

26. SEARCH FOR NEW NEUROPROTECTIVE DRUGS AGAINST SOMAN-INDUCED CENTRAL NEUROPATHOLOGY: ANTIOXIDANTS

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ABSTRACT

Injecting rodents with soman (GD), a potent organophosphate nerve agent, quickly induces seizures evolving into status epilepticus and death at lethal dosage. Central neuropathology is especially prominent in some structures such as in the limbic and thalamic areas. Glutamatergic neurotransmission is a key player in the onset of the neurological insult. Oxygen free radicals are most probably involved although evidence for their production and/or toxic role during soman intoxication is still scarce. 21-aminosteroid (lazaroids) are potent inhibitors of iron-catalyzed lipid peroxidation in neural tissue and have been developed for the acute treatment of central nervous system injury and ischemia. We focused on two typical representatives of this family, U-74389G and U-83836E, and tested their neuroprotective properties in mice pretreated with HI-6 (50 mg/kg, i.p.) and challenged with a convulsant dose of soman (172 µg/kg). In these preliminary studies, pretreatment or treatment with these molecules could not prevent seizures. Pretreatment or treatment with U-74389G (5 and 10 mg/kg) or U-83836E (14 mg/kg) led to inconsistent neuroprotection in hippocampus following soman intoxication. Similarly U-74389G could not prevent or reverse the increase of [³H]PK11195 binding, a ligand of peripheral benzodiazepine receptor, used as a neuropathological index. U-83836E by itself increased the binding of this ligand leading to a difficulty in assessing its potential benefit. Conversely, U-74389G (5 and 10 mg/kg) tended to reduce malondialdehyde levels in cortices of intoxicated animals suggesting a dissociation between neuroprotection and antioxidant properties. Further studies are in progress.

INTRODUCTION

The highly toxic organophosphorous nerve agent soman (pinacolyl methylphosphonofluoridate) is a potent irreversible inhibitor of the enzyme acetylcholinesterase (AChE). Following experimental exposure, rodents suffer limbic seizures of quick onset, rapidly generalized and evolving into status epilepticus. The build-up of acetylcholine concentrations at central synapses due to the inhibition of AChE is essential to seizure onset [9,16]. A secondary increase of other neurotransmitters, especially excitatory amino acids such as L-glutamate (Glu), is a key process in neuropathology development. Glu is well known to be involved in several central nervous system pathologies [5,20]. Histopathological examination of rodent brains, hours after injection of soman, revealed severe neuronal damage in different regions of the brain, especially the hippocampus, piriform cortex, neocortex and thalamus [e.g. 13,15]. Even though the role of Glu appeared prominent, as evidenced by the neuroprotection afforded by ionotropic glutamate receptors [10-12], little is known about the precise biochemical mechanisms that lead to neuronal death. It is believed that Glu mediates neuronal injury via several mechanisms and that reactive oxygen species (ROS) production is involved in the neurotoxicity [3,8]. Evidence of oxidative stress during soman intoxication is scarce [7,14]. However, a recent study pointed out an increased level of lipidperoxidation products in rat brain as early as 30 min. after the injection of a convulsing dose in areas where lesions are described (e.g. hippocampus and thalamus) [7]. This working hypothesis led us to investigate the potential benefits of the administration of two antioxidants from the 21-aminosteroid family, U-74389G and U-83836E. This family of non-glucocorticoid compounds has been developed as cytoprotective lipid peroxidation inhibitor for the treatment of traumatic and ischemic central nervous system injuries [6]. Some of them have also been tested in seizure experimental models [1,2]. The compound's ability to inhibit lipid peroxidation resides in the combination of a chemical antioxidant action and a decrease in the fluidity of membrane phospholipids [6].

In the present study, our preliminary results suggest that neither U-74389G nor U-83836E appear to significantly prevent hippocampal neuronal damage (cresyl violet staining) or glial reaction (ω3 binding studies) induced by the injection of a convulsant dose of soman. Conversely, U-74389G tended to decrease lipidperoxidation in cortex.

