapeutic window (i.e. delayed administration) of 2 h (Fig. 4). Similarly, phenelzine provided a concentration-dependent neuroprotection of retinal ganglion cells against 3-AP with a therapeutic window of 3 h (Fig. 5). Phenelzine was at least 2 times more potent than MPG, demonstrating the avidity of the hydrazine function for sequestering aldehydes, as previously shown with hydralazine (Burcham et al., 2002; Kaminskas et al., 2004). (–)Deprenyl (100–600 μM), which is a monoamine oxidase inhibitor lacking a hydrazine function, was inactive in protecting retinal ganglion cell cultures from 3-AP toxicity.

In the case of acrolein (50 μM) induced 24-hour LDH release, both MPG (300 μM) and phenelzine (100 μM) provided greater than 95% protection (Fig. 6). In contrast, neither agent protected against $H_2O_2$ (50 μM) toxicity (Fig. 6).

## 3. Discussion

In ischemic brain injury, the loss of mitochondrial integrity is the pivotal event leading to neuronal cell death in the penumbra (Kroemer et al., 1998). With the opening of the

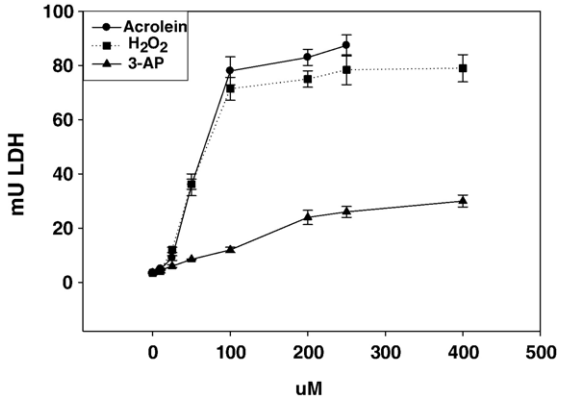


**Fig. 3 – Concentration response for 24 h LDH release induced by acrolein, 3-AP and $H_2O_2$. Media LDH was assayed 24 h after addition of the aldehydes or $H_2O_2$.**

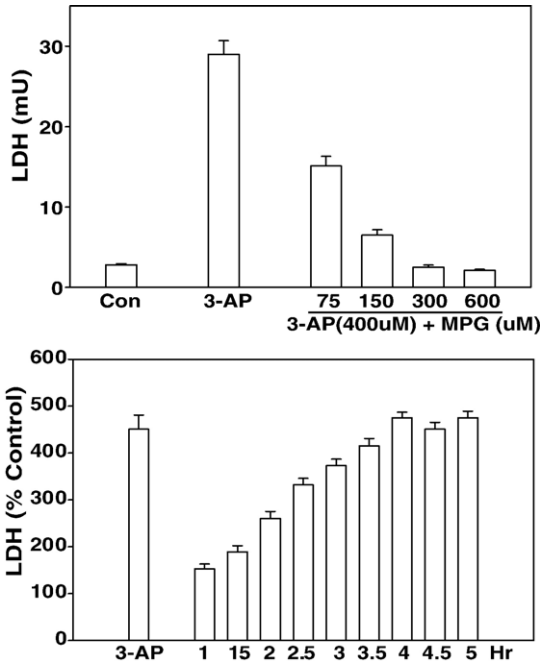


**Fig. 4 – Concentration response and delayed treatment studies for *N*-(2-mercaptopropionyl)glycine (MPG) protection of retinal ganglion cells from 3-AP-induced LDH release. 3-AP was 400 μM, and MPG was added as a co-treatment. For the delayed treatment (1–5 h) studies, MPG was 400 μM. Media LDH was assayed 24 h later.**

mitochondrial permeability transition pore, mitochondrial swelling leads to both a massive efflux of cytochrome c into the cytoplasm and to cellular energy failure. The cytochrome c in turn activates the caspase cascade (Fig. 7). The combined energy failure and caspase activation represent a time point at which pharmacological intervention may not be possible.

