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Two medical therapies very effective shortly after high levels of soman poisoning in rats, but only one with universal utility

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ABSTRACT

A treatment regimen consisting of HI-6, scopolamine, and physostigmine (termed the physostigmine regimen) has been based on the serendipitous discovery that it exerts powerful antidotal effects against high levels of soman poisoning if it is administered 1 min after exposure. A medical therapy with corresponding efficacy, but without the time limitation of the latter regimen, has been developed through studies of microinfusions of anticonvulsants into seizure controlling sites in the forebrain of rats. From these studies procyclidine emerged as the most potent anticonvulsant, and its potency was further enhanced when being combined with the antiepileptic levetiracetam during systemic administration. In the present study, the capacity of HI-6, levetiracetam, and procyclidine (termed the procyclidine regimen) was tested against that of the physostigmine regimen. The results showed that both regimens were very effective against supralethal doses of soman (3, 4, 5 × LD_{50}) when given 1 and 5 min after intoxication. When the treatments were administered 10 and 14 or 20 and 24 min after soman exposure, only the procyclidine regimen was able to terminate seizures and preserve lives. When used as prophylactic therapies, both regimens protected equally well against seizures, but only the procyclidine regimen provided neuroprotection. The procyclidine regimen has apparently capacities to serve as a universal therapy against soman intoxication in rats.

1. Introduction

Intoxication by the organophosphorus nerve agent soman, an irreversible acetylcholinesterase (AChE) inhibitor, causes a progression of toxic signs including miosis, hypersalivation, respiratory distress, tremor, seizures/convulsions, coma, and death. In survivors, soman can evoke sustained seizure activity resulting in neuropathology in vulnerable brain areas like the piriform cortex, amygdala, and hippocampus. A large body of evidence relates brain damage to secondary massive release of glutamate, a neurotransmitter with excitotoxic potential (Carpentier et al., 2010).

Exposure to nerve agents requires immediate medical treatment. For this purpose, military personnel are issued with autoinjectors containing countermeasures for self-administration or "buddy aid". Antidotes against nerve agents are based on drugs acting at the muscarinic receptors and $GABA_A$ receptors (McDonough and Shih, 1997). In addition, partial protection against nerve agents can be obtained by the use of reversible (carbamate) AChE inhibitors shielding a portion of AChE from irreversible inhibition by nerve agents prior to nerve agent exposure. Furthermore, reactivation of any unaged AChE by an oxime is regarded as important immediate treatment after nerve agent exposure.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic agent (atropine) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). Such treatment regimens can, however, reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions, unless atropine is given early and at a high dose (McDonough and Shih, 1997). To overcome this shortcoming, several nations have provided their personnel with autoinjectors containing diazepam or avizafon (both benzodiazepine analogs with similar anticonvulsant action).

The choice of oxime in autoinjector therapy differs among countries. Currently, there is no universal oxime that effectively can reactivate AChE inhibited by any known nerve agent. This oxime-nerve agent specificity makes the choice of a single oxime difficult; alternatively plural oximes have to be considered. Most studies indicate that obidoxime is more efficient than HI-6 (1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium) against tabun, whereas HI-6 is a better drug than obidoxime against soman. However, HI-6 is assessed to be a promising broad spectrum oxime against nerve intoxication (Aas, 2003; Kassa, 2002). Some countries use atropine

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along with obidoxime (e.g., Germany, Norway, The Netherlands, Finland). A future autoinjector regimen containing atropine, HI-6, and diazepam (presently used by Canada) is proposed by nations within NATO (Aas, 2003).

