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The neuroprotective activity of 8-alkylamino-1, 4-benzoxazine antioxidants

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Abstract

Antioxidant 8-alkylamino-1,4-benzoxazines, (*R,S*)-(3-*tert*-butyl-8-phenylethylamino-3,4-dihydro-2*H*-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24429) and (*R,S*)-(3-cyclopentyl-8-benzylamino-3,4-dihydro-2*H*-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24718), were prepared according to a two-step one-pot electrochemical procedure. These compounds had been selected from a previous study of structure/activity. Both compounds (1–100 μM) prevented the fall in ATP levels caused by 24 h of hypoxia in astrocytes. Both compounds (1 and 10 mg/kg i.p.) were powerful neuroprotective agents in protecting against the lesions induced by 15 μg *S*-bromo-willardiine injected into the cortex or white matter of 5-day old mice pups. In contrast, exifone, an antioxidant compound, was inactive at these doses. S 24429 and S 24718 appear to be novel neuroprotective agents, which are effective in a model of brain damage mimicking the lesions underlying cerebral palsy.

Keywords: Antioxidant; Neuroprotection; Astrocyte; Leukomalacia; Cerebral palsy; Alkylamino-1,4-benzoxazine

1. Introduction

The production of free radicals from complex III of mitochondria has been claimed to be a critical factor in setting the life span of different species (Pearl, 1928; Harman, 1956; Harman, 1972; Harman et al., 1976; Cutler, 1984; Prinzinger, 1993; Sohal et al., 1990; Ku et al., 1993; Barja et al., 1994), and there is good evidence that free radical production may be increased in neurodegenerative disease (Phillis, 1994; Benzi and Moretti, 1995; Gerlach et al., 1995; Mattson, 1995; Cowley et al., 1996; Wolz and Krieglstein, 1996). Antioxidants have been claimed to be useful agents in the treatment of Parkinson's disease, Alzheimer's disease and cerebral aging, amyotrophic lateral sclerosis (ALS), and in reperfusion-driven damage after ischaemia (Harman, 1976; Phillis, 1994; Benzi and Moretti, 1995; Gerlach et al., 1995; Mattson, 1995; Cowley et al., 1996; Wolz and Krieglstein, 1996). However, the relationship between oxidative stress and activation of transcription factors such as nuclear factor-κB is cell-type dependent (Schoonbroodt and Piette, 2000).

The above diseases are associated with aging, but excitotoxicity may contribute to cerebral damage in the new born. Despite improvement in neonatal mortality and morbidity in the last 40 years, rates of cerebral palsy are not declining and may even be increasing in some western countries. Recently hypothesized etiologies of cerebral palsy have gone beyond hypoxic–ischemic mechanisms to include multiple preconceptional and prenatal factors, such as hypoxia/perfusion failure, genetic factors, growth factor deficiency, excess free radical production and maternal infection that produces excess cytokines.

Reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radicals can be neurotoxic by damaging critical cellular components necessary for cell viability. Insults such as ischemia and reperfusion, glutamate receptor over-activation and microglial activation lead to excess production of reactive oxygen species (Phil-

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lis, 1994; Benzi and Moretti, 1995; Gerlach et al., 1995; Mattson, 1995; Cowley et al., 1996). Therefore, antioxidant molecules could have therapeutic benefits in brain insults underlying cerebral palsy. Thus, a well-characterized animal model of excitotoxic lesions in newborn mice has been used (Marret et al., 1995a,b, 1996; Dommergues et al., 1998, 2000; Gressens et al., 1999a,b; Redecker et al., 1998a,b; Tahraoui et al., 2001).