A number of pharmacological approaches have been investigated to intervene via mitochondrial stabilization and at the premitochondrial phase of cell injury. Stabilization of the mitochondrial transition pore with compounds like cyclosporine A provides significant protection (Han et al., 2003; Domanska-Janik et al., 2004); however, this approach is limited by a narrow therapeutic window of only 30 min (Domanska-Janik et al., 2004).

While a vast array of potential toxic mediators, involved in the premitochondrial phase of cell injury, have been characterized in models of brain ischemia–reperfusion injury, the case favoring a pivotal role of reactive aldehydes as mediators of cell death in the penumbra is strong (Seiler, 2000; Fig. 7). Specific points include:

1. Ischemia–reperfusion injury results in an early and dramatic augmentation of polyamine metabolism with induction of both ornithine decarboxylase and polyamine oxidase (Paschen, 1992; Baskaya et al., 1997; Liu et al., 2005).
2. Putrescine (Rohn et al., 1990) and 3-AP (Ivanova et al., 1998) accumulate in the penumbra within 2 h of an ischemic insult, supporting a dramatic augmentation of the polyamine interconversion pathway (Seiler, 2000).

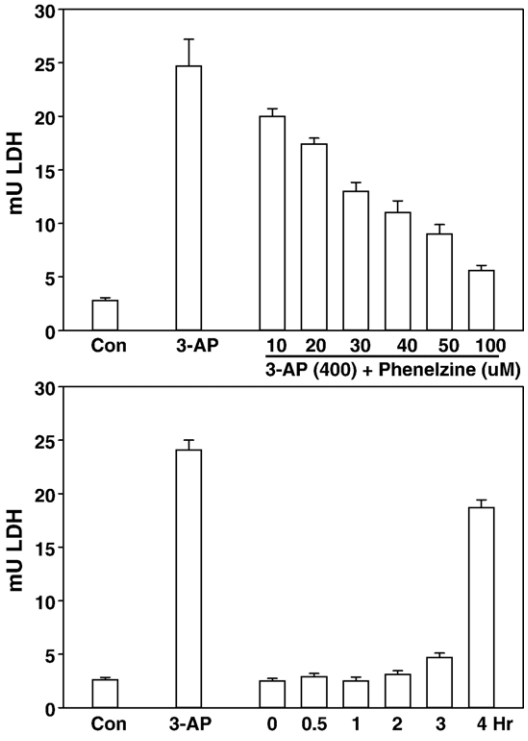


**Fig. 5 – Concentration response and delayed treatment studies for phenelzine protection of retinal ganglion cells from 3-AP-induced LDH release. 3-AP was 400 μM, and phenelzine was added as a co-treatment. For the delayed treatment (1–4 h) studies, 3-AP was 400 μM and phenelzine was 100 μM. Media LDH was assayed 24 h later.**

3. Neutralization of 3-AP and other reactive aldehydes via the formation of thioacetal adducts with MPG provides neuroprotection in the rat MCAO stroke model (Ivanova et al., 2002) and in the rat model of global brain ischemia (Huang and Huang, 1990).
4. 3-AP accumulates in lysosomes within 3 to 4 h leading to efflux of proteolytic enzymes that compromise mitochondrial integrity (Li et al., 2003). The exact mechanism of 3-AP damage to lysosomes has not been completely elucidated but may involve damage to the sulphydral-rich proton pump, sequestration of intracellular cysteine resulting in oxidative stress and aldehyde cross-linking of critical structural proteins (Li et al., 2003; Pisoni et al., 1990).
5. Microinjection of 3-AP into the brain kills neurons (Ivanova et al., 1998).
6. Inhibition of polyamine oxidase with the specific inhibitor MDL 72527 (Dogan et al., 1999a) or with the nonspecific inhibitor chloroquine (Ivanova et al., 1998) provides neuroprotection in the rat MCAO stroke model. In addition to inhibiting polyamine oxidase, chloroquine is a lysomotropic agent (Lu et al., 2002) that can increase lysosomal pH thereby inhibiting 3-AP entry.
7. Decreasing aldehyde production via inhibition of diamine oxidase ($IC_{50}$=14.9 nM; Holt and Baker, 1995) and semicarbazide-sensitive amine oxidase (IC50=10 μM; Yu and Zuo, 1997) with aminoguanidine provides neuroprotection in the rat MCAO model (Cockroft et al., 1996; Zhang et al., 1996; Ivanova et al., 1998). The data obtained with aminoguanidine are not simple to interpret since this compound inhibits several other enzymes and directly sequesters reactive aldehydes (Ivanova et al., 2002).
8. 3-AP protein adducts are increased in the CSF of patients with traumatic brain injury and subarachnoid hemorrhage (Ivanova et al., 2002). Inhibition of polyamine oxidase also has been shown to provide neuroprotection in a preclinical model of traumatic brain injury (Dogan et al., 1999b).
9. Polyamine oxidase and acrolein are elevated in the plasma of stroke patients (Tomitori et al., 2005).

