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A novel neuroprotectant PAN-811 protects neurons from oxidative stress

Research Article

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Abstract: Hydrogen peroxide (H_2O_2), a major non-radical reactive oxygen species (ROS) could elicit intracellular oxidative damage and/or cause extracellular free calcium influx by activating the NMDA receptor or through calcium channels. In the present study, NMDA receptor antagonist MK-801 fully blocked H_2O_2-induced neuronal cell death, whereas green tea (GT) extract containing-antioxidants only partially suppressed the neurotoxicity of H_2O_2. These suggest that majority of ROS overproduction is downstream of H_2O_2-induced calcium influx. A novel neuroprotectant PAN-811 was previously demonstrated to efficiently attenuate ischemic neurotoxicity. PAN-811 hereby fully blocks H_2O_2-elicited neuronal cell death with a more advanced neuroprotective profile than that of GT extract. PAN-811 was also shown to protect against $CaCl_2$-elicited neurotoxicity. Efficient protection against oxidative stress–induced neurotoxicity by PAN-811 indicates its potential application in treatment of ROS-mediated neurodegenerative diseases.

Keywords: *PAN-811 • Hydrogen peroxide • Neuroprotection • Chelation • Antioxidant*

1. Introduction

The reactive oxygen species (ROS) is implicated in aetiology of both acute and chronic neurodegenerative diseases. Various cell types including neurons produce large amounts of ROS under ischemia-reperfusion conditions, such as stroke [1,2]. Alzheimer's disease (AD) is a chronic neurodegenerative disease. The fact that AD occurs commonly in aged people indicates an important contribution of accumulated age-related risk factors to the initiation and development of the disease, although heritage could be an intrinsic determinant in familial AD. It has been well documented that the AD brain is characterized by significantly increased regional oxidative stress during AD [3-6]. Hydrogen peroxide (H_2O_2) is one of the major ROS that is overproduced in neuronal disorders. H_2O_2 elicits its toxic effects through either the ferrous ion (Fe^{2+})-dependent (Fenton reaction) or superoxide-driven (Haber-Weiss reaction) formation of the highly reactive hydroxyl radical, which leads to alteration of lipids, proteins and DNA, and a change in the redox status of the cytosol [1]. In contrast, recent research efforts have demonstrated that an excessive intracellular free calcium ($[Ca^{2+}]_i$) caused by either NMDA receptor [7,8] and/or non-selective cation channel [9,10] activations also actively mediates H_2O_2-induced neurotoxicity.

PAN-811 (or triapine) was originally developed for cancer therapy due to its bioactivity in inhibiting ribonucleotide reductase (RNR), a key enzyme in DNA replication, through its intrinsic characteristic of Fe^{2+} chelation [11]. In addition to Fe^{2+}, PAN-811 also binds

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to Cu^{2+} and Zn^{2+}, and thus suppresses the growth of a tumor [12]. Our previous results demonstrated that PAN-811 significantly reduces both ischemia-elicited excessive $[Ca^{2+}]_i$ and ROS possibly *via* dual individual mechanisms of Ca^{2+} chelation and free radical scavenging [13]. Therefore, we hypothesized that PAN-811 is able to block H_2O_2-induced neurotoxicity. The effect of PAN-811 was examined in H_2O_2-stressed cerebral neurons. Excitatory neurotoxicity seemed to dominate H_2O_2-induced neuronal cell death in our model since NMDA receptor antagonist MK-801 achieved more significant protection than green tea (GT) extract that contains antioxidants. In this system, PAN-811 fully blocked H_2O_2-induced neuronal cell death at concentrations of 5 - 10 μM (EC_{50}: 0.55 μM). PAN-811 was examined in $CaCl_2$-treated neuronal cultures, using MK-801 as a positive control. PAN-811 at a concentration of 2 μM completely blocked $CaCl_2$-induced neuronal cell death. These results indicate a potent neuroprotective activity of PAN-811 and underlying mechanism for its neuroprotection.

