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In vitro properties of 5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone: A novel permeability transition pore inhibitor

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Abstract

Despite the increasing implication of the permeability transition pore (PTP) in the pathophysiology of neurodegenerative diseases, few selective PTP inhibitors have been reported so far. Here, we evaluate the pharmacological properties of a novel PTP inhibitor, BBMP (5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone). This drug was discovered from the screening of a compound library against the PTP using a functional assay with isolated mitochondria. Similarly to cyclosporin A, the drug prevented Ca^{2+}-induced permeability transition and mitochondrial depolarization. BBMP appeared more potent that minocycline in both swelling and membrane potential assays displaying pIC_{50} values of 5.5 ± 0.1 and 5.6 ± 0.0, respectively. Unlike minocycline, BBMP dose-dependently prevented DNA fragmentation induced by KCl 25/5 mM shift and serum deprivation in cerebellar granule neurons with a pIC_{50} of 5.7 ± 0.6. The inhibition of PTP-mediated cytochrome *c* release observed in isolated mitochondria at 10 and 100 μM may explain its neuroprotective properties in vitro. These data suggest that the mitochondrial PTP is potentially involved in neuronal cell death and that PTP inhibitors, like BBMP, may possess a therapeutic potential in neurodegenerative disorders.

Keywords: Permeability transition; Apoptosis; Neuroprotection; Mitochondrion

1. Introduction

Neuronal apoptotic cell death is a shared pathway occurring in neurodegenerative diseases such as Parkinson's disease (Mattson, 2000). There is no effective treatment today for preventing or reducing the risks of neurodegenerative diseases. One potential new approach for pharmacologic intervention is the blockade of neuronal cell death, for example, by inhibition of caspases, kinases (e.g., c-Jun NH_2-terminal protein kinase), p53-induced transcriptional activation and stabilization of the mitochondrial function with antioxidants and drugs which suppress mitochondrial release of pro-apoptotic factors such as cytochrome *c* (Waldmeier and Tatton, 2004).

Although neglected for a long time, the mitochondrial megachannel or permeability transition pore (PTP) has been recognized as a major component in the pathways leading to cell death (Susin et al., 1998; Halestrap et al., 1998; Lemasters et al., 1998; Bernardi, 1999; Kroemer and Reed, 2000). Various cell death triggering factors converge to mitochondria and facilitate the opening of these high-conductance, nonspecific pores: Ca^{2+} overload during excitotoxicity, pro-apoptotic proteins like Bax, oxidative stress, ganglioside, nitric oxide. The pore openings destroy the functional integrity of mitochondria giving rise to membrane potential collapse, membrane breakage, increase of reactive oxygen species, uncoupling of respiration and ATP depletion. The loss of ionic gradient and subsequent water influx increases the matrix volume and provokes large amplitude swelling. Swelling then leads to outer membrane disruption and to the release of apoptosis mediators (e.g.,

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apoptosis inducing factor, cytochrome *c*) as well as endogenous caspases (Hengartner, 2000; Olson and Kornbluth, 2001; Bortner and Cidlowski, 2002). There is another hypothesis concerning a mechanism of release of mitochondrial factors independent of the PTP and without matrix swelling (Kroemer and Reed, 2000; Brustovetsky et al., 2003). In this model, the voltage-dependent anion channel VDAC is affected by the pro-apoptotic proteins Bax and tBid and may account for a selective outer membrane permeabilization and release of cytochrome *c*.

Despite the role of the PTP in neuronal cell death still being debated (Nicholls and Budd, 2000), several lines of evidence indicate that the PTP plays a key role in the pathogenesis of neurodegenerative diseases in which cell death and apoptotic features have been described. The PTP inhibitor cyclosporin A and in particular its non-immunosuppressive analog *N*-MeVal4-cyclosporin A have been found to delay neuronal cell death in vitro (Khaspekov et al., 1999; Friberg et al., 1999) and to be neuroprotective in animal models of head trauma, cerebral ischemia (Friberg et al., 1998; Kuroda et al., 1999; Yoshimoto and Siesjo, 1999) and chronic neurodegenerative diseases like Parkinson's disease (Matsuura et al., 1997). More recently, the antibiotic minocycline has been shown to inhibit the PTP and cytochrome *c* release (Zhu et al., 2002) and to prevent neurodegeneration in cultured cell lines and animal models of Parkinson's disease and amyotrophic lateral sclerosis (Du et al., 2001; Zhu et al., 2002). This finding highlights the possible use of PTP inhibitors for providing prophylactic or curative pharmacotherapy for Parkinson's disease.