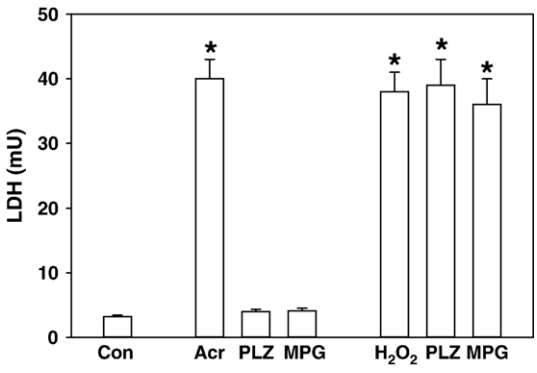


**Fig. 6 – Neuroprotection by phenelzine (PLZ) and MPG against acrolein (Acr, 50 μM) toxicity but not against $H_2O_2$ (50 μM) toxicity. Phenelzine and MPG were added as co-treatments. Media LDH was assayed 24 h later. *$p<0.05$ vs. controls (Con).**

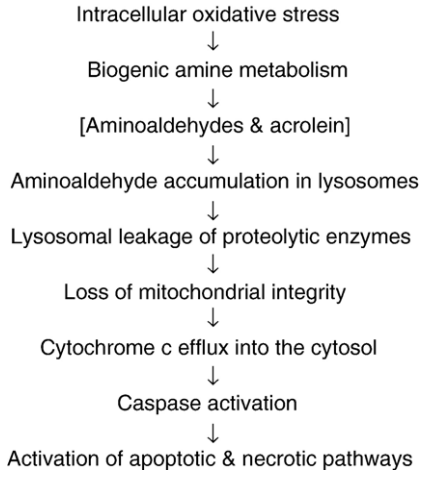


**Fig. 7 – Schematic summary of putative premitochondrial role of reactive aldehydes in apoptotic and necrotic pathways.**

In the gerbil model of reversible transient forebrain cerebral ischemia, hippocampal ornithine decarboxylase enzyme activity increases 7- to 16-fold (Paschen et al., 1990; Rao et al., 1995) and the concentration of putrescine increases approximately 4-fold (Paschen et al., 1988), supporting activation of polyamine synthesis and the polyamine interconversion pathway (Seiler, 2000). Immunocytochemical studies of the gerbil model have

demonstrated that the ornithine decarboxylase induction occurs in CA1 hippocampal neurons that are destined to undergo apoptosis (Maeda et al., 1998), suggesting that the generation of reactive aldehydes is intracellular and not from extracellular sources. Blockade of polyamine synthesis via inhibition of ornithine decarboxylase also provides hippocampal CA1 neuroprotection in this model (Kindy et al., 1994). Our data demonstrate the neuroprotective actions of phenelzine in the gerbil global ischemia model, with up to a 3-hour delay in drug treatment. This action may involve phenelzine inhibition of the amine oxidases, diamine oxidase ($IC_{50}$=1.95 μM; Holt and Baker, 1995) and semicarbazide-sensitive amine oxidase ($IC_{50}$=20 nM; Lizcano et al., 1996): each of which can generate reactive aldehydes (Seiler, 2000; Sowa et al., 2004). However, it is clear from our work that the hydrazine function of phenelzine readily forms hydrazones with reactive aldehydes at physiological pH, similar to previous reports for hydralazine (Burcham et al., 2002; Kaminskas et al., 2004). Our *in vitro* studies also show that phenelzine avidly neutralizes 3-AP even when its administration is delayed up to 3 h after 3-AP addition to cultured cells. While our data have been generated with the highly toxic aldehydes acrolein and 3-AP, it is important to note that phenelzine will also neutralize other aldehydes generated in the penumbra. This could include aldehydes from augmented polyamine metabolism, from oxidation of biogenic amines by amine oxidases, from lipid peroxidation, from oxidation of amino acids by myeloperoxidase and from glycation reactions (Toninello et al., 2004).