In a recent study (Myhrer et al., 2013), a comparative assessment was made of the antidotal capabilities of atropine/obidoxime/diazepam (termed the obidoxime regimen), atropine/HI-6/avizafone (termed the HI-6 regimen), and a recently recommended regimen consisting of scopolamine/HI-6/physostigmine (termed the physostigmine regimen). The latter therapy is supposed to be applied just 1 min after nerve agent exposure and has been suggested to represent next generation of countermeasures, because it will reduce reliance on pretreatment and requirement for 3 autoinjectors (Wetherell et al., 2007). The results from our study show that each regimen administered 2 times (1 and 5 min after exposure to soman doses of 2, 3, or $4 \times LD_{50}$) can effectively prevent or terminate epileptiform activity within 10 min (Myhrer et al., 2013). However, the regimens differ markedly in life saving properties with the physostigmine regimen ranking highest followed by the HI-6 and obidoxime regimens. Pretreatment with pyridostigmine increases the potency of the HI-6 regimen, but not the obidoxime regimen. The physostigmine regimen has outstanding antidotal capacity, but the very narrow time window (<10 min) makes it unsuitable for use in the field (Myhrer et al., 2013). Conceptually, delayed administration of physostigmine will aggravate actions of soman by inhibiting residual active AChE. It has, however, been shown that physostigmine administered along with obidoxime 10 min after soman has some anticonvulsant effect in guinea pigs (Joosen et al., 2011), but determination of time limits of physostigmine in rats has not been carried out. The ultimate aim would be to develop a regimen effective shortly after high levels of soman poisoning that also can be effective at late points of time after exposure.

It has previously been suggested to work out specifications for what subreceptors in what sites of the forebrain that preferentially should be affected by countermeasures to obtain optimal efficacy (Myhrer, 2007). Without such specifications pharmacological research on anticonvulsants against nerve agents can take the form of a seemingly endless number of trial and error. In epilepsy research, more than 30,000 compounds have been screened for antiepileptic properties (Crepeau and Treiman, 2010). In a series of studies, we have mapped critical pharmacological receptors in specified brain areas. Through this process, procyclidine turned out to have the highest impact of the drugs tested in seizure controlling sites in the forebrain of rats (Table 4 in Myhrer, 2010). Enhancement of procyclidine's excellent antidotal properties (anticholinergic and antiglutamatergic) would make up a novel and interesting approach. Levetiracetam with a unique profile in preclinical models of epilepsy has been shown to increase the potency of other antiepileptic drugs up to 19-fold (Kaminski et al., 2009). In a recent study, we demonstrated that the combination of levetiracetam and procyclidine can effectively terminate soman-induced seizures 20 or 40 min after onset in rats pretreated with pyridostigmine or HI-6, respectively. This therapy could also save the lives of rats that were about to die 5–10 min after seizure onset (Myhrer et al., 2011).

The purpose of the present study was to determine the antidotal capacities of the physostigmine regimen (HI-6/scopolamine/physostigmine) and a procyclidine regimen (HI-6/levetiracetam/procyclidine) against moderate or supralethal levels of soman poisoning (1.3, 1.6, 3, 4, or $5 \times LD_{50}$) when given twice with 4 min interval at different points of time (1, 10, or 20 min) after exposure. Additionally, the importance of including HI-6 in the combination of levetiracetam and procyclidine was investigated. The prophylactic potency of the regimens was also examined.

2. Materials and methods

2.1. Animals

Male Wistar rats from a commercial supplier (Taconic Breeding Laboratories, Denmark) weighing 300–330 g served as subjects. The experiments were approved by the National Animal Research Authority. The animals were housed individually and had free access to commercial rat pellets and water. The rats were handled individually 3 days preoperatively and 3 days postoperatively, being allowed to explore a table top (80 × 60 cm) for 3 min a day. The climatized vivarium (21 °C) was illuminated from 0700 to 1900 h.

2.2. Surgery

The rats were anesthetized ip with diazepam (4.5 mg/kg) and fentanyl fluanisone (2 mg/kg). Of 2 stainless screws, one was lowered 1 mm into the parietal cortex (1 mm behind bregma, 3 mm lateral to midline), and the contralateral one served as ground. The screws were fixed with dental cement (Durelon; ESPE, Seefeldt, Germany). The rats were given a recovery period of 7 days.