2. Experimental Procedures

2.1 Embryonic cerebral neuronal culture, compound treatment and H_2O_2 stress

Mixed cortical and striatal neurons from embryonic 17-day Sprague-Dawley rats were seeded onto poly-D-lysine (50 μg/ml) coated 96-well plates at a density of 50,000 cells/well [13]. To facilitate the oxidative stress experiment, the neurons were originally cultured in Neurobasal medium (NB) with B27 supplement containing anti-oxidants (AO) for one week, followed by replacing 50% of the medium 2 or 3 times with NB containing B27 without AO (Invitrogen), resulting in medium containing 12.5-25% AO. The neurons were cultured for over 12 days at 37°C, 5% CO_2. The neurons were generally treated with vehicle, 7:3 polyethylene glycol 300:ethanol (PEG: EtOH), PAN-811 (Kimia), MK-801 or extract of GT [13] overnight and then stressed with H_2O_2. To determine the protective window of PAN-811, neurons were also treated simultaneously with PAN-811 and H_2O_2 for co-treatment experiments. Similarly, neurons were treated with PAN-811 and $CaCl_2$ for the same time period in the Ca^{2+}-elicited toxic model.

2.2 Photomicroscopy

At the end of experiment, the cultures were photographed under an inverted phase contrast microscope (IX 70, Olympus) at magnification of 300x.

2.3 Lactate dehydrogenase (LDH) Assay

An aliquot of 35 μl of culture supernatant was incubated with 17.5 μl mixed Substrate, Enzyme and Dye Solutions (Sigma) at room temperature (RT) for 30 minutes. The preparations were then measured by spectrophotometry at a wavelength of 490 nm using a 96-well plate reader (Model 550, Bio-Rad). Conditioned culture medium from medium replacements was used as blanks for background.

2.4 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay

Neurons in 50 μl of culture medium were incubated with 10 μl of MTS at 37°C for 90 minutes. The preparations were then measured by spectrophotometry at a wavelength of 490 nm using a 96-well plate reader (Mode 550, Bio-Rad). Conditioned culture medium from medium replacements was used as blanks for background.

2.5 Data Analysis

The data were expressed as a percentage of the mean ± SD (n = 4-6) of the untreated and non-stressed control group. Statistical evaluation was performed using VassarStats with one- or two-factor ANOVA (significance level of 1%) followed by the Tukey HSD test. Pair comparison was carried out by t-test.

3. Results

3.1 PAN-811 provides potent neuroprotection against H_2O_2-elicited toxicity

Mixed cortical and striatal neurons were treated overnight with different concentrations of PAN-811 and then stressed with 35 μM H_2O_2 for 24 h. The neurons under non-stressed conditions showed phase-brilliant cell bodies (Figure 1A). In contrast, the neurons that received a stress with 35 μM H_2O_2 formed tiny fragments. Pretreatment with PAN-811 at all testing concentrations resulted in well-preserved neuronal morphology. H_2O_2 stress resulted in a 2.5-fold increase (with $P<0.01$, compared to untreated/non-insulted group) in LDH release, which directly indicates cell membrane damage and indirectly shows neuronal cell death quantitatively (Figure 1B). PAN-811 at a concentration as low as 1 μM suppressed LDH release by 78% (with $P<0.01$, compared to untreated/H_2O_2-insulted group) and at a concentration of 10 μM achieved maximal protection (90% of that in untreated and H_2O_2-insulted group) with an EC_{50} of ~0.55 μM.

