Molecular and cellular pharmacology

JM-20, a novel benzodiazepine–dihydropyridine hybrid molecule, protects mitochondria and prevents ischemic insult-mediated neural cell death in vitro


Abstract

The ischemic stroke cascade is composed of several pathophysiological events, providing multiple targets for pharmacological intervention. JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5]benzodiazepine) is a novel hybrid molecule, in which a benzodiazepine portion is covalently linked to a dihydropyridine ring, forming a new chemical entity with potential multisite neuroprotective activity. In the present study, JM-20 prevented PC-12 cell death induced either by glutamate, hydrogen peroxide or KCN-mediated chemical hypoxia. This molecule also protected cerebellar granule neurons from glutamate or glutamate plus pentylenetetrazole-induced damage at very low micromolar concentrations. In rat liver mitochondria, JM-20, at low micromolar concentrations, prevented the Ca$^{2+}$-induced mitochondrial permeability transition, as assessed by mitochondrial swelling, membrane potential dissipation and organelle release of the pro-apoptotic protein cytochrome c. JM-20 also inhibited the mitochondrial hydrolytic activity of F1F0-ATP synthase and Ca$^{2+}$ influx. Therefore, JM-20 may be a multi-target neuroprotective agent, promoting reductions in neuronal excitotoxic injury and the protection of the mitochondria from Ca$^{2+}$-induced impairment as well as the preservation of cellular energy balance.

1. Introduction

Ischemic stroke is a major cause of death and the leading source of adult-onset disability in many countries (Mukherjee and Patil, 2011). The recombinant tissue plasminogen activator is currently the only approved drug for use in humans during the acute phase of ischemic stroke, and alternative treatments remain limited (Howells and Donnan, 2010). Despite encouraging pre-clinical results, none of the evaluated candidates have resulted in consistent clinical improvements. This may be due to the multiplicity of mechanisms involved in the neuronal damage cascade following brain ischemia, which contrasts with the more simplistic vision of the proposed neuroprotectants (Minnerup and Schäbitz, 2009). Accumulating pre-clinical evidence indicates that a highly selective ligand for a given target does not always result in a clinically efficacious drug, particularly in pathologies that involve multiple factors, such as cerebral stroke. Therefore, drugs acting at a single site in the ischemic cascade, such as Ca$^{2+}$ channel blockers, glutamate antagonists, GABA agonists, antioxidants/free radical scavengers, phospholipid precursors, nitric oxide signal-transduction down-regulators, and anti-inflammatory agents, have generally failed in the bench to bedside translation process (Ginsberg, 2008).
Emerging neuroprotective approaches have begun to consider mitochondrial bioenergetic dysfunction. Evidence suggests that the mitochondria play a pivotal role in ischemic neuronal injury either by integrating noxious signals involved in their structural and functional damage or by pathway amplification, which eventually leads to cell death (Christophe and Nicolas, 2006; Mazzeo et al., 2009; Perez-Pinzon et al., 2012). Therefore, there has been increasing interest in the identification of new classes of compounds that simultaneously target several toxic processes in ischemic neuronal cells, including those that act at the mitochondrial level.

Recently, we obtained a new family of 1,5-benzodiazepines that structurally differ from the currently available 1,5-benzodiazepines due to the presence of a 1,4 dihydropyridine moiety fused to the benzodiazepine ring. JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5]benzodiazepine) is a member of this family of compounds (Fig. 1) and has an anxiolytic profile that is similar to that of diazepam (Figueroed et al., 2014). Its dihydropyridine moiety does not appear to interfere with the GABAgergic activity associated with its benzodiazepine portion but may confer Ca²⁺ channel blocking properties, indicating that JM-20 may act as a potential neuroprotector.

Moreover, mitoprotection is another potential mechanism that may be involved in the neuroprotective effects of JM-20 because diazepam and nimodipine, both of which exhibit structural features that are similar to those of JM-20, have been reported to protect neuronal cells in brain ischemia models via mitochondrial mechanisms (Taya et al., 2000; Sarnowska et al., 2009). In this context, we hypothesized that JM-20 may protect neural cells from different toxic mediators that play major roles in the ischemic cascade by acting as