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NEUROPROTECTIVE STRATEGIES FOR TREATMENT OF LESIONS PRODUCED BY MITOCHONDRIAL TOXINS: IMPLICATIONS FOR NEURODEGENERATIVE DISEASES

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Abstract—Neuronal death in neurodegenerative diseases may involve energy impairment leading to secondary excitotoxicity, and free radical generation. Potential therapies for the treatment of neurodegenerative diseases therefore include glutamate release blockers, excitatory amino acid receptor antagonists, agents that improve mitochondrial function, and free radical scavengers. In the present study we examined whether these strategies either alone or in combination had neuroprotective effects against striatal lesions produced by mitochondrial toxins. The glutamate release blockers lamotrigine and BW1003C87 significantly attenuated lesions produced by intrastriatal administration of 1-methyl-4-phenylpyridinium. Lamotrigine significantly attenuated lesions produced by systemic administration of 3-nitropropionic acid. Memantine, an *N*-methyl-D-aspartate antagonist, protected against malonate induced striatal lesions. We previously found that coenzyme Q_{10} and nicotinamide, and the free radical spin trap n-tert-butyl-α-(2-sulfophenyl)-nitrone (S-PBN) dose-dependently protect against lesions produced by intrastriatal injection of malonate. In the present study we found that the combination of MK-801 (dizocipiline) with coenzyme Q_{10} exerted additive neuroprotective effects against malonate. Lamotrigine with coenzyme Q_{10} was more effective than coenzyme Q_{10} alone. The combination of nictotinamide with S-PBN was more effective than nicotinamide alone.

These results provide further evidence that glutamate release inhibitors and *N*-acetyl-D-aspartate antagonists can protect against secondary excitotoxic lesions *in vivo*. Furthermore, they show that combinations of agents which act at sequential steps in the neurodegenerative process can produce additive neuroprotective effects.

These findings suggest that combinations of therapies to improve mitochondrial function, to block excitotoxicity and to scavenge free radicals may be useful in treating neurodegenerative diseases.

Key words: Parkinson's disease, Huntington's disease, mitochondria, free radicals, excitotoxicity, coenzyme Q_{10}.

Neurodegenerative diseases are illnesses such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and cerebellar degenerations. Much interest has focused on the role of excitotoxicity and oxidative damage in these illnesses.[2,9] One way slow or weak excitotoxicity could occur is as a consequence of a defect in energy metabolism.[1,3] This defect may lead to partial neuronal depolarization, relief of the voltage-dependent Mg^{2+} block of the NMDA receptor, and persistent receptor activation by ambient glutamate levels followed by free radical generation (Figure 1). Substantial experimental evidence supporting such a mechanism has been obtained *in vitro*, and more recently *in vivo*.[1,3]

We and others found that several agents which inhibit the mitochondrial electron transport chain produce secondary excitotoxic neuronal degeneration *in vivo*.[4,5,26] These compounds include 1-methyl-4-phenylpyridinium (MPP^+, the active metabolite of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), which is a selective inhibitor of complex I,[26] and malonate and 3-nitropropionic acid (3-NP), which are reversible and irreversible inhibitors of succinate dehydrogenase, respectively.[4,5] MPTP treated animals have been used to model Parkinson's disease, while both malonate and 3-NP treatment model many of the features of Huntington's disease. We and others found that the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 can attenuate MPP^+ and malonate induced striatal lesions.[4,14,26] Malonate lesions were also attenuated by the glutamate release inhibitor, lamotrigine.[15] Two compounds which are putative enhancers of mitochondrial function, coenzyme Q_{10} and nicotinamide, block both malonate

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Abbreviations: MK-801, dizocipiline; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; NBQX, 6-nitro-7-sulphanoylbenzo (f)-quinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; 3-NP, 3-nitropropionic acid; S-PBN, n-tert-butyl-α-(2-sulfophenyl)-nitrone; TTC, triphenyltetrazolium chloride.