2.3. Drugs

The drug doses chosen were derived from previous studies of anticonvulsant effects against soman-evoked seizures in rats; HI-6 dimethanesulphonate 125 mg/kg, scopolamine hydrobromide 1 mg/kg, physostigmine salicylate 0.1 mg/kg, procyclidine hydrochloride 20 mg/kg, levetiracetam 50 mg/kg, pyridostigmine bromide 0.1 mg/kg (McDonough and Shih, 1993; Myhrer et al., 2011, 2013; Shih et al., 1999). The drugs were dissolved in 0.9% saline and were administered intramuscularly. The injection site alternated between the left and right muscle in the hind leg. The soman doses were $1.3 \times LD_{50}$ (100 μg/kg), $1.6 \times LD_{50}$ (128 μg/kg), $3 \times LD_{50}$ (240 μg/kg), $4 \times LD_{50}$ (320 μg/kg), or $5 \times LD_{50}$ (400 μg/kg) resulting in convulsions and death in all rats of our strain (Sterri et al., 1980). Soman was given subcutaneously. All drugs were purchased from Sigma (St Louis, MO, USA), except HI-6 dimethanesulphonate that was a gift from Defence Research and Development (Suffield, Medicine Hat, Canada). Soman was purchased from TNO (Netherlands Organisation for Applied Scientific Research), The Netherlands.

2.4. Experimental design

For overview, see Table 1. When the physostigmine regimen and the procyclidine regimen were used as prophylactic treatment, they were administered 20 min before a soman dose of $1.3 \times LD_{50}$. When used as postexposure treatment, the regimens were given 1 and 5 min after a soman dose of 3, 4, or $5 \times LD_{50}$, respectively, 10 and 14 min after a soman dose $1.6 \times LD_{50}$, or 20 and 24 min after a soman dose of $1.3 \times LD_{50}$ in rats pretreated with pyridostigmine.

2.5. Histology

The rats were anesthetized as described for surgery, perfused intracardially with 10% formalin, and the brains were post-fixed in 10% formalin for at least 24 h. The brains were dehydrated and embedded in paraffin (Schmued et al., 1997). The sections were cut 5 μm thick and dried in an incubator (37 °C) for 12 h before they were stained with hematoxylin and eosin (HE) or Fluoro-Jade B (Schmued and Hopkins, 2000). Since Fluoro-Jade staining requires perfusion of the brain, only live rats could be used for this purpose. Rats that recently died or rats about to die were decapitated, and the brain sections were stained with HE. Because Fluoro-Jade has been considered to be the compound most suitable for the detection of neuronal degeneration (Schmued et al., 1997), this fluorescent staining technique was used to supplement the more conventional HE staining technique. A degenerating neuron presumably expresses a strong basic molecule, since it has an affinity for the strongly acidic Fluoro-Jade (Schmued et al., 1997). The Fluoro-Jade method has previously been described in detail (Schmued et al., 1997; Schmued and Hopkins, 2000). In order to make a distinct contrast between degenerated neurons and intact ones the sections were co-stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) resulting in a blue fluorescence of cellular nuclei. Such nuclear staining is seen in all viable cells. A 0.01% stock solution of DAPI (10 mg/100 ml distilled water) was prepared and 2 ml of this stock solution was added to 98 ml of the Fluoro-Jade B staining solution. Blue counterstained normal cell nuclei can be visualized when excited by ultraviolet (330–380 nm) light (Schmued and Hopkins, 2000). Fluoro-Jade B staining is seen with blue excitation filter, whereas DAPI is visualized by filter set 49 from Zeiss with excitation at 365 nm and emission at 445/50 nm. One picture was superimposed on the other in order to see both simultaneously. A digital microscope camera (AxioCam, Zeiss, Jena, Germany) was used to make photomicrographs. This technique allows processing of the photographs so that elements of particular interest can be made clearer by adjusting contrasts.

2.6. Evaluation of neuropathology

A grading system of 0–4 previously described (McDonough et al., 1995), was used to determine severity of neuronal damage in the piriform cortex, the