The exact molecular composition of the PTP is not clear but several laboratories have isolated a protein complex which mediates the permeability transition and is constituted of VDAC (voltage-dependent anion channel), ANT (adenine nucleotide translocator), cyclophilin-D, creatine kinase, hexokinase and the peripheral benzodiazepine receptor (Halestrap et al., 1998; Marzo et al., 1998). The binding sites of PTP inhibitors identified so far are cyclophilin-D for cyclosporin A (Halestrap et al., 1998), VDAC for Ro 68-3400 (Cesura et al., 2003) and ANT for bongkrekic acid (Marzo et al., 1998). Trifluoperazine (Hoyt et al., 1997) and ubiquinone 0 (Fontaine et al., 1998) have also been reported to prevent PTP openings but their molecular mechanism of action is less clear.

In this study, we report the discovery of a novel PTP inhibitor, BBMP (5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone), and its in vitro effects on mitochondrial swelling, membrane depolarization and cytochrome *c* release from isolated mitochondria. The activity of this compound is further evaluated using serum deprivation as stimulus and reduced potassium from 25 to 5 mM, which induces apoptosis in cerebellar granule neurons. The pharmacological properties of BBMP are also compared to that of the reference drugs cyclosporin A and minocycline (Fig. 1).

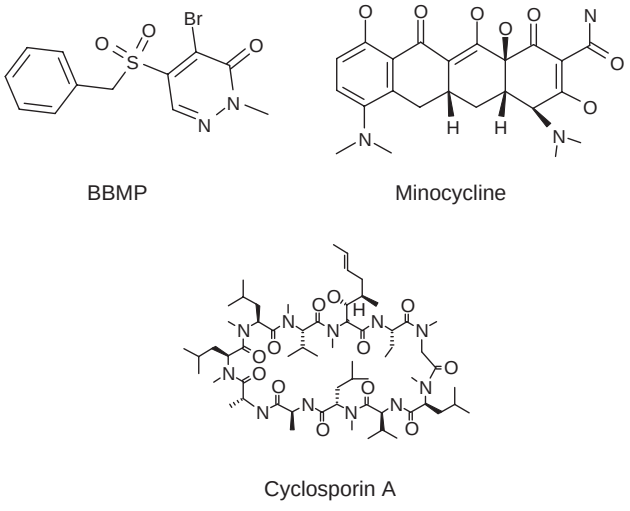

Fig. 1. Chemical structures of BBMP and the reference compounds evaluated in this study.

2. Materials and methods

2.1. Chemicals

Cyclosporin A and minocycline were purchased from Sigma–Aldrich (Belgium). Tetramethylrhodamine (TMRM) was obtained from Molecular Probes (The Netherlands). All other reagents were of the highest purity available from commercial vendors. The chemicals were dissolved in dimethyl sulfoxide and the final concentration was 1%.

2.2. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from Sprague Dawley rats by standard differential centrifugation. Briefly rats were decapitated and the livers were homogenized in an ice-cold HEPES 5 mM pH 7.4, mannitol 210 mM, sucrose 70 mM, EGTA 0.5 mM, BSA 2 mg/ml. The homogenate was centrifuged at 600×*g* for 5 min and the supernatant was removed and centrifuged at 15,000×*g* for 5 min to pellet the mitochondrial fraction. The pellets were washed three times, resuspended in HEPES 5 mM pH 7.4, mannitol 210 mM, sucrose 70 mM and used immediately.

2.3. Measurements of mitochondrial permeability transition

Permeability transition was assessed photometrically in 96-well plates by suspending mitochondria in 0.2 ml (100 μg per assay) at room temperature in HEPES 5 mM pH 7.4, mannitol 210 mM, sucrose 70 mM supplemented with succinate 10 mM, Na_2HPO_4 1 mM, rotenone 2 μM and oligomycine 1 μg/ml (Blattner et al., 2001). After 5 min of incubation in the presence of various concentrations of the test compounds, 200 μM $CaCl_2$ was added and the swelling rate was recorded at 550 nm for 5 min using an Optimax microplate reader (Molecular Devices, U.K.). The parameter used to evaluate the compounds is the inhibition of the initial swelling rate of untreated isolated mitochondria. Data of swelling inhibition were fitted using a sigmoidal curve and pIC_{50} values were calculated from each curve (GraphPad, Prism).