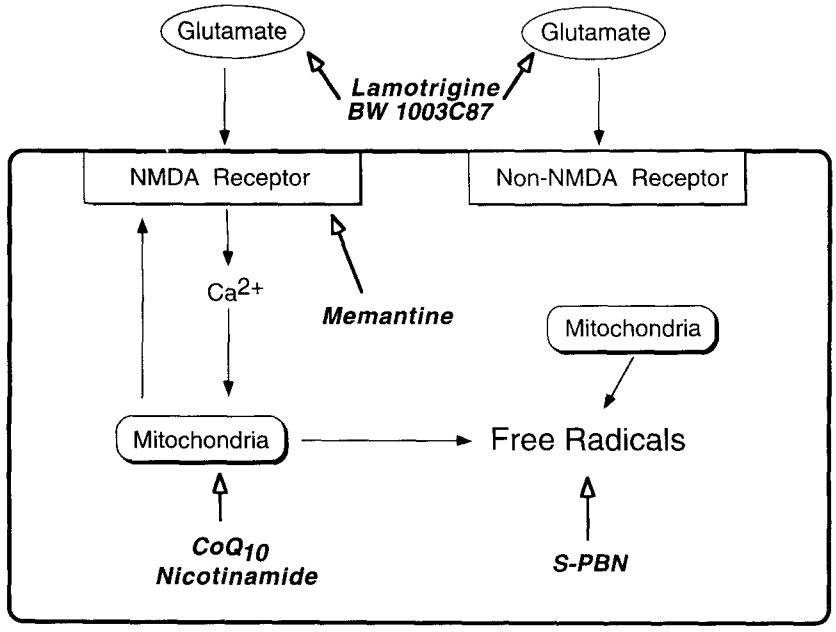


Fig. 1. Diagram showing a cascade which may result in neuronal death in neurodegenerative diseases, as well as possible sites of intervention. Impaired mitochondrial function may lead to membrane depolarization followed by activation of voltage-dependent NMDA receptors, calcium influx, and generation of free radicals. Potential sites of intervention are glutamate release inhibitors (lamotrigine, BW1003C87), NMDA receptor antagonists (memantine), agents to improve mitochondrial function (coenzyme Q_{10}, nicotinamide) and free radical scavengers (S-PBN). Combinations of these agents may exert additive neuroprotective effects.

induced lesions and ATP depletions.[6] Treatment with the free radical spin trap n-tert-butyl-α-(2-sulfophenyl)-nitrone (S-PBN), a free radical scavenger, also significantly attenuates malonate lesions, and the combination of S-PBN and MK-801 produces additive neuroprotective effects.[25]

In the present experiments we extended these findings by examining whether glutamate release inhibitors can attenuate both MPP^+ and 3-NP neurotoxicity, and whether the NMDA antagonist, memantine,[18] can attenuate malonate neurotoxicity. We also examined whether combinations of coenzyme Q_{10} with either lamotrigine, MK-801 or S-PBN exert additive neuroprotective effects. Lastly, we examined whether the combination of nicotinamide with S-PBN could exert additive neuroprotective effects.

EXPERIMENTAL PROCEDURES

Stereotaxic injections

Male Sprague–Dawley rats (Charles River, Cambridge, MA) weighing 75–100 g (MPP^+ lesions) or 300–325 g (malonate lesions) were anesthetized with pentobarbital (50 mg/kg i.p.). Nine or 10 animals were used in each group. S-PBN was obtained from Aldrich (Milwaukee, WI), Nicotinamide from Sigma (St Louis, MO), MK-801 from Research Biochemicals (Natick, MA) and coenzyme Q_{10} tablets from Vitaline Formulas (Ashland, OR). Lamotrigine and BW 1003C87 were generous gifts of Burroughs–Wellcome (Research Triangle, NC), and memantine of Merz (Frankfurt/Main, Germany).

Malonate (Sigma, St Louis, MO) and MPP^+ (Research Biochemicals, Natick, MA) were dissolved in 0.1 M phosphate-buffered saline (pH 7.4). Intrastriatal injections were made with a 10-μl-Hamilton syringe fitted with a 26 gauge blunt-tipped needle into the left striatum at the coordinates 0.5 mm anterior to the bregma, 2.6 mm lateral to the midline, and 5 mm ventral to the dura. Injection volumes were 1.5 μl consisting of 3 μmol malonate or 1 μl consisting of 120 nmol MPP^+. All injections were made over 1 min, and the needle was left in place for an additional 1 min, before being slowly withdrawn.