METHODS

Chemicals

Soman (97,9 % pure, CPG) was obtained from the Centre d'Etudes du Bouchet (France). [³H]PK11195 (specific activity = 86 Ci/mmol) was obtained from NEN™ Life Science, Inc. (France). The 21-aminosteroids U-74389G and U-83836E were obtained from Biomol Research Laboratories (TEBU, France). U-74389G was first dissolved in CS-4 sterile vehicle and then loaded into a pre-formed emulsion (i.e., Lipoven®) and the pH adjusted to ca. 7 with 100 mM pH 7 phosphate buffer. U-83836E was dissolved at the required concentration in normal saline. MDA was prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (ICN Biomedicals, Inc., France) at 95°C for 10 min.

Animals

Adult male mice, weighing 25-30 g were obtained from Elevage Janvier France. Animals were maintained on a 12h/12h light-dark cycle and given food and water *ad libitum*. The experimental protocol and procedures used meet the French and European community guidelines and have been approved by the Animal Care Committee (CRSSA).

Intoxication and determination of the LD₅₀ of soman in the model with HI-6 pretreatment

The oxime HI-6 (50 mg/kg, i.p.) was given to the animals 5 min. prior to soman challenge with a convulsing dose (172 µg/kg, s.c.). LD₅₀ was determined by the moving-average interpolation method applied to the number of dead mice 24 hr after soman challenge [19,21].

Determination of the neuroprotective activity of U-74389G and U-83836E

Neuroprotection afforded by the drugs was assessed by different means:

- Measurement of ω3 site densities in hippocampus of soman-treated mice ([10] with modifications)

48 h after soman treatment, mice were decapitated and the hippocampi dissected, frozen and stored at -80°C until assay. Hippocampi were weighed, Polytron homogenized in 20 % (w/v) of a 120 mM NaCl, 50 mM Tris-HCl (pH 7.4) buffer. Aliquots of 25 µL were used for protein determination using the method of Lowry et al. Aliquots of 100 µL of homogenate (ca. 500 µg of protein) were incubated with 2 nM [³H]PK11195 in 1 mL (final volume) of Tris-saline buffer for 30 min. at +25°C. The incubate was decanted by vacuum filtration through GFB filters; the filters were rinsed 3 times with 3.5 mL of cold Tris-saline buffer and the bound radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined using 1 µM PK11195. Binding was measured in duplicate.

- Histological analysis by cresyl violet staining

48 hours after soman intoxication, mice were decapitated and their brain rapidly removed. They were immediately frozen in dry ice cooled isopentane. Serial coronal brain tissue sections (10 µm) were obtained with a cryostat. A classic cresyl violet staining procedure was then applied. Presence or absence of hippocampal lesions was used as a marker for neuroprotection.

Measurement of the antioxidant effect of U-74389G

The animals were killed by decapitation 48 h after the injection of soman and the brain rapidly dissected on ice. For this preliminary study, it was divided into two parts, cortex and the remaining portion. They were weighed and homogenized using a Teflon pestle in ice-cold 0,2 M phosphate buffer pH 7.4 (10% w/v). Aliquots of 500 µL of homogenates were diluted 1:2 with distilled water and 50 µL of a 2% Butylated hydroxytoluene solution in ethanol were added to prevent further oxidation. The homogenates were frozen and kept at -80°C. The day of the experiment, they were thawed and an aliquot was treated with 50% trichloroacetic acid, centrifuged at 2000 g for 10 min. at +4°C. Malondialdehyde (MDA) and other end products derived from peroxidation of polyunsaturated fatty acids and related esters were assayed as thiobarbituric acid reactive substances (TBARS). Briefly, an aliquot of the supernatant was collected and mixed with 0.67% thiobarbituric acid (TBA) and incubated for 10 min. at +95°C. TBARS were measured by spectrofluorimetry (λ_{exc} 532 nm, λ_{em} 553 nm).

Data analysis and statistics

Data of MDA (TBARS) content and [³H]PK11195 binding values are expressed as mean ± SEM. All statistical analyses (for groups with at least n=5) were performed using nonparametric tests (Kruskal-Wallis test followed by post-hoc Mann-Whitney test using the corrective Bonferroni's procedure).