Another class of compounds that may act via sequestering reactive aldehydes is spin trap agents like α-phenyl-*N*-*tert*-butylnitrone (PBN) which is neuroprotective in models of ischemia–reperfusion injury (Green and Ashwood, 2005). In this regard, PBN can generate *N*-t-butyl hydroxylamine (Atamna et al., 2001; Hipkiss, 2001) which has been shown by HPLC to trap aldehydes (Atamna et al., 2001). Similarly, mercaptopropionylglycine has been demonstrated with electrospray mass spectrometry to form a thioacetal adduct with 3-AP (Ivanova et al., 2002), and in this report we demonstrate by GC–MS that phenelzine can form a hydrazone with 3-AP. As a result of the low levels of 3-AP produced *in vivo*, no direct evidence of adduct formation with aldehyde sequestering agents *in vivo* is yet available. This will be an important piece of evidence to validate the importance of aldehyde sequestration *in vivo*. It is important to note, however, that the actions of aldehyde-sequestering agents are complex in that they can (i) directly sequester free intracellular and extracellular aldehydes and aminoaldehydes; (ii) enter lysosomes and sequester lysomotropic aminoaldehydes, such as 3-AP (Li et al., 2003; Wood et al., 2006b); and (iii) "trap" protein bound aldehydes (Burcham and Pyke, 2006). Since 3-AP has a $pK_a$ of about 9.3 (Li et al., 2003) and phenelzine has a $pK_a$ of about 5.2 (Do Amaral et al., 1966), both compounds will concentrate in lysosomes for which the pH is around 4.5. In the case of phenelzine, our data demonstrate that it potently protects against both 3-AP and acrolein toxicity *in vitro* and provides significant neuroprotection in the ischemic penumbra of the gerbil global ischemia model. In contrast, no protection against $H_2O_2$ was provided by MPG or phenelzine, both of which have been shown to provide significant neuroprotection *in vivo* in ischemia–reperfusion models (Huang and Huang, 1990; Ivanova et al., 2002). These data would support previous suggestions that $H_2O_2$ only plays a minor role in cell death in the ischemic penumbra (Ivanova et al., 1998; Seiler, 2000).

With regard to specificity, phenelzine has a number of pharmacological actions which might contribute to its neuroprotective actions *in vivo* (Sowa et al., 2004). While we cannot definitively exclude other actions of phenelzine acting to provide neuroprotection *in vivo*, the lack of activity of deprenyl in our cellular assay is consistent with the hypothesis that aldehyde sequestration is the main mode of action of phenelzine. Deprenyl is an MAO inhibitor that does not possess a nucleophilic group to sequester aldehydes.

In summary, a number of pharmacological approaches are emerging that support the hypothesis that aldehyde-sequestering agents will provide a new generation of therapeutics for neurological disorders (Shapiro, 1998; Burcham et al., 2002). The data presented here demonstrate that compounds with a hydrazine function, like phenelzine, might prove to be useful drug candidates based on their avidity for sequestering reactive aldehydes. We have synthesized a number of analogues of phenelzine which contain a substituted hydrazine function and found that these compounds are weak inhibitors of monoamine oxidase but retain neuroprotective properties in the gerbil ischemia model and in the DSP4 neurotoxicity model (Todd et al., 1999; Sloley et al., 2001; Ling et al., 2001; Sowa et al., 2003, 2005). We are currently evaluating the aldehyde-sequestering properties of these compounds.

