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Communications

# Histamine-3 receptor antagonists reduce superoxide anion generation and lipid peroxidation in rat brain homogenates

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## Abstract

Using a cyanide model to induce neurotoxic effects in rat brain homogenates, we examined the neuroprotective properties of three $H_3$ antagonists, namely clobenpropit, thioperamide and impentamine, and compared them to aspirin, a known neuroprotective agent. Superoxide anion levels and malondialdehyde concentration were assessed using the nitroblue tetrazolium and lipid peroxidation assays. Clobenpropit and thioperamide significantly reduced superoxide anion generation and lipid peroxidation. Impentamine reduced lipid peroxidation at all concentrations used, but only reduced superoxide anion generation at a concentration of 1 mM. In the lipid peroxidation assay, all the drugs compared favourably to aspirin. This study demonstrates the potential of these agents to be neuroprotective by exerting antioxidant effects.

## Introduction

Over-production of free radicals is implicated in ageing and in various degenerative disorders (Nagy 2001; Kim et al 2002). Reduction of oxygen by the transfer of a single electron produces the superoxide anion, a source of hydrogen peroxide ($H_2O_2$), which has the ability to inhibit enzymes and, through reactions like the Fenton, produce hydroxyl radicals (Finkelstein et al 1980; Cheeseman & Slater 1993; McCord 2000). Membrane phospholipids are subject to lipid peroxidation (De Zwart et al 1999), a process that converts membrane lipids to hydroperoxides and finally to malondialdehyde, a marker of oxidative damage (Gutteridge & Halliwell 1990; Halliwell 1992). Antioxidants can alter these damaging processes by quenching free radicals and acting as oxygen scavengers (Gülçin et al 2002).

The histamine $H_3$ receptor is an autoreceptor, controlling the synthesis and release of histamine in cerebral neurons (Arrang et al 1983, 1987), but also acts as a heteroreceptor, regulating the release of dopamine and other neurotransmitters (Hamami et al 2004). Previous studies have suggested that $H_3$ receptors are under tonic dopaminergic influence (Ryu et al 1994). Reducing $H_3$-mediated inhibition of dopamine release with $H_3$ receptor antagonists could thus have therapeutic benefits in Parkinson's disease, a neurological disorder resulting from dopamine deficiency due to the destruction of dopaminergic neurons in the basal ganglia of the brain (Piggott et al 1999).

It is thus imperative to determine whether or not $H_3$ receptor antagonists can act as free radical scavengers and have a dual therapeutic effect, increasing dopamine levels and protecting dopamine neurons against free radical damage.

In the present study, a cyanide model was used to induce neurotoxicity. Cyanide inhibits a number of antioxidant enzymes (Ardelt et al 1989) and it was proposed that it generates reactive oxygen species, such as superoxide anion, through intracellular calcium influx, giving rise to lipid peroxidation and subsequent neuronal damage (Johnson et al 1987). The ability of $H_3$ antagonists to quench free radicals and to reduce lipid peroxidation could thus be determined using this model.

Superoxide anion generation was evaluated with the nitroblue tetrazolium (NBT) assay and malondialdehyde formation was used as an index for lipid peroxidation (De Zwart et al 1999; Mahadik et al 1999). Aspirin was used as the positive control as it was

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shown in previous studies in this laboratory to significantly curb increases in superoxide anion generation and lipid peroxidation in the millimolar concentration range (Daya et al 2000; Maharaj et al 2003).

## Materials and Methods

### Chemicals

Thioperamide maleate, impentamine ½fumarate and clobenpropit dihydrobromide were obtained from the Division of Medicinal Chemistry, Vrije Universiteit, Amsterdam, The Netherlands. All other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA), SAARCHEM (Pty) Ltd (Krugersdorp, South Africa) or Merck (Darmstadt, Germany) and were of the highest chemical purity.

### Animals

Adult male Wistar rats approximately 3 months old, weighing 250–300 g, were used in this study. Animals were housed in groups of five to six per cage under standardized conditions to minimize stress. Food and water were provided ad libitum. The experimental protocol followed was approved by the Rhodes University Animal Ethics Committee.