Nicotinamide, S-PBN, MK-801, BW 1003C87 and memantine were dissolved in water and the pH adjusted to 7.4. S-PBN (100 mg/kg) was administered intraperitoneally (i.p.) at 1 h before, 2 and 5 h after stereotaxic surgery. MK-801 (4 mg/kg) and BW 1003C87 (15 mg/kg) were injected i.p. 0.5 h before surgery. Nicotinamide (200 mg/kg) was administered sequentially i.p. at 0.5 h before, at the time point, 2 and 4 h after stereotaxic surgery. Lamotrigine (12 or 16 mg/kg) was administered orally by gavage 0.5 h before surgery. In experiments with coenzyme Q_{10}, animals received either normal rat chow or oral coenzyme Q_{10} at 200 mg/kg per day in 30 g/day of rat chow for nine days before surgery. The rats then remained on coenzyme Q_{10} for one more day, before being switched to normal rat chow. Since anesthesia induced hypothermia may produce neuroprotection, the body temperature of the animals was maintained at 37.5°C in an incubator during the period of anesthesia.

Quantification of lesion volume

Animals were decapitated at seven days and the brains were rapidly removed, placed in cold 0.9% saline for 10 min, and sectioned coronally into slices at 2-mm-intervals. Slices were stained in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC, Sigma, St Louis, MO) solution at room temperature in the dark for 30 min followed by fixation in

Table 1. Effects of treatment with lamotrigine and BW 1003C87 on lesions produced by 120 nmol of MPP^+

Treatment	Lesion volume (mm^3)
Vehicle	20.1 ± 0.8 ($n = 10$)
Lamotrigine 12 mg/kg	14.3 ± 1.7* ($n = 10$)
Lamotrigine 16 mg/kg	11.6 ± 1.9** ($n = 10$)
BW 1003C87 15 mg/kg	13.0 ± 1.4* ($n = 10$)

*$P < 0.05$; **$P < 0.01$ as compared with vehicle treated controls.

phosphate-buffered 4% paraformaldehyde.[7] The lesioned area (noted by pale staining) was measured on the posterior surface of each section using an Apple Macintosh© based image analysis system (Sony color video camera, Software: ColorSnap© (Computer Friends Inc., Portland, Oregon) and IPLab Spectrum© (Signal Analytics, Vienna, Virginia). The lesions were evaluated by an experienced histologist blinded to the experimental conditions. We previously verified the reliability of the TTC measurements in animals injected with malonate on adjacent sections stained with either TTC or Nissl stain.[6]

Systemic 3-nitropropionic acid treatment

3-NP was diluted in water and adjusted to pH 7.4 with NaOH and administered at a dose of 10 mg/kg i.p. every 12 h. With this dosing regimen the animals become acutely ill after 4–5 days and show large striatal lesions.[5] At the same time points, 12 mg/kg of lamotrigine dissolved in peanut oil at 12 mg/ml (treated group) or vehicle alone (peanut oil, control group) was administered s.c. Since there was variability in the times at which animals became ill, they were clinically examined 3 h after the injections and one animal of each group was decapitated when either a vehicle-treated control or a lamotrigine-treated animal became acutely ill. Twelve animals per group were studied. Striatal lesions in both hemispheres were assessed using TTC staining (see Quantification of Lesion Volume) and the mean lesion volume per hemisphere was used for calculations.

Data analysis

Data are expressed as mean ± S.E.M. In studies of stereotaxic malonate or MPP^+ injections statistical comparisons were made using one-way ANOVA followed by Fisher PLSD (protected least significant difference) *post-hoc* test to compare group means. In the study of systemic 3-NP treatment vehicle-treated and lamotrigine-treated animals were compared by χ^2 test and Mann–Whitney U-test.

Animal guidelines

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

RESULTS

Pretreatment with lamotrigine at 12 or 16 mg/kg i.p. or BW 1003C87 at 15 mg/kg on MPP^+ induced striatal lesions (Table 1). Lamotrigine produced dose-dependent significant neuroprotective effects. A comparable degree of neuroprotection occurred with BW 1003C87 at 15 mg/kg i.p. Further studies examined the effects of treatment with lamotrigine on striatal lesions produced by systemic administration of 3-NP. As shown in Table 2 treatment with lamotrigine significantly attenuated the lesions. We also investigated whether the non-competitive NMDA antagonist memantine could produce neuroprotective effects against malonate induced striatal lesions. As shown in Table 3 memantine produced dose-dependent neuroprotective effects.

Table 3. Effects of pretreatment with memantine on lesions lesions produced by 3 μmol of malonate

Treatment	Lesion volume (mm^3)
Vehicle	34.0 ± 0.7 ($n = 10$)
Memantine 10 mg/kg	19.4 ± 4.0* ($n = 9$)
Mamantine 20 mg/kg	10.5 ± 3.8** ($n = 9$)

*$P < 0.05$; **$P < 0.01$ compared to vehicle.