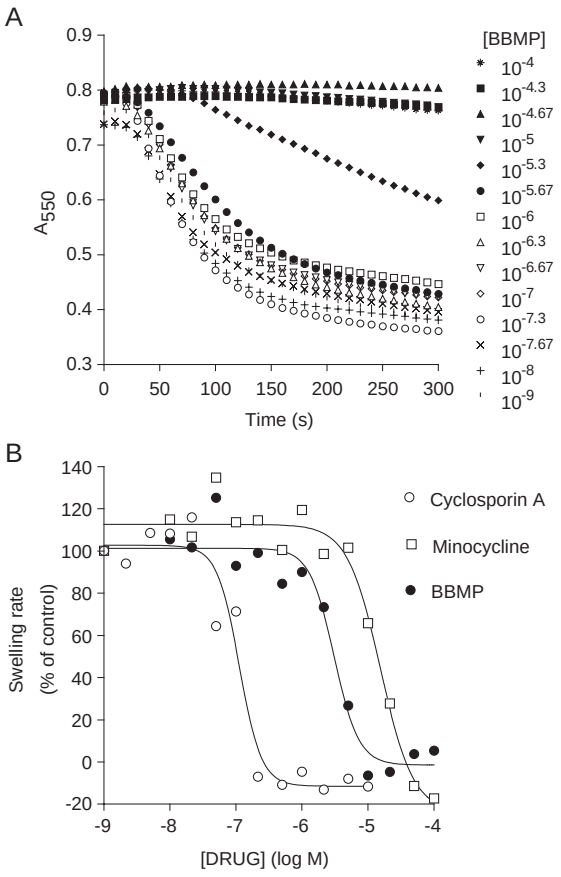

Fig. 2. Inhibitory effects of Cyclosporin A, minocycline and BBMP on the permeability transition induced by Ca^{2+} (200 μM). The permeability transition was measured as the change in absorbance of isolated mitochondria at 550 nm. The kinetics of a typical swelling assay performed with BBMP is shown in panel (A). BBMP, cyclosporin A and minocycline were added to the suspension of mitochondria for 5 min prior Ca^{2+} addition. Dose–response curves are shown in panel (B).

2.4. Fluorescence measurements

Fluorescence was measured in 96-well plates in a Polarstar multiwell fluorescence plate reader (BMG, Germany). Assays were conducted at room temperature in the same buffer as above and mitochondria (100 μg) were added in 0.2 ml of buffer. Fluorescence of tetramethylrhodamine (TMRM) was measured with excitation at 544 nm and emission at 590 nm (Blattner et al., 2001). The net change of fluorescence signal was expressed as the percentage of initial fluorescence before depolarization (F_0) that is $(F_{\text{final}} - F_0) \times 100/F_0$.

2.5. Measurements of cytochrome c release

A rat/mouse cytochrome *c* immunoassay (Quantikine M, R&D Systems, France) was used to quantify the amount released from isolated rat liver mitochondria. Mitochondria (100 μg) were preincubated for 5 min with the test drugs or vehicle in 0.2 ml of the swelling buffer at 25 °C followed by the addition of $CaCl_2$ (200 μM) for PTP activation. After 5 min, the suspension was centrifuged at 13,000 rpm for 1 min. The supernatants were transferred to fresh tubes, stored overnight at −20 °C and then further diluted 1:100 with the kit buffer and processed as defined in the manufacturer protocol.

2.6. Primary neuronal cultures

Cultures of granule neurons were obtained from dissociated cerebella of 7-day-old (P7) rats. Cells were seeded on precoated poly-(L-lysine) 96-well plates at a density of 1×10^5 cells per well in Basal Medium Eagle (BME) containing 25 mM KCl and 10% Fetal Bovine Serum (FBS). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. At day 1 (Day in vitro—DIV 1), deoxyuridine 10 μM was added to avoid non-neuronal cells proliferation. At day 7 (DIV 7), medium was replaced with serum-free BME containing 5 mM KCl during 16 h in the absence or presence of the test drug.