For several risk factors of cerebral palsy, it has been established that excitatory amino acids could represent a common final pathway leading to neuronal cell damage and death. Brain damage is induced with intracerebral administration of glutamatergic agonists acting on alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors (*S*-bromo-willardiine). When *S*-bromo-willardiine is injected on post-natal day 5 (P5), neuronal death is observed in all cortical layers, producing brain lesions similar to those observed in full-term human infants. Furthermore, *S*-bromo-willardiine injections administered on P5 induced periventricular white matter cystic lesions, which mimicked several aspects of human cystic periventricular leukomalacia, observed most frequently in very premature human infants (Tahraoui et al., 2001). While MK-801, an NMDA antagonist, had no effect on neuronal death induced by *S*-bromo-willardiine, cotreatment with MK-801 and *S*-bromo-willardiine resulted in a significant reduction of the white matter lesion size (Tahraoui et al., 2001). These data suggest that the *S*-bromo-willardiine-induced lesion of the white matter involves both NMDA and AMPA–kainate receptors, while the cortical plate lesion observed in this model is purely mediated by AMPA–kainate receptors. In the neonatal white matter, NMDA receptors are transiently present on microglia/macrophages (Tahraoui et al., 2001), while oligodendrocytes and their precursors express AMPA–kainate receptors (Follett et al., 2000).

Some antioxidant agents may have serious side effects. Exifone (Fig. 1) has been shown to be a neuroprotective agent in a range of models (Allain et al., 1988; Bentue-Ferrer et al., 1989; Porsolt et al., 1987). However, the antioxidant exifone was associated with severe hepatotoxicity (Denjean et al., 1990). In an attempt to find safe and effective neuroprotective agents, the synthesis of new 8-alkylamino-1,4-benzoxazine derivatives was reported (Largeron and Fleury, 1998; Fig. 1). From their capacity to inhibit oxidative stress-induced neuronal degeneration in hippocampal cells in vitro, some of these compounds were found to be potent neuroprotective antioxidants, with activity close to that of standard α-tocopherol (Largeron et al., 1999).

On the basis of structure–activity relationships within the studied series, the 3-alkyl functionality appeared to be essential for activity, the most suitable substituents being alkyl chains such as *tert*-butyl, methyl or cyclopentyl. From the combined results of both toxicity and neuroprotection expressed in terms of the safety index, 8-benzyl-

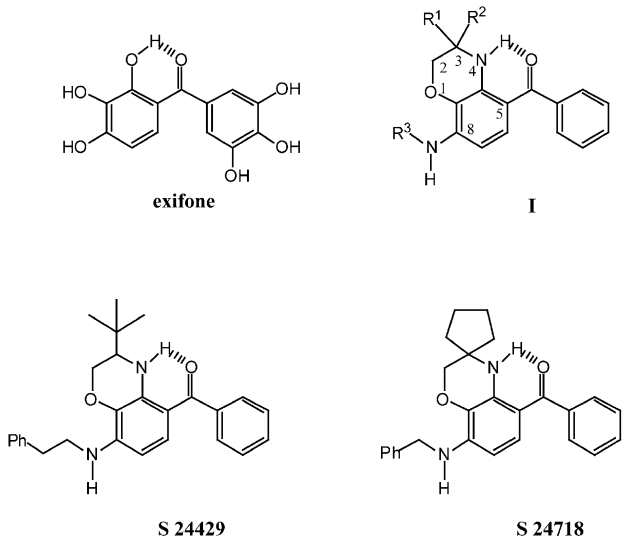

Fig. 1. Core structure of 8-alkylamino-1,4-benzoxazines (I), and structures of S 24429, S 24718 and exifone.

amino- and 8-phenylethylamino-substituted-3-alkyl-1,4-benzoxazines were selected as the most promising compounds. The neuroprotective effects of (*R,S*)-(3-*tert*-butyl-8-phenylethylamino-3,4-dihydro-2*H*-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24429) and (*R,S*)-(3-cyclopentyl-8-benzylamino-3,4-dihydro-2*H*-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24718) were tested, against hypoxia-induced ATP depletion in cultured astrocytes and in an animal model of excitotoxic lesions in newborn mice.

2. Methods

2.1. Astrocytes in tissue culture

Astrocytes cultures were obtained from neonate rats using a technique described by Booher and Sensenbrenner (1972). Cultures were used for 3 weeks after preparation. The oxygen-free culture medium (DMEM) was added and different concentrations of drug were then added. Hypoxia was carried out in an anaerobic chamber for 24 h. The ATP concentration was measured by luminescence (Sigma) and expressed in picomoles of ATP/mg of cell proteins. Experiments were carried out in triplicate.