Neuronal injury produced by mitochondrial toxins may involve a cascade of deleterious effects, with energy depletion leading to activation of NMDA receptors, calcium influx and the generation of free radicals.[3] It may therefore be possible to produce additive neuroprotective effects by intervening at more than one step of the pathologic process. We previously found that both coenzyme Q_{10} and nicotinamide exert neuroprotective effects against malonate lesions.[6] In the present study we examined whether a combination of coenzyme Q_{10} with the glutamate release blocker, lamotrigine, could exert additive neuroprotective effects. Coenzyme Q_{10} was administered orally for nine days before the lesions. The neuroprotective effects of coenzyme Q_{10} alone was not significant ($P < 0.10$) while treatment with lamotrigine alone at the $P < 0.05$ level was significant (Table 4). The combination of coenzyme Q_{10} with lamotrigine was significantly better than by coenzyme Q_{10} alone (Table 4).

We also examined whether coenzyme Q_{10} pretreatment could exert additive neuroprotective effects in combination with either the free radical scavenger S-PBN, or the non-competitive NMDA antagonist MK-801. As shown in Table 5 coenzyme Q_{10} alone produced significant neuroprotective effects, as did S-PBN alone, but there were no additive effects when the two compounds were administered together. MK-801 was effective when administered alone, and the combination of MK-801 with coenzyme Q_{10}

Table 2. Effects of lamotrigine on striatal lesions produced by 10 mg/kg of 3-NP every 12 h

Treatment	Number of animals No lesion	Number of animals Lesion	Lesion volume (mm^3)
Vehicle	0	12	62.1 ± 7.2
Lamotrigine	8	4	7.8 ± 3.6***

***$P < 0.001$ as compared to vehicle.

Table 4. Effects of coenzyme Q_{10} (CoQ_{10}) alone, lamotrigine alone or the combination of coenzyme Q_{10} (CoQ_{10}) with lamotrigine on striatal lesions produced by 3 μmol of malonate

Treatment	Lesion volume (mm^3)
Vehicle	27.2 ± 2.5 ($n = 10$)
CoQ_{10} (200 mg/kg/day)	19.5 ± 3.5 ($n = 9$)
Lamotrigine (12 mg/kg)	13.9 ± 2.1* ($n = 10$)
CoQ_{10} and lamotrigine	9.9 ± 3.5**† ($n = 9$)

*$P < 0.05$, **$P < 0.01$ compared to vehicle. †$P < 0.05$ compared to coenzyme Q_{10} treatment alone.

produced additive neuroprotective effects which were significantly better than those achieved by coenzyme Q_{10} or MK-801 alone. Examples of TTC stained sections in each of the three groups as compared with controls are shown in Figure 2.

Lastly, we examined whether nicotinamide pretreatment could exert additive neuroprotective effects when administered in combination with the free radical scavenger S-PBN. Both nicotinamide and S-PBN produced significant neuroprotective effects when administered alone (Table 6). The combination of nicotinamide with S-PBN produced neuroprotective effects which were significantly greater than those produced by nicotinamide alone (Table 6).