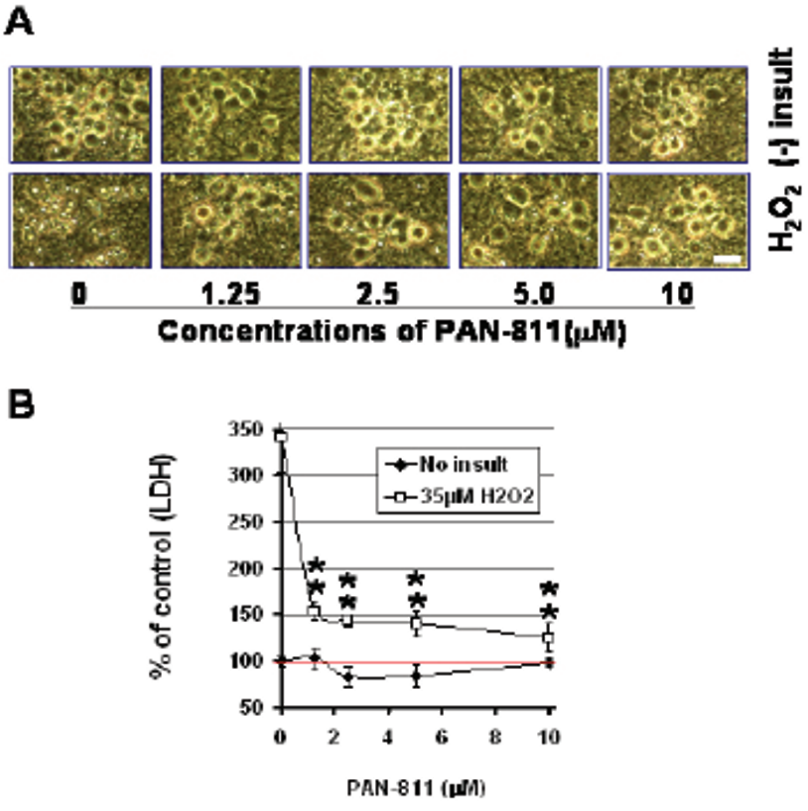

Figure 1. Neuroprotective activity of PAN-811.
Neurons were cultured in NB medium containing 25% AO for 12 days, treated with different concentrations of PAN-811 overnight and then stressed with 35 μM H_2O_2 for 24 h by taking neurons under unstressed condition as control. (A) The cultures were photographed under an inverted phase-contrast microscope. Scale bar = 25 μm. (B) Cell death was quantified with LDH analysis and expressed as a percentage of mean ± SD of the no-insult and untreated control group. **P<0.01 compared with untreated and H_2O_2-insulted group by ANOVA.

3.2 A pretreatment, but not co-treatment, with PAN-811 is neuroprotective

To understand the neuroprotective window, the neurons were either treated with 10 μM PAN-811 24 h prior to, or treated simultaneously with, H_2O_2 stress. Twenty-four hour stress with H_2O_2 resulted in strong neuronal cell death (Figure 2A and 2D). Pretreatment with PAN-811 resulted in well-preserved neuronal morphology (Figure 2A). However, PAN-811 administered into the culture simultaneously with H_2O_2 did not show any protection against H_2O_2–induced neuronal cell death (Figure 2D). Identical with these data, LDH readings under H_2O_2 stress conditions alone were 23- and 13-fold of untreated/non-stressed control group (P<0.01) in pretreatment (Figure 2B) and co-treatment (Figure 2E) experiments, respectively. Pretreatment with 10 μM PAN-811 reduced LDH release by 84% whereas a co-treatment with same concentration of PAN-811 only reduced LDH release by 28% with only a marginally statistical difference (P<0.05) from the untreated and H_2O_2 stressed group. On the other hand, H_2O_2 stress reduced MTS readings (indirect determination of cell viability by mitochondria-based color reaction) by 86% and 100% in pretreatment (Figure 2C) and co-treatment (Figure 2F) experiments, respectively. Pretreatment with PAN-811 increased the MTS reading by 38% with significant statistical difference (P<0.01) from the untreated and H_2O_2 stressed group. In contrast, a co-treatment with PAN-811 did not show any significant preservation in the MTS reading (no significantly statistical difference from the untreated and H_2O_2 stressed group).

3.3 NMDA receptor activation dominates H_2O_2-elicited neurotoxicity

To understand the underlying mechanism of neuroprotective activity provided by PAN-811, the toxic pathways of the H_2O_2 stress were investigated by using NMDA receptor antagonist MK-801 and GT extract containing free radical scavengers. The neurons were pretreated overnight with 5000-fold diluted vehicle (for