### Homogenate preparation

Rats were killed by cervical dislocation and the brains were rapidly excised and homogenized (10% w/v) with 0.1 M phosphate buffered saline, pH 7.4, to prevent lysosomal damage of the tissue. The homogenate was used for the lipid peroxidation and NBT assay.

### Sample preparation

Stock solutions of KCN and the $H_3$ antagonists were dissolved in Milli-Q water. One millimolar KCN and suprapharmacological concentrations (0–1 mM) of the $H_3$ antagonists were used.

### NBT assay

The NBT assay as described by Ottino & Duncan (1997) with nitroblue diformazan (NBD) as a standard was used in this set of experiments. A standard curve was generated by measuring the absorbance at 560 nm of appropriate concentrations of NBD dissolved in acetic acid. Brain homogenates (1 mL) containing 0, 0.25, 0.5 and 1 mM KCN in the absence and presence of 0, 0.25, 0.5 and 1 mM impentamine, thioperamide and clobenpropit were incubated with 0.4 mL of a 0.1% NBT solution in an oscillating water bath at 37°C for 60 min. Extraction of NBD was carried out by centrifugation of the samples at $2000 \times g$ and resuspension of the resulting pellets in 2 mL glacial acetic acid. The absorbance of the solutions was then measured at 560 nm and converted to micromoles diformazan using the generated standard curve. Final results are expressed as $\mu$mol mg$^{-1}$ protein.

### Protein assay

Protein estimation was performed using the method described by Lowry et al (1951).

### Lipid peroxidation

Lipid peroxidation was evaluated using the thiobarbituric acid-reactive substance (TBARS) production assay (Ottino & Duncan 1997), involving the reaction of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA) to yield a pink complex. A calibration curve was generated by measuring the absorbance of appropriate aliquots of 1,1,3,3-tetramethoxypropane at 5 nmol increments and plotting it against the molar equivalent weight of MDA in the assayed complex. Brain homogenates (1 mL) containing 0, 0.25, 0.5 and 1 mM KCN alone or in combination with 0, 0.25, 0.5, 1 and 1.5 mM impentamine, thioperamide and clobenpropit were incubated in a shaking water bath at 37°C for 60 min. After incubation, 0.5 mL butylated hydroxytoluene (0.5 g L$^{-1}$ in absolute methanol) and 1 mL 25% trichloroacetic acid were added to the mixtures. The samples were centrifuged at $2000 \times g$ for 20 min at 4°C to remove insoluble proteins. Two millilitres of the protein free supernatant was removed from each mixture and 0.5 mL 0.33% TBA was added. Mixtures were then heated for 60 min at 95°C in a water bath. After cooling, the TBA–MDA complexes were extracted with 2 mL butanol. The absorbance was read at 532 nm and MDA levels were determined from the generated standard curve. Final results are represented as nmol MDA mg$^{-1}$ tissue.

### Statistical analysis

The NBT assay and lipid peroxidation samples were tested a maximum of six times in order to determine the statistical difference (n = 6). The results were analysed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls Multiple Range Test. The level of significance was accepted at $P < 0.05$ (Zar 1974).

## Results

### NBT assay

The results of the NBT assay (Table 1, Figure 1) showed that KCN induced a significant concentration-dependent rise in superoxide anion generation in rat brain homogenates. Clobenpropit and thioperamide significantly curbed the 1 mM KCN-induced superoxide anion generation in a dose-dependent manner at all concentrations used (Table 1, Figure 1), with a reduction in superoxide anion generation of 50% at 1 mM of the drugs. Impentamine only reduced the 1 mM KCN-induced rise in superoxide anion generation significantly at the 1 mM concentration (Table 1, Figure 1).