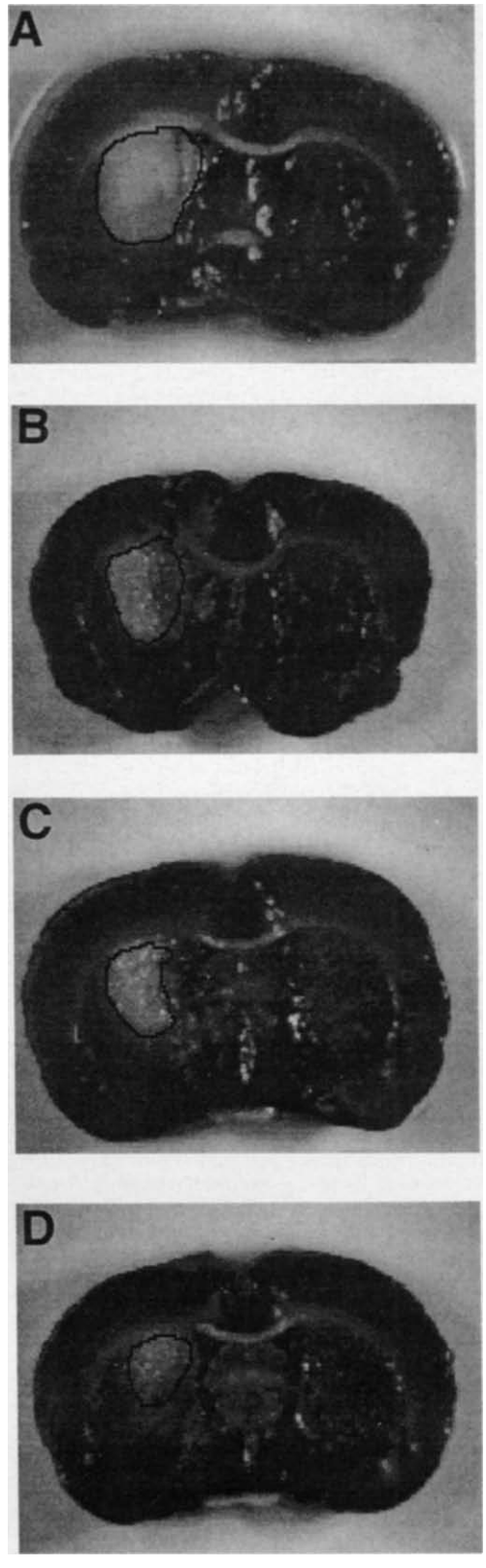
DISCUSSION

A promising strategy for treatment of neurodegenerative diseases is to use glutamate release inhibitors. One such compound is riluzole which recently showed promise in the treatment of amyotrophic lateral sclerosis.[8] Another is lamotrigine which is a useful anti-epileptic in man.[21] Prior studies in animals showed that it blocks kainic acid lesions and focal ischemic lesions.[13,23] We found that it blocks malonate induced neurotoxicity.[15] In the present studies we extended these results to show that both lamotrigine and a related compound BW 1003C87, attenuate striatal lesions produced by intrastriatal administration of MPP^+. This is consistent with a recent report that lamotrigine protects against MPTP toxicity.[16] Lamotrigine was also effective in attenuating lesions produced by systemic administration of

Table 5. Effects of S-PBN alone, coenzyme Q_{10} alone, MK-801 alone, and the combination of coenzyme Q_{10} with S-PBN or MK-801 on striatal lesions produced by 3 μmol of malonate

Treatment	Lesion volume (mm^3)
Vehicle	30.8 ± 4.2 ($n = 10$)
S-PBN	22.2 ± 3.2* ($n = 10$)
CoQ_{10}	18.2 ± 2.9** ($n = 10$)
CoQ_{10} and S-PBN	19.1 ± 4.4** ($n = 9$)
MK-801	17.5 ± 4.8** ($n = 9$)
CoQ_{10} and MK-801	8.6 ± 2.1***† ($n = 10$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle. †$P < 0.05$ compared with coenzyme Q_{10} or MK-801 treatment alone.



eation of the lesioned area produced by striatal injection of malonate in a saline-treated control (A) an animal treated with coenzyme Q_{10} alone (B), an animal treated with MK-801 alone (C) and an animal treated with the combination of coenzyme Q_{10} with MK-801 (D).

3-NP. This is consistent with our previous results that removal of the cortico-striatal glutamatergic projection significantly attenuates 3-NP neurotoxicity.[5] Other researchers have shown that the non-NMDA antagonist NBQX [6-nitro-7-sulphanoylbenzo(f) quinoxaline-2,3-dione] can attenuate 3-NP toxicity.[28] Taken together these results strongly implicate excitotoxicity in the pathogenesis of 3-NP lesions.

A number of glutamate receptor antagonists have been developed and are now in clinical trials for stroke in man. Since it has a lower affinity for the receptor, it has been argued that memantine may produce less behavioral toxicity than some other NMDA antagonists, allowing use-dependent blockade during conditions of receptor activation.[22] In the present study we found that memantine was effective in attenuating lesions produced by malonate, consistent with previous studies using MK-801.[4,14]