2.7. DNA fragmentation analysis

DNA fragmentation was measured photometrically by quantification of cytosolic histone-associated DNA fragments with an enzyme-linked immunosorbent assay (ELISA) kit for cell death detection (Roche, Germany) as described in Piret et al. (2004). The soluble DNA was extracted from each well, bound by an anti-histone antibody immobilized in an ELISA plate and recognized photometrically by an anti-DNA antibody. Data were fitted using a sigmoidal curve (GraphPad, Prism).

3. Results

3.1. Mitochondrial swelling experiments

There is a large body of literature describing the mitochondrial PTP as an attractive target for neuroprotection in Parkinson's disease and other brain disorders. Consequently, we decided to perform a screening campaign of a proprietary compound library against the PTP. The assay was designed in a 96-well format and used the decrease in absorbance of a mitochondrial suspension upon addition of Ca^{2+} (200 μM) as a readout (Fig. 2A). We worked with liver mitochondria for screening because the ease of the preparation, high yield of purification and well-known properties. The fact that the Ca^{2+}-induced swelling was inhibited by the reference inhibitors cyclosporin A and minocycline (Fig. 2B) validated this assay. The pIC_{50} values of cyclosporin A and minocycline were calculated from dose–response curves (Fig. 2B

Table 1
Inhibition of the Ca^{2+}-induced permeability transition in rat liver mitochondria

	pIC_{50}	*n*
Cyclosporin A	7.2±0.1	8
BBMP	5.5±0.1	5
Bongkrekic acid	5.3±0.1	3
Ubiquinone 0	4.9±0.0	3
Trifluoperazine	4.9±0.2	3
Minocycline	4.9±0.1	3

The permeability transition was measured as the change in absorbance of isolated mitochondria at 550 nm. Data of swelling inhibition were fitted using a sigmoidal curve and pIC_{50s} were calculated from each curve.

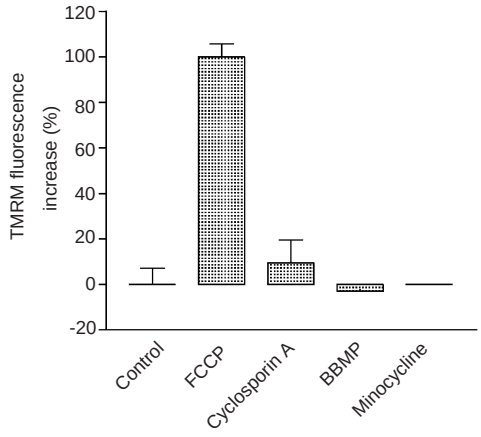

Fig. 3. Effects of test drugs on the mitochondrial membrane potential measured with the fluorescent dye tetramethylrhodamine (TMRM). BBMP, cyclosporin A and minocycline at 10 μM and the uncoupler FCCP at 1 μM were added to isolated mitochondria and TMRM fluorescence excited at 544 nm was recorded as described under Material and methods. Each bar represents the mean and the standard deviation of 3 individual experiments.

and Table 1) and are identical to previous published data (Elimadi et al., 1997; Zhu et al., 2002). During our screening campaign, we identified a small molecule, BBMP, (5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone) which is one of the most potent PTP inhibitors discovered so far although less active than cyclosporin A (see Table 1). Fig. 1A illustrates the time course of absorbance of rat mitochondria treated with increasing concentrations of BBMP. This compound did not activate the permeability transition by itself but prevented the mitochondrial swelling in a concentration-dependent manner, with a pIC_{50} value of 5.5±0.1 (Fig. 2B and Table 1).

3.2. Mitochondrial membrane potential measurements

Another event characteristic of the permeability transition is the collapse of mitochondrial membrane potential. To monitor membrane depolarization, suspensions of mitochondria were incubated with TMRM (1 μM). TMRM is a cationic fluorescent probe diffusing across mitochondrial membranes in response to a negative membrane potential and becomes quenched (Blattner et al., 2001). Membrane depolarization leads to an increase of basal fluorescence as exemplified with the uncoupler FCCP at 1 μM (Fig. 3). Uncouplers like FCCP are also detected as "false positive" PTP inhibitors in the swelling assay because depolarization prevents Ca^{2+} uptake into mitochondria and thereby PTP activation. Thus, the PTP inhibitors were also tested for their ability to change basal fluorescence levels. The addition of BBMP, cyclosporin A or minocycline at 10 μM to the mitochondrial suspension did not lead to a fluorescence increase (Fig. 3). These results clearly indicate that BBMP as well as the