**Table 1** The results of the NBT assay

| Compound | Concentration of test compound (mM) | Superoxide anion formation ± s.e.m. (n = 6) (μmol diformazan mg$^{-1}$ tissue) |
|---|---|---|
| Water (control) | | 6.29 ± 0.44 |
| KCN | 0.25 | 8.51 ± 0.28 |
| | 0.50 | 11.05 ± 0.37 |
| | 1.00 | 17.31 ± 0.76 |
| 1 mM KCN + aspirin | 0.25 | 14.04 ± 0.73 |
| | 0.50 | 12.05 ± 0.45 |
| | 1.00 | 9.43 ± 0.57 |
| 1 mM KCN + thioperamide | 0.25 | 14.03 ± 0.85 |
| | 0.50 | 10.32 ± 0.40 |
| | 1.00 | 8.22 ± 0.56 |
| 1 mM KCN + impentamine | 0.25 | 19.01 ± 0.41 |
| | 0.50 | 17.72 ± 0.54 |
| | 1.00 | 14.30 ± 0.30 |
| 1 mM KCN + clobenpropit | 0.25 | 13.01 ± 0.48 |
| | 0.50 | 10.68 ± 0.57 |
| | 1.00 | 7.90 ± 0.29 |

**Table 2** The results of the TBARS assay

| Compound | Concentration of test compound (mM) | Malondialdehyde formation ± s.e.m. (n = 6) (nmol MDA mg$^{-1}$ tissue) |
|---|---|---|
| Water (control) | | 0.19 ± 0.01 |
| KCN | 0.25 | 0.23 ± 0.01 |
| | 0.50 | 0.30 ± 0.01 |
| | 1.00 | 0.33 ± 0.01 |
| 1 mM KCN + aspirin | 0.25 | 0.33 ± 0.02 |
| | 0.50 | 0.33 ± 0.02 |
| | 1.00 | 0.26 ± 0.01 |
| 1 mM KCN + thioperamide | 0.25 | 0.20 ± 0.01 |
| | 0.50 | 0.19 ± 0.01 |
| | 1.00 | 0.17 ± 0.01 |
| 1 mM KCN + impentamine | 0.25 | 0.28 ± 0.01 |
| | 0.50 | 0.22 ± 0.01 |
| | 1.00 | 0.21 ± 0.01 |
| 1 mM KCN + clobenpropit | 0.25 | 0.21 ± 0.01 |
| | 0.50 | 0.19 ± 0.01 |
| | 1.00 | 0.15 ± 0.01 |

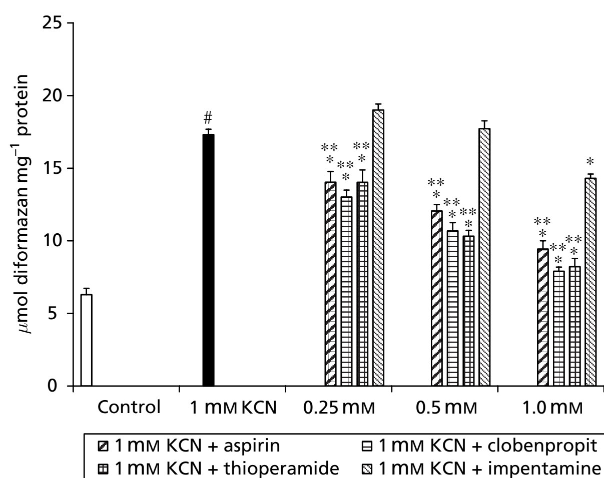


**Figure 1** The effect of histamine $H_3$ antagonists on 1 mM KCN-induced superoxide generation in whole rat brain homogenates. Each bar represents the mean ± s.e.m.; n = 6. $^{\#}P < 0.001$ compared to control, $*P < 0.001$ compared to KCN, $**P < 0.001$ compared to 0.25, 0.5 and 1 mM impentamine.

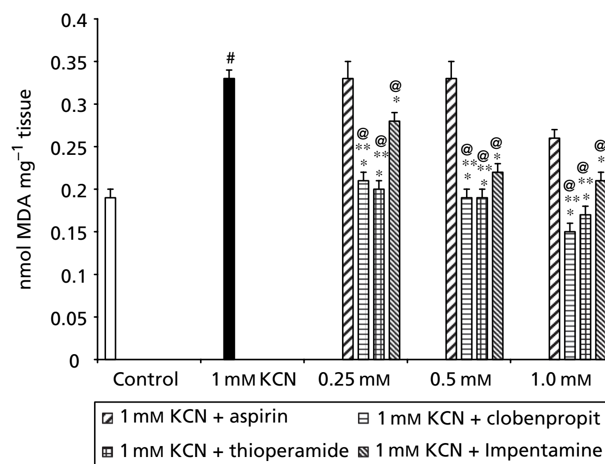


**Figure 2** The effect of histamine $H_3$ antagonists on 1 mM KCN-induced lipid peroxidation in whole rat brain homogenates. Each bar represents the mean ± s.e.m.; n = 6. $^{\#}P < 0.001$ compared to control, $*P < 0.001$ compared to KCN, $**P < 0.05$ compared to 0.25, 0.5 and 1 mM impentamine and @$P < 0.05$ compared to 0.25, 0.5 and 1 mM aspirin.