After the meninges had been cleaned off, the brain tissue was forced gently through a nylon sieve (180 μm). DMEM supplemented with 10% fetal calf serum (Hyclone, USA), 2 mM glutamine and 50 μg/ml of gentamicin was used for the dissociation of cerebral tissue and development of astrocytes. Cultures were used for 3 weeks after preparation. The oxygen-free culture medium (DMEM) was added and different concentrations of the product were then added. Hypoxia was carried out in an anaerobic chamber for 24 h. ATP was extracted and measured by a Luciferin–luciferase method using a Sigma kit. Statistical

Table 1
Effects of S 24429 and S 24718 on the fall in ATP levels in astrocytes maintained in hypoxic conditions for 24 h ($n = 4$)

	ATP pmol/mg protein					
	Mean	S.E.M.				
Normoxia	5969[a]	271				
Hypoxia	4668	269				
	1 μM	S.E.M.	10 μM	S.E.M.	100 μM	S.E.M.
S 24429	6118[a]	414	6409[a]	448	6169[a]	245
S 24718	6003[a]	287	6092[a]	384	5881[a]	294

[a] $P < 0.01$ vs. hypoxia.

analysis calculations were made using the Mann–Whitney nonparametric test.

2.2. Neonatal mice

At postnatal day 5, Swiss mouse pups were anesthetized with ether for intracerebral (i.c.) and i.p. injections. Intracerebral injections were performed using a 26-gauge needle mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin, in the frontoparietal area of the right hemisphere 2 mm from the midline in the lateral-medial plane, and 3 mm (in the rostro-caudal plane) from the junction between sagittal and lambdoid sutures. Two 1-μl boluses were injected at a 30-s interval.

Fifteen micrograms *S*-bromo-willardiine (Tocris), diluted in phosphate buffer saline (PBS), were injected i.c. Immediately following i.c. injection, 1 or 10 mg/kg S 24449 ($n = 8$ and 6, respectively) or S 24718 ($n = 8$ and 15, respectively) or exifone ($n = 6$), diluted in PBS containing 10% dimethylsulfoxide (DMSO), were administered i.p. Controls received i.p. PBS containing 10% DMSO alone ($n = 19$). S 24429 and S 24718 were synthesized and purified as previously described (Largeron et al., 1999).

Five days later, the surviving pups were sacrificed and the brains were fixed in formalin. Coronal serial sections, 15-μm thick, were cut, and every third section was stained with cresyl violet. The brain was completely and serially sectioned from the frontal pole to the occipital lobes. In theory, neocortical and white matter lesions can be defined by the maximal length of three orthogonal axes: the lateral-medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle) and the fronto-occipital axis (in a sagittal plane). Due to the difficulty of accurately evaluating the degree of damage to neurons in neocortical layers in the epicenter of the lesion focus, the radial axis did not appear as an objective measure of the lesion size. In preliminary studies (Gressens et al., 1999a,b; Gressens, unpublished), we had shown an excellent correlation between the maximal size of the radial and fronto-occipital diameters of the excitotoxic lesions. Based on these observations, the entire brain was serially sectioned in the coronal plane. This permitted an accurate and reproducible determination of the maximal sagittal fronto-occipital diameter (which is equal to the number of sections where the lesion was present multiplied by 15 μm) and was as an index of the volume of the lesion. Statistical analyses were performed by analysis of variance (ANOVA) with Dunnet's multiple comparison of means test. Results were expressed as means ± S.E.M.

3. Results

8-Alkylamino-1,4-benzoxazines, S 24429 and S 24718, were prepared according to a two-step one-pot electrochemical procedure previously reported (Fig. 1; Largeron and Fleury, 1998). The compounds were sparingly soluble in aqueous media, which limited the concentrations which could be used.

The neuroprotective effects of S 24429 and S 24718 against hypoxia-induced ATP depletion were assessed in vitro in cultures of astrocytes. Both S 24429 and S 24718 were fully effective in preventing the fall in ATP levels caused by 24-h hypoxia (Table 1; Fig. 2). In this model, the hypoxia causes only a small depletion of ATP, of 22%, which is a mild insult.

In newborn mice, there were no deaths within the experimental groups. *S*-bromo-willardiine injection induced tonic and tonico-clonic seizures in most of the pups. These epileptic manifestations were observed during the first 8 h following intracerebral injection and were not modified in terms of frequency, severity or phenotype by i.p. treatments.