RESULTS AND DISCUSSION

Pretreatment with HI-6 increases the survivability of the animals and allows higher soman challenges. In this model, LD₅₀ was 278 µg/kg (95% confidence limits: 248-311 µg/kg), higher than LD₅₀ without HI-6 (ca. 110 µg/kg). This model is derived from the rat model previously described by Shih [17]. All animals injected with soman (172 µg/kg, s.c.) developed classic signs of nerve agent intoxication including spontaneous tremor, limbic seizures and hypersecretion. The first visually observed limbic-type movements appeared on average 4.2 ± 0.5 min. (mean ± SEM, n=18) after soman injection. Most survived at least 48 hr. At 24 and 48 hr, histological examination of mouse

brains often revealed obvious damage to hippocampus, especially in CA1 and CA3 subsectors (Figure 1). Cresyl violet staining does not easily reveal lesions in other parts of the brain (e.g. thalamic area).

Neither U-74389G nor U-83836E, injected as pretreatment 30 min. prior to soman challenge or as treatment 15 min. post-challenge, interfered with seizure onset and status epilepticus. This is not surprising given the neurochemical basis of soman-induced seizures and consistent with previous studies using other convulsant agents [1,2]. Pretreatment with U-74389G (5 and 10 mg/kg) or U-83836E (14 mg/kg) led to inconsistent neuroprotection in hippocampus following soman intoxication (Figure 2). Owing to the variability of hippocampal lesions in soman-intoxicated mice compared to other damaged brain structures, it is therefore difficult to clearly conclude. Other histological analyses are currently being conducted. Increase of the binding of [³H]PK11195, a ligand of the peripheral benzodiazepine receptors [4], is considered to be a suitable marker of soman-induced brain lesions [10]. U-74389G could not prevent or reverse the increase of [³H]PK11195 binding. In the case of U-83836E we found that the drug itself could increase [³H]PK11195 binding thus rendering a more complex analysis of the results. The results obtained with both lazardoids are thus consistent with the histological findings and suggest a limited effectiveness (Figure 3). Antioxidant properties of U-74389G were evaluated in cortex and our first results are in favor of a reduction of TBARS following administration of this lazardoid (Figure 4). This suggests a dissociation between neuroprotection and antioxidant properties of the molecule. Such a dissociation has been reported for U-83836E in vitro [18].

CONCLUSIONS

Neither U-74389G nor U-83836E appears to significantly prevent hippocampal neuronal damage or glial reaction induced by the injection of a convulsant dose of soman despite a tendency to decrease lipidperoxidation (studied with U-74389G). Studies are in progress to confirm these findings especially the relative dissociation between the antioxidant and neuroprotective properties of U-74389G.

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KEYWORDS

soman, organophosphorous compound, seizures, neuroprotection, 21-aminosteroid

FIGURES AND TABLES

Figure 1 - Light microscopy pictures showing soman-induced hippocampal neuronal loss or modifications. Brain tissue coronal sections (10 μm) from a control mouse (A) and those injected with soman (172 $\mu\text{g}/\text{kg}$) after 24 hr (B) and 48 hr (C). The loss of neurons in the pyramidal cell layer of the CA1 hippocampal area is obvious. Cresyl violet acetate staining. Scale bar = 400 μm .