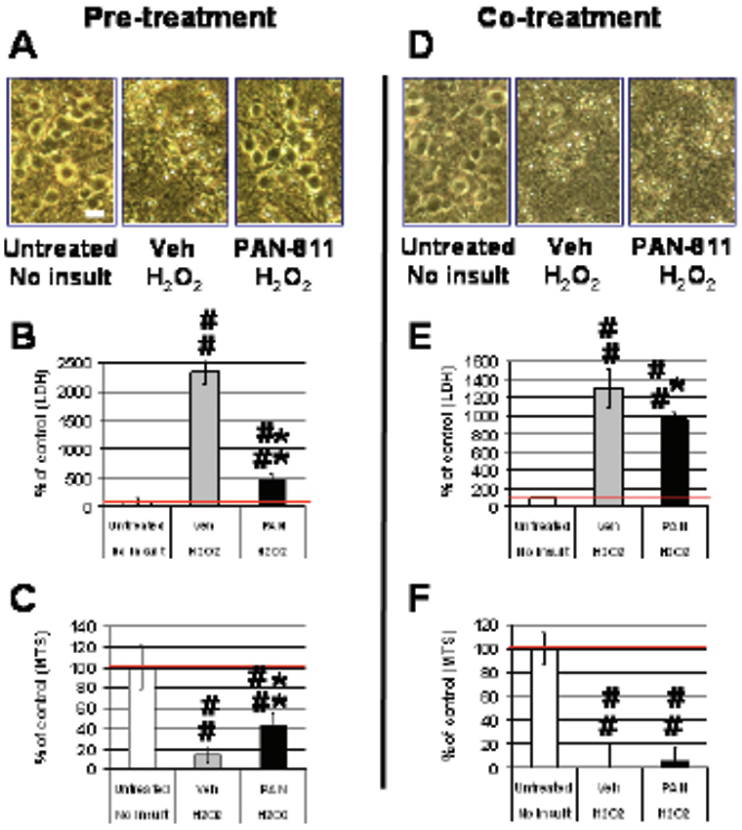

Figure 2. Neuroprotective window of PAN-811.
Mixed cortical and striatal neurons were cultured in NB medium containing 12.5% AO for 14 days, and treated for 1 day with 1:5,000 vehicle (Veh) or 10 μM PAN-811 either 1 day early (Pre-treatment) or at the same time (Co-treatment) with an insult of 35 μM H_2O_2. Photos (A and D) were taken 1 day post-insult. Scale bar = 25 μm. Neuronal cell death and survival were quantified with LDH (B and E) and MTS (C and F) analyses, respectively, and expressed as a percentage of mean ± SD of the no-insult and untreated control group. ##P<0.01 compared with untreated/non-insulted control group; *P<0.05 and **P<0.01 compared with untreated/ H_2O_2 group.

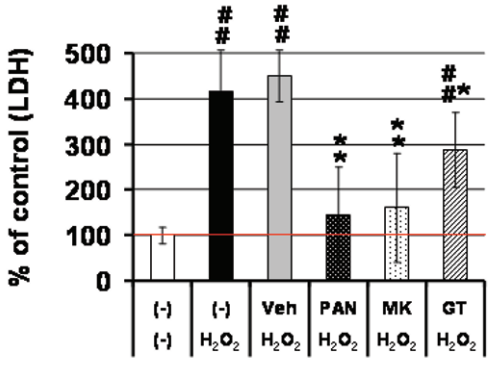

Figure 3. Comparison of PAN-811 with MK-801 and GT in neuroprotection.
Neurons were cultured in NB medium containing 25% AO for 12 days, treated overnight with 1:5,000 vehicle, 5 μM PAN-811, 5 μM MK-801 or 1:200 diluted GT extract, and then stressed with 70 μM H_2O_2. Neuronal cell death was quantitatively evaluated by LDH analysis and expressed as a percentage of mean ± SD of the untreated and no-insult control group. ##P<0.01 compared with untreated/non-insulted control group; *P<0.05 and **P<0.01 compared with vehicle-treated/ H_2O_2–stressed group.