Histological lesions induced by *S*-bromo-willardiine affected both the cortical plate and the white matter (Fig. 3).

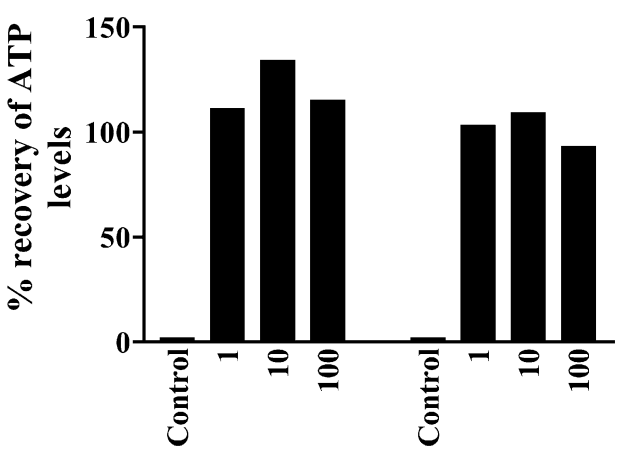

Fig. 2. Depletion of ATP by 24-h hypoxia is prevented by S 24429 (1, 10, 100 μM, left panels) and S 24718 (1, 10, 100 μM, right panels). The data are calculated from the means in Table 1, by comparing the protective effects of the drugs with the falls in ATP in hypoxic conditions.

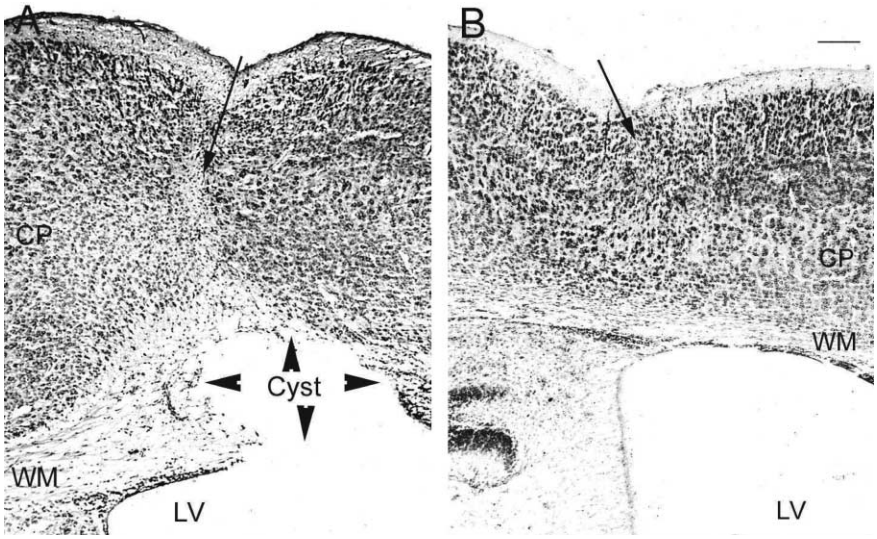

Fig. 3. S 24718 prevents *S*-bromo-willardiine-induced neuronal death and white matter cysts. Cresyl violet-stained sections showing typical brain lesions induced by *S*-bromo-willardiine injected at postnatal day 5 and studied at the age of postnatal day 10. (A) Brain injected with *S*-bromo-willardiine alone, showing the typical neuronal loss in layers II–VI (arrow) and the white matter cystic lesion (arrowheads). (B) Brain cotreated with *S*-bromo-willardiine and S 24718; note the absence of detectable white cystic matter lesion and the limited neuronal death in the cortical plate (arrow). Bar: 70 μm.

Cotreatment with S 24429 or S 24718 protected in a dose-dependant manner both the white matter and the cortical plate against the insult (Figs. 3 and 4). Both doses (1 and 10 mg/kg) of S 24718 were neuroprotective, while only the highest dose of S 24429 yielded a significant protection in this model (Figs. 3 and 4). These results were in good agreement with those previously reported from our preliminary in vitro evaluation (Largeron et al., 1999), since S 24718 was more potent than S 24429. In contrast, at the tested doses (1 and 10 mg/kg), exifone was not neuroprotective against *S*-bromo-willardiine-induced brain lesions (Fig. 4).