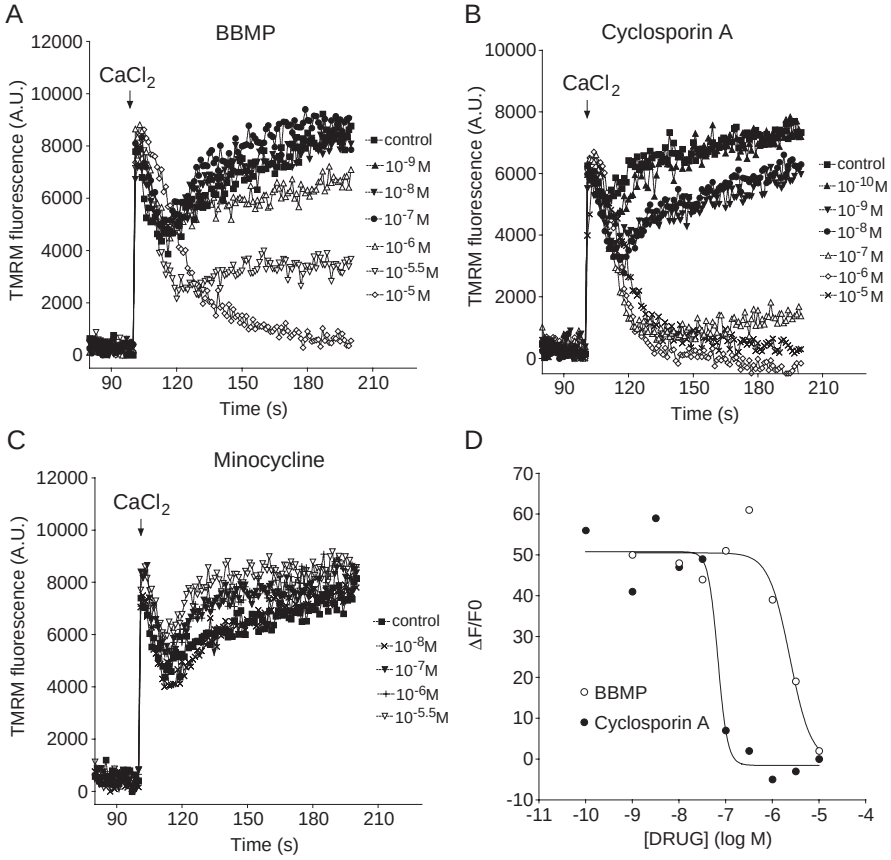

Fig. 4. Effects of test drugs on Ca^{2+}-induced membrane depolarization. Kinetics of the depolarization were measured after the addition of Ca^{2+} (200 μM) to mitochondria preincubated for 5 min with various concentrations of cyclosporin A, BBMP and minocycline. The traces shown in panels (A–C) are representative kinetics of three independent experiments. The dose–response curves in panel (D) represents the inhibition of the Ca^{2+}-induced fluorescence ($\Delta F/F_0$) increase with various concentrations of the test drugs.

reference compounds neither collapse the membrane potential nor induce a permeability transition. In contrast, the inclusion of $CaCl_2$ (200 μM) in the assay produced a mitochondrial depolarization and increased the TMRM fluorescence (Fig. 4A–C). To ascertain if this event could be attributed to the onset of the PTP, mitochondria were treated with the PTP blocker cyclosporin A (1 μM). As expected, this treatment prevented the Ca^{2+}-induced fluorescence increase, and within 1 min, the basal fluorescence level was recovered up to 100% in the presence of cyclosporin A (Fig. 4B). Similarly to its effect in the swelling assay, BBMP was active in the Ca^{2+}-induced membrane depolarization model with a pIC_{50} value of 5.6±0.0 (n=3) calculated from a dose–response curve as shown in Fig. 4D. As with BBMP, the pIC_{50} values for cyclosporin A calculated from both absorbance and fluorescence assays were perfectly correlated with values of 7.2±0.1 (n=8) and 7.2±0.0 (n=3), respectively. Minocycline which was less effective in preventing the mitochondrial swelling did not inhibit the membrane depolarization up to 3 μM (Fig. 4C) and interfered with the potential probe at higher concentrations (data not shown). Although we cannot definitively exclude the possibility that prevention of PTP openings by BBMP may be due to inhibition of Ca^{2+} uptake, a transient depolarization was however detected in the presence of active doses of BBMP and cyclosporin A following Ca^{2+} addition, which is induced by Ca^{2+} influx through the Ca^{2+} uniporter (Fig. 4A–B). In contrast, ruthenium red, a blocker of the Ca^{2+} uniporter, prevented this Ca^{2+}-mediated depolarization by inhibiting Ca^{2+} uptake (data not shown).