PAN-811), 5 μM PAN-811, 5 μM MK-801 or 200-fold diluted GT extract and then stressed with 70 μM H_2O_2 (to facilitate mechanism study) for another day. LDH release in this experiment was examined (Figure 3). H_2O_2 stress resulted in 3.2 and 3.5 folds increase in LDH readings for untreated and vehicle-treated groups, respectively (P<0.01 compared to untreated and no stress group). A pretreatment with MK-801 brought LDH release down to about normal level with no significant difference from control. In contrast, a pretreatment with GT only achieved 41% protection with significant statistical difference from the H_2O_2 stressed group (P<0.05). These results indicate excitatory neurotoxicity elicited by H_2O_2 dominated neuronal cell death and intracellular ROS accumulation could be mainly Ca^{2+} influx-dependent. In comparison, a pretreatment with PAN-811 fully blocked H_2O_2 induced LDH release, showing no significant difference from the untreated/ non-stressed group. The full protection by PAN-811 implied that it predominantly suppresses $[Ca^{2+}]_i$ level.

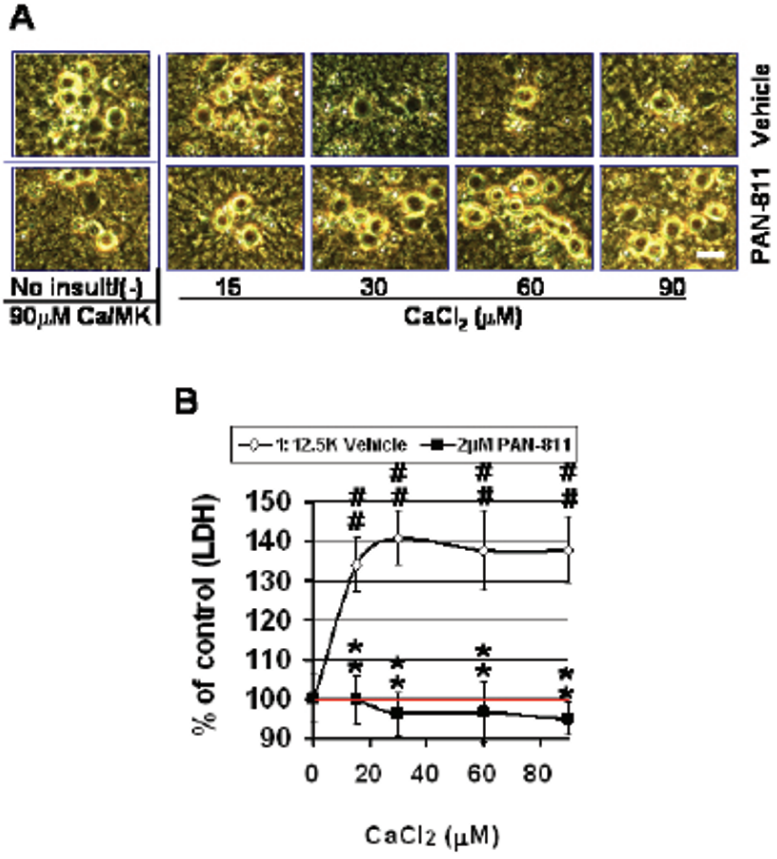

Figure 4. PAN-811 blocks $CaCl_2$-induced neurotoxicity.
Neurons were cultured in 12.5% AO and treated with different concentrations of $CaCl_2$ and 2 μM PAN-811. The cultures treated with 1:12,500 diluted vehicle and 10 μM MK-801 were taken as the carrier and positive controls, respectively. (A) The cultures were photographed under an inverted phase-contrast microscope. Scale bar = 25 μm. (B) The cell death was evaluated with LDH analysis and expressed as a percentage of mean ± SD of the no-insult and untreated control group. ##P<0.01 compared with untreated/no stress control group; **P<0.01 for pair comparison under a concentration of $CaCl_2$ by t-test.

3.4 PAN-811 blocks $CaCl_2$-induced neuronal cell death

Excessive extracellular free calcium ($[Ca^{2+}]_e$) is able to elicit neuronal cell death [14]. To further confirm the effect of PAN-811 on Ca^{2+}, neurons were co-treated with different concentrations of $CaCl_2$ and 2 μM PAN-811 for 10 days. A co-treatment with 90 μM $CaCl_2$ and 10 μM MK-801 was used as a positive control. $CaCl_2$ stress resulted in dose-dependent neuronal cell death in vehicle-treated groups (Figure 4A). A treatment with 2 μM PAN-811 or 10 μM MK-801 during the $CaCl_2$ stress period resulted in well-preserved neuronal and neurite morphology and density. $CaCl_2$ stress elicited a dose-dependent LDH release (Figure 4B). MK-801 at 10 μM suppressed LDH release to control levels (data not shown). Co-treatment with 2 μM PAN-811 fully blocked the LDH release under each $CaCl_2$ concentration (without significant statistical difference from the untreated/no insult group).