### Lipid peroxidation

The results of the lipid peroxidation assay (Table 2, Figure 2) showed that KCN induced a significant concentration-dependent rise in lipid peroxidation in rat brain homogenates. This rise in MDA concentration was significantly reduced by all three $H_3$ antagonists in a dose-dependent manner (Table 2, Figure 2). At 1 mM, thioperamide and clobenpropit reduced the MDA levels to a value lower than the basal control value.

## Discussion

In Parkinson's disease it is the free radicals that ultimately bring about neuronal death of the dopaminergic neurons in the substantia nigra (Koutsilieri et al 2002). Previous studies have shown that induced denervation of dopaminergic neurons results in a marked increase in the density of histamine $H_3$ receptors in the striatum and substantia nigra of the rat, suggesting that these receptors are under tonic dopaminergic influence (Ryu et al 1994).

The $H_3$ receptor is known to be involved in the release of dopamine (Rodrigues 1996). Reducing the $H_3$-mediated inhibition of dopamine release with histamine $H_3$ receptor antagonists and inhibiting oxidative stress by reducing free radical concentrations could have therapeutic application in neurological disorders such as Parkinson's disease (Esbenshade et al 2004; Witkin & Nelson 2004).

The present study thus examined the ability of three $H_3$ antagonists, namely clobenpropit, thioperamide and impentamine, to quench superoxide anion generation and reduce lipid peroxidation in a cyanide model. Since 1 mM KCN has been shown to significantly induce superoxide anion generation and lipid peroxidation (Maharaj et al 2004), as shown in Tables 1 and 2, this concentration of the toxin was used to determine whether or not the $H_3$ antagonists possessed any free radical scavenging properties. Suprapharmacological concentrations (0–1 mM) of the $H_3$ antagonists were tested for antioxidant effects due to the high concentration (1 mM KCN) of toxin being used. Previous in-vitro studies indicated that aspirin, a well-known non-steroidal anti-inflammatory drug, significantly curbed increases in superoxide anion generation and lipid peroxidation at the same concentrations and was therefore used as a positive control (Daya et al 2000; Maharaj et al 2003).

Clobenpropit and thioperamide significantly reduced superoxide anion generation as well as lipid peroxidation across the concentration range used. Thioperamide and clobenpropit, at a concentration of 1 mM, were able to reduce lipid peroxidation to values lower than the control (Table 2, Figure 2), thus suggesting that the drugs are able to reduce the free radical concentration from normal processes in the brain and not only from the induced free radical generation. Impentamine only reduced superoxide anion generation at a concentration of 1 mM, but effectively reduced lipid peroxidation at lower concentrations as well. The difference between aspirin, clobenpropit and thioperamide in reducing superoxide anion generation was not significant (Figure 1), but all three $H_3$ antagonists significantly reduced lipid peroxidation in comparison with aspirin (Figure 2). Relative to the positive control, aspirin, these compounds are thus more effective in reducing lipid peroxidation than scavenging superoxide anions, suggesting alternative mechanisms of action.

The results of this study show that clobenpropit and thioperamide are more potent in scavenging free radicals than the well-known antioxidant aspirin and that these drugs have the potential to be used in the treatment of neurodegenerative disorders.

## Conclusion

Neurodegenerative diseases such as Parkinson's disease are having an immense effect on an ageing society and it is important to identify beneficial agents that might be useful in reducing the risks of these diseases. It is evident that the $H_3$ antagonists used in this study have potential benefits as neuroprotectants through a dual mechanism of action, namely inhibition of oxidative stress and increasing dopamine levels by antagonism of the $H_3$ receptor. Further research is being done to investigate their mechanism of action and to prove their efficacy and safety in vivo.

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