## 4. Experimental procedures

### 4.1. *Materials*

Dulbecco's Minimal Essential Medium (DMEM) was purchased from GIBCO, Long Island, NY. Fetal bovine serum (FBS) was from Hyclone, Logan, UT. The Cytotoxicity Detection Kit (LDH) was obtained from Roche Applied Science, Indianapolis, IN. The protein BCA kit was purchased from Pierce, Rockford, IL and 3-aminopropanal diethyl acetal from TCI America, Portland, OR. All other reagents were purchased from Sigma Chemicals, St. Louis, MO.

### 4.2. *3-Aminopropanal (3-AP) synthesis*

For the synthesis of 3-AP, 20 mmol of 3-aminopropanal diethyl acetal was stirred with 5 ml of 1.5 N HCl for 5 h at 25 °C. The reaction mix was next applied to a column (3×6 cm) containing Dowex-50, in the $H^+$ form. 3-AP was eluted with 2 N HCl and concentrated in a Savant concentrator. The 3-AP was characterized by 1H NMR (500 MHz, $D_2O$): δ 7.61 (br, 1H), 3.22 (q, 2H), 3.15 (q, 2H), 2.15 (d, 1H), 1.98 (d, 1H) and quantitated with the Purpald reaction. For GC–MS analysis, the 3-AP was derivatized with pentafluorobenzyl hydroxylamine (2 mg in 100 μl pyridine; 80 °C for 30 min) to form the oxime followed by derivatization of the free amino group with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluroacetamide plus 1% *tert*-butyldimethylchlorosilane (Wood et al., 2006a). The reaction product (M.Wt.=73+195+114=382) demonstrated a predominant $[MH]^+$ of 383 in ammonia PCI and a predominant $[M\text{-}HF]^-$ ion of 362 in ammonia NCI GC–MS.

### 4.3. *Retinal cell cultures*

The rat retinal cell line E1A-NR.3 (Seigel et al., 2004) was grown in DMEM, containing 10% FBS. For neurotoxicity assays, cells were plated in 48 well tissue culture plates and exposed to acrolein or 3-AP for 24 h. Media was collected and assayed for LDH using the Roche assay kit. Rabbit muscle LDH was used for the standard curve. Drug treatments with phenelzine and MPG were as co-treatments except in cases where delayed drug administration was investigated. Drugs and aldehydes were dissolved in PBS and sterile filtered prior to addition to cultures.

### 4.4. *Gerbil transient forebrain ischemia*

Male Mongolian gerbils (50–60 g) were anesthetized with halothane and a mixture of $N_2O$ and $O_2$ prior to making a small midline incision made in the ventral neck region. The ischemia group underwent a 5 min bilateral occlusion of the common carotid arteries after which vessel patency and re-establishment of blood flow were visually confirmed. Three hours post reperfusion, animals received either PBS or phenelzine (15 mg/kg, s.c.). Once daily dosing with phenelzine (15 mg/kg) continued for 7 days. On day 8, 24 h after the last phenelzine dose, animals were euthanized and the brains flash frozen in isopentane on dry ice. Frozen brains were cut coronally into 20 μm sections which were air dried prior to storage at – 80 °C. Slides were stained with H&E for neuronal cell counts of the CA1 region of the hippocampus. Cell counts were performed blinded.

### 4.4. *Hydrazone formation*

Phenelzine or [$^2H_4$]phenelzine (500 nmol) and 3-AP (500 nmol) were incubated in PBS at room temperature for 2 h. The reaction mix was next dried in a Savant concentrator prior to the addition of 50 μl of acetonitrile and 50 μl of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluroacetamide with 1% *tert*-butyldimethylchlorosilane. The samples were heated for 60 min at 80 °C and subsequently analyzed by GC–MS using ammonia PCI, which allows monitoring $[MH]^+$ ions of 306 and 310 for phenelzine and [$^2H_4$] phenelzine, respectively (Wood et al., 2006a).

### 4.5. *Statistical analysis*

All data are presented as mean ± SEM for groups of 6 to 8. Each experiment has been repeated at least 6 times over the last year to determine if the immortalized cell line might change its characteristics over time. This has not been an issue. Data were analyzed by one-way ANOVA followed by the Dunnett's *t* test for comparisons to control.

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