4. Discussion

H_2O_2 is a major non-radical ROS that is overproduced in different neurodegenerative diseases. H_2O_2 can inhibit the uptake of glutamate and enhances the release of glutamate, resulting in NMDA receptor overstimulation [7,15]. Consequently, H_2O_2 results in $[Ca^{2+}]_i$ increase [16]. Antagonists of the NMDA receptor show significant suppression of neurotoxic effects related to H_2O_2 [6]. Identical to these findings, our study demonstrates that NMDA receptor antagonist MK-801 protected well against H_2O_2–induced neuronal cell death. In contrast, GT containing free radical scavengers only partially suppressed the neurotoxicity. Thus, intracellular ROS accumulation could be predominantly downstream of Ca^{2+}influx in our experimental condition.

Non-specific Ca^{2+} channel activations are also indicated to be involved in the H_2O_2 toxic mechanism since non-specific Ca^{2+} channel antagonists fangchinoline and

tetrandrine significantly block H_2O_2-induced neurotoxicity [17]. H_2O_2 stress also causes Ca^{2+} release from intracellular stores, resulting in its accumulation in the cytoplasm [18]. In these conditions where Ca^{2+} originates from other sources rather than through activated NMDA receptors, MK-801 will be not sufficient to block H_2O_2-elicited toxicity. PAN-811 manifests a different mechanism from MK-801 in neuroprotection since PAN-811 directly chelates Ca^{2+} in a cell-free system [13]. To support this, PAN-811 in the present study protected neurons from $CaCl_2$-induced cell death no matter whether PAN-811 chelates Ca^{2+} extracellularly or intracellularly. In fact, PAN-811 is capable of entering the cell and nucleus since it has to bind to nucleus-located RNR to inhibit the bioactivity of this enzyme [11]. Thus the advantage of PAN-811 over MK-801 is to suppress $[Ca^{2+}]_i$ unrelated to the path from which it comes.

The underlying mechanism for the full neuroprotection provided by PAN-811 could be due to the antioxidative activity of PAN-811. Excitatory neurotoxicity-induced increase in $[Ca^{2+}]_i$ was found to be nontoxic in conditions preventing free radical generation [19,20]. Excessive ROS could be the executive factor for the cell toxicity of excessive $[Ca^{2+}]_i$. In contrast to these findings, in the present study, GT extract containing free radical scavengers could also partially suppress H_2O_2-induced neurotoxicity. Thus ROS production should not be an exclusive downstream signal of intracellular Ca^{2+} accumulation. Our previous study showed that PAN-811 is able to scavenge stable free radical diphenylpicrylhydrazyl and this activity seemed to be independent from its chelation activity since the experiment was carried out in a Ca^{2+}-free and cell free system [13].

Neuroprotective agents have been developed to suppress the toxicity of ROS. Vitamin E (α-tocopheral), the major lipid-soluble antioxidant can act directly with a variety of oxy radicals, including the peroxy radical (ROO•), CCl_3•, HO• as well as the superoxide radical (O_2•$^-$) [21-24], and protects against lipid peroxidation [25]. Vitamin C is water soluble and, along with vitamin E, can quench free radicals as well as singlet oxygen. Vitamin C has been shown to react directly with O_2•$^-$ [26,27], HO• [28] and singlet oxygen [29]. *Ginkgo biloba* protected neurons against Aβ-induced neuronal cell death [30]. On the other hand, the NMDA receptor antagonist memantine has been approved in phase III clinical trial in AD treatment [31]. However, each of the above reagents generally affects only one aspect of toxic pathways. PAN-811 has demonstrated dual independent functions in suppressing $[Ca^{2+}]_i$ and ROS. PAN-811 could be a potent drug candidate for neuroprotection.

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