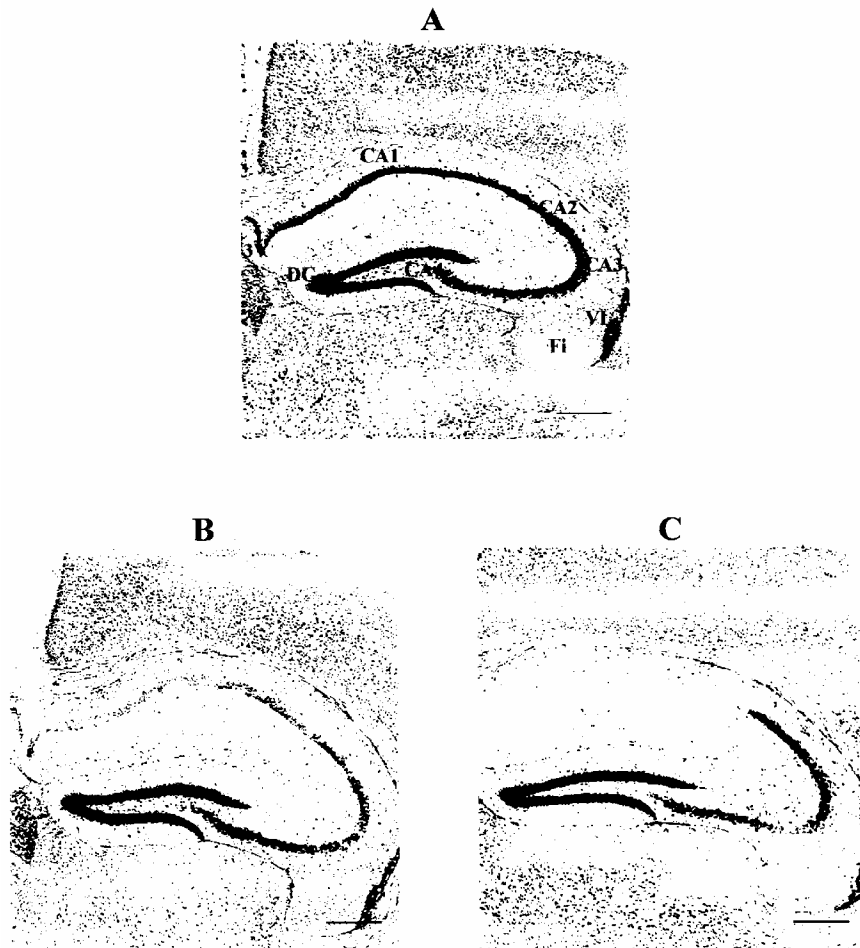


Figure 2 - Light microscopy pictures showing soman-induced CA1 pyramidal cell loss or modifications 48 hr after the challenge and the lack of clear improvement by 21-aminosteroid pretreatment. Brain tissue coronal sections (10 μm) from a control mouse (B), and those injected with soman (172 $\mu\text{g}/\text{kg}$) alone (A) or receiving additionally, 30 min. previously, an intraperitoneal injection of U-74389G (10 mg/kg) (C) or U-83836E (14 mg/kg) (D). Note the loss of neurons in the pyramidal cell layer of the CA1 hippocampal area (between arrows). Cresyl violet acetate staining. Scale bar = 100 μm .