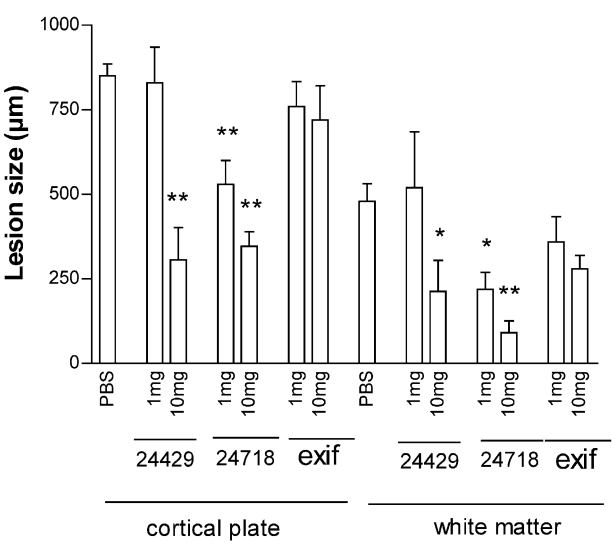

Fig. 4. The histograms represent the mean length of the neocortical lesion in the sagittal fronto-occipital axis ± S.E.M. Asterisks indicate difference from control ($^{*}p < 0.05$, $^{**}p < 0.01$ in ANOVA with Dunnet's multiple comparison test). PBS: control animals injected with *S*-bromo-willardiine into the cortex and white matter and i.p. with physiological saline; all the other experimental groups were cotreated with *S*-bromo-willardiine and the indicated drug at the doses indicated in mg/kg, i.p. (exif, exifone).

4. Discussion

Both S 24429 and S 24718 were fully effective in preventing ATP depletion in cultured astrocytes following 24-h hypoxia. The failure of this test to discriminate between the drugs is due to only mild hypoxia being used, which caused only 22% fall in ATP levels. This mild level of hypoxia is chosen to be physiologically relevant, because there is no liberation of LDH in the present experimental conditions. The model therefore represents a model of metabolic stress. In this model, severe hypoxia causes profound falls in ATP levels (> 50%); but in our experience, of > 20 neuroprotective agents of different classes, no drug has prevented this fall (Cecchelli et al., in preparation). Nevertheless, even if the mechanism of action of the drugs cannot be defined from this experiment, the results are concordant with earlier data against oxidative stress in hippocampal cells maintained in tissue culture, where pronounced neuroprotection was observed at these concentrations. Thus, the drugs have the possibility to exert an effect in both neurones and astrocytes, which may be of particular relevance for lesions of white matter.

In this respect, *S*-bromo-willardiine injection caused marked neurotoxic effects in both cortex and white matter in a newborn mouse model, where the cortical lesion is an accepted model of neonatal hypoxic–ischemic brain lesion and the white matter lesion is a model of periventricular leukomalacia, two brain lesions found in patients with cerebral palsy (Marret et al., 1995a,b, 1996; Dommergues

et al., 1998, 2000; Gressens et al., 1999a,b; Redecker et al., 1998a,b; Tahraoui et al., 2001). In this model, systemically injected S 24429 and S 24718 significantly protected the developing mouse brain from neocortical gray and white matter damage induced by *S*-bromo-willardiine. The protective effects were dose-dependent and S 24718 was more potent than S 24429. Both compounds were more potent than the reference compound exifone, where the effects did not reach statistical significance. Glutamatergic lesions in white and gray matter involve both neurones and glia, so the effectiveness of S 24429 and S 24718 is of interest and confirms protective effects in both neurones and astrocytes. The present in vivo model did not permit to evaluate the capacity of tested drugs to cross the blood–brain barrier since this barrier is not fully competent at this developmental stage (Farrell and Risau, 1994). However, the compounds are lipophilic and would be expected to penetrate the blood–brain barrier.

Assuming that similar mechanisms are operant in human newborns, the present study and others (Largeron et al., 1999) suggest that 8-alkylamino-1,4-benzoxazine derivatives, including S 24429 and S 24718, are efficient neuroprotective agents and may be considered as potential candidates for the treatment and prevention of cerebral palsy, and might be active in other neurodegenerative diseases.

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