Neuronal injury in neurodegenerative diseases may involve energy impairment followed by activation of NMDA receptors, leading to free radical generation.[3] It may therefore be possible to utilize combinations of compounds which intervene at various steps in this cascade to achieve improved neuroprotection (Fig. 1). In the present experiments we therefore examined whether agents which improve energy metabolism can exert additive neuroprotective effects with a glutamate release inhibitor, an NMDA antagonist or with a free radical scavenger. Coenzyme Q_{10}, or ubiquinone, is an essential component of the electron transport chain where it serves as an electron donor and acceptor.[24] It protects against glutamate neurotoxicity in cultured cerebellar neurons,[11] and is a free radical scavenger.[12] Nicotinamide is a precursor to NADH, and therefore, plays an essential role in both electron transport and in the activity of dehydrogenase enzymes. We found that the protection against striatal lesions provided by pretreatment with coenzyme Q_{10} or nicotinamide is dose-dependent. The combination of the two compounds was more effective than either compound alone, and both compounds were effective in preventing malonate induced ATP depletion.

Another approach to the treatment of neurodegeneration is to utilize free radical scavengers. A substantial amount of evidence has implicated oxidative stress in the pathogenesis of excitotoxic neuronal injury.[9,10,20] Free radical spin traps are compounds which react with free radicals to form more stable adducts such as nitroxides.[17] We found that the free radical spin trap S-PBN significantly attenuates malonate striatal lesions, and that it reduces malonate induced generation of hydroxyl radicals.[25] Furthermore, it produced additive neuroprotective effects when administered with the NMDA receptor antagonist MK-801, showing that agents which block excitotoxicity and which scavenge free radicals work together in blocking the neurodegenerative cascade.

In the present experiments we examined whether combinations of coenzyme Q_{10} or nicotinamide with either lamotrigine, MK-801 or S-PBN could exert additive neuroprotective effects. We found that the combination of lamotrigine with coenzyme Q_{10} produced neuroprotective effects which were significantly more effective than those of coenzyme Q_{10} alone. The combination of coenzyme Q_{10} with MK-801 produced neuroprotective effects, which were significantly better than those produced by coenzyme Q_{10} or MK-801 alone. The addition of S-PBN to coenzyme Q_{10} produced no additional neuroprotective effect, suggesting that the free radical scavenging effects of coenzyme Q_{10} are comparable to those of S-PBN. Lastly we found that the combination of nicotinamide with S-PBN produces neuroprotective effects, which were significantly better than those produced by nicotinamide alone. This demonstrates that some agents which improve energy metabolism have neuroprotective effects which are enhanced by free radical scavengers.

The present results have implications for the treatment of neurodegenerative diseases. MPTP has been used to model Parkinson's disease,[27] while intrastriatal injections of malonate or systemic administration of 3-NP closely replicate the neurochemical and histologic features of Huntington's disease.[4,5] In the present experiments we provide evidence that glutamate release inhibitors are efficacious against both MPP^+ and 3-NP neurotoxicity, consistent with our previous results with malonate,[15] and that of others showing that lamotrigine blocks MPTP neurotoxicity.[16] These results suggest that glutamate release inhibitors might be a useful treatment strategy in the treatment of Parkinson's disease or Huntington's disease, as has recently been achieved in the treatment of amyotrophic lateral sclerosis.[8]

Table 6. Effects of treatment with nicotinamide alone, S-PBN alone or the combination of nicotinamide and S-PBN on lesions produced by 3 μmol of malonate

Treatment	Lesion volume (mm^3)
Vehicle	32.8 ± 3.9 ($n = 10$)
Nicotinamide (200 mg × 4)	21.4 ± 3.8* ($n = 10$)
S-PBN (100 mg/kg × 3)	13.9 ± 4.5** ($n = 9$)
Nicotinamide and S-PBN	5.8 ± 3.5***† ($n = 10$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle.
†$P < 0.05$ compared with nicotinamide treatment alone.

CONCLUSIONS

We recently found that coenzyme Q_{10} therapy in Huntington's disease patients resulted in significant reductions in lactate concentrations in 13 of 15 treated patients.[19] The present results provide a rationale for testing coenzyme Q_{10} both alone and in combination with glutamate release inhibitors and NMDA receptor antagonists. They also suggest that strategies using a combination of nicotinamide with a free radical scavenger might be useful. The use of glutamate release inhibitors and NMDA receptor

antagonists at neuroprotective dose levels may be limited by CNS side effects.[22] A combination of therapies might allow one to utilize some compounds at doses which are lower than would otherwise be possible, and thereby avoid adverse CNS side effects. Various combinations of agents to improve mitochondrial function, to block excitotoxicity, and to scavenge free radicals may therefore be useful therapeutic strategies in the treatment of neurodegenerative diseases.

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