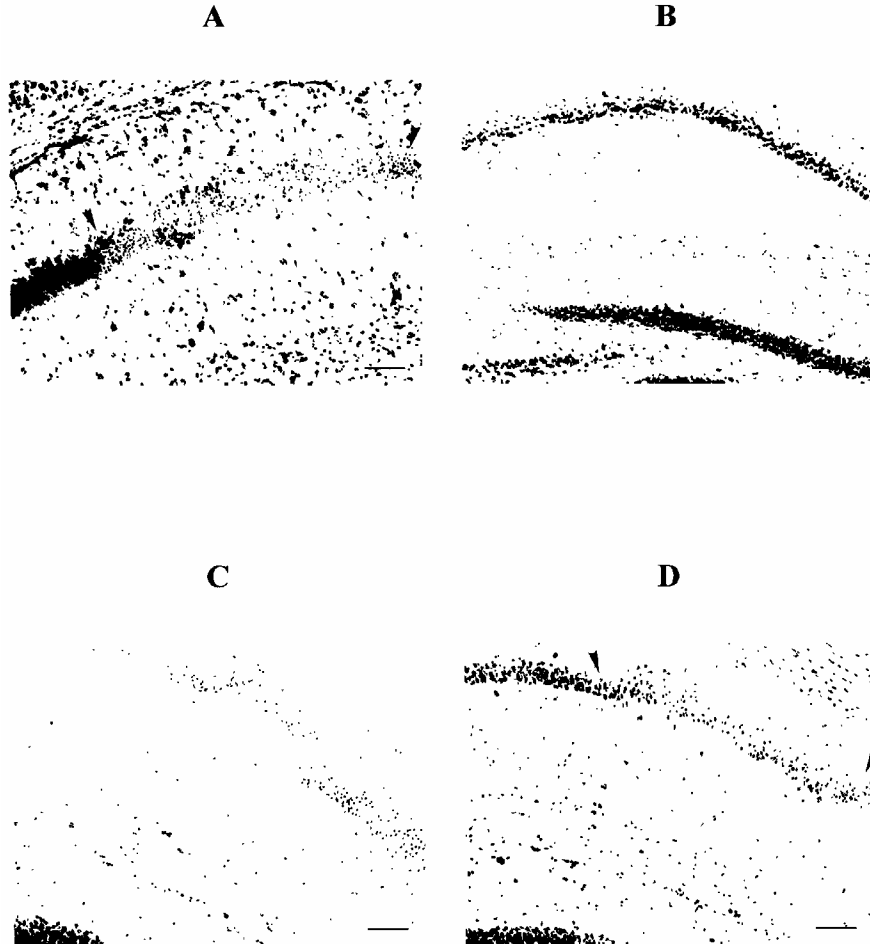


Figure 3 - [³H]PK11195 specific binding on mouse hippocampal homogenates 48 hr after the indicated treatment. Control groups U-74389G and U-83836E only received the drug (10 mg/kg). Pretreatment and treatment are injected 30 min. prior to and 15 min. after the soman challenge (172 μg/kg) respectively. Data are presented as mean ± SEM of n separate determinations. For each of the molecules, experimental groups are compared using the Kruskal-Wallis nonparametric test followed by post-hoc pairwise comparisons with the Mann-Whitney test corrected by the Bonferroni's procedure (k=10). Comparison to control (saline) ** p < 0,001 (α 1%), *** p<0,0001 (α 1%).

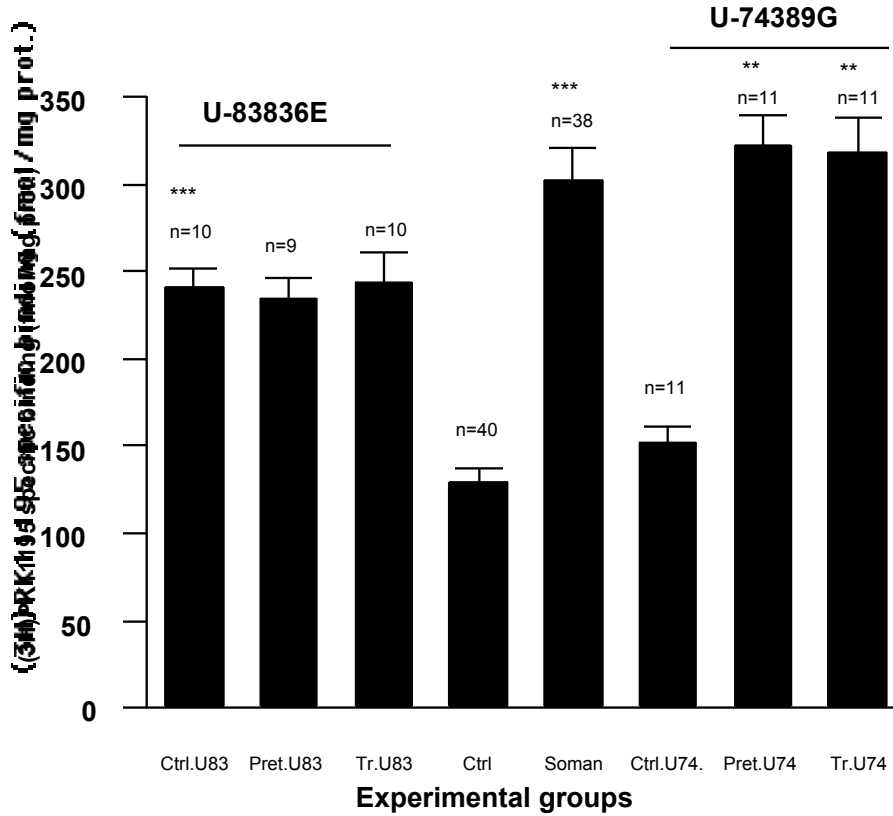


Figure 4 - Effect of U-74389G on TBARS (MDA) level in mouse neocortex 48 hr after a soman challenge (172 $\mu\text{g}/\text{kg}$). Results are presented as mean \pm SEM of n separate determinations.