3.3. Cytochrome c release from isolated mitochondria

Activators of permeability transition, such as Ca^{2+}, stimulate the release of pro-apoptotic proteins from the mitochondrial interspace. When mitochondria were challenged by Ca^{2+}, cytochrome c release was detected in our preparations. Cyclosporin A at 10 μM completely inhibited the Ca^{2+}-induced swelling and thus the cytochrome c release under the PTP assay condition (Fig. 5). We also observed an inhibition of cytochrome c release of 60% with BBMP at 10 μM and 100 μM. Minocycline was also active in this in vitro model at 100 μM while the release was weakly

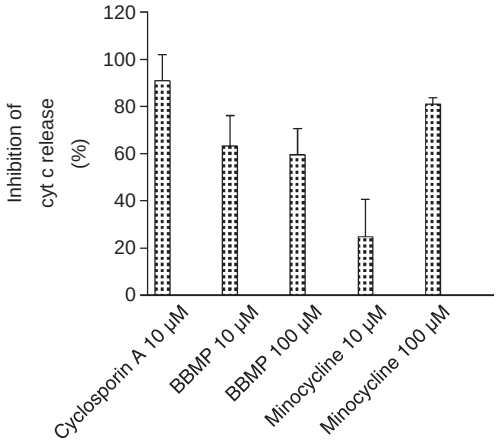

Fig. 5. Inhibition of cytochrome c release in isolated mitochondria. Liver mitochondria were incubated with cyclosporin A, BBMP and minocycline and cytochrome c release was induced by Ca^{2+} (200 μM). Data represent the mean±S.D. of two to three independent experiments.

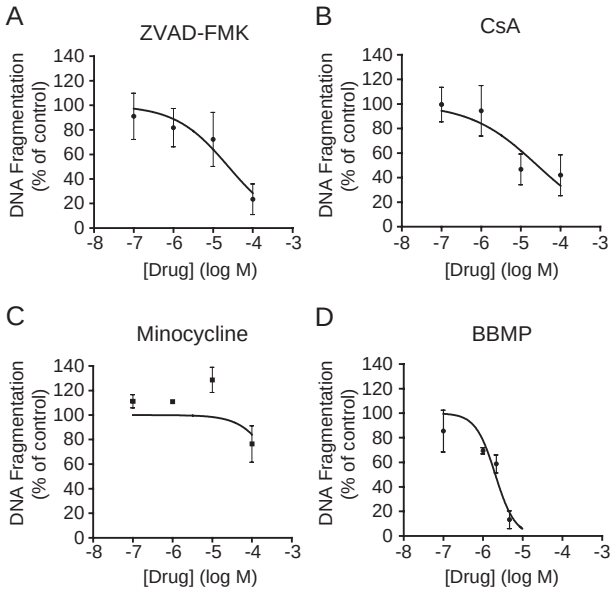

Fig. 6. Effects of test drugs on DNA fragmentation of rat cerebellar granule neurons (DIV 7) induced by serum deprivation and a KCl 25/5 mM shift. Production of histone-DNA fragments was measured photometrically 16 h after the beginning of the insult. The cells were treated with the test drugs at the beginning of serum deprivation. Histone-DNA fragments were expressed as percent of absorbance obtained with serum deprivation and KCl shift. Values are the means±S.D. from three independent experiments.

inhibited at 10 μM, a concentration found to be less active against the PTP in the swelling assay.

3.4. DNA fragmentation in cerebellar granule neurons after serum deprivation and KCl 25/5 mM shift

In order to evaluate the neuroprotective activity of BBMP and the reference drugs, we used a well-recognized in vitro model of apoptosis on cerebellar granule neurons. Cell death was initiated by serum deprivation and KCl 25/5 mM shift which are known to promote an apoptotic pathway associated with DNA fragmentation (D'Mello et al., 1993) and cytochrome c release (Wigdal et al., 2002). Thus, a marked increase in DNA fragments concentration was detected after serum deprivation and at low K^+ concentration (5 mM) after 16 h. The pan-caspase inhibitor ZVAD-fmk and the PTP inhibitor cyclosporin A were used to validate this cell death assay. Cells treated with these drugs were completely protected against DNA fragmentation (Fig. 6A–B). The pIC_{50} values measured for these compounds were 4.6±0.2 and 4.6±0.6, respectively. Treatment with minocycline at a concentration up to 100 μM did not lead to a reduced level of fragmentated DNA in serum deprived and KCl shifted cerebellar granule cells (Fig. 6C). In contrast, BBMP provided a complete protection in the micromolar range with a pIC_{50} of 5.7±0.6 (Fig. 6D).

4. Discussion

The present study demonstrated that BBMP, (5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone), is a novel inhibitor of the permeability transition as shown in Ca^{2+}-induced mitochondrial swelling, depolarization and

cytochrome *c* assays. A correlation between potency for inhibition in swelling and depolarization assays was observed. At the time of the study, most of the pIC_{50} values for PTP blockers reported in the literature were below 5.5 (Elimadi et al., 1997; Plemper van Balen et al., 2002; Zhu et al., 2002 and Table 1). Thus, BBMP is one of the most potent PTP inhibitors identified so far although less potent than cyclosporin A. More recently, Cesura et al. (2003) have discovered using a similar approach small molecules which inhibited the PTP with potencies comparable to that of cyclosporin A.

Although the molecular architecture of the pore remains uncertain, the PTP is thought to include the peripheral benzodiazepine receptor (Castedo et al., 2002). Therefore, we tested the ability of BBMP to displace the selective radioligand [^{3}H]PK11195 from this receptor. Our results indicate that this protein is not the binding site within the PTP complex (not shown). Thus, further work is required to characterize the molecular site by which BBMP blocks the PTP openings and to provide experimental evidences that this molecule acts directly on the PTP. The Ca^{2+}-induced permeability transition may also generate free radicals (Maciel et al., 2001) which in turn stimulate more channel openings. We found that the molecule was inactive against a set of 13 targets including G-protein-coupled receptors and channels and did not exhibit antioxidant properties as measured by lipid peroxidation of rat brain homogenates (not shown). Therefore, it is unlikely that this molecule prevents PTP openings by decreasing the level of reactive oxygen species.

PTP activation releases cytochrome *c* into the cytosol and thereby activates caspases and apoptotic cell death (Susin et al., 1998; Hengartner, 2000). In this study, the inhibition of cytochrome *c* release observed with BBMP may be explained by the blockade of the PTP. Furthermore, inhibition of the PTP by cyclosporin A correlated well with inhibition of cytochrome *c* release. BBMP also exhibited protective effects in isolated neurons in the micromolar range when DNA fragmentation was induced by serum deprivation and KCl 25/5 mM shift in cerebellar granule cells. This neuroprotective activity seems to be mediated by the PTP since cyclosporin A was also effective in this model and may be attributed to the prevention of cytochrome *c* release observed in isolated mitochondria.

Unlike BBMP, minocycline was unable to prevent DNA fragmentation in vitro in cerebellar granule cells submitted to serum deprivation and KCl shift. Although minocycline-mediated neuroprotection was recently found to be associated with inhibition of the permeability transition and cell death in vitro (Zhu et al., 2002), recent experimental data question the role of mitochondria and the neuroprotective effect of minocycline. In rat cerebellar granule cells, prevention of cell death by minocycline was shown to be mediated through the inhibition of free radical generation in oxidative stress induced by 6-hydroxy-dopamine (Lin et al., 2003) and inhibition of p38 MAPkinase (Lin et al., 2001) but there was no protection when neurotoxicity was induced by the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Du et al., 2001).

In conclusion, our results obtained with BBMP using in vitro models indicate that this novel PTP blocker may be a useful tool to understand the molecular pathways leading to cell death and the role of the PTP in this process. In addition, the inhibition of DNA fragmentation and thus cell death observed in isolated neurons with BBMP suggest that drugs preventing PTP activation and mitochondrial depolarization may have potential application in neurodegenerative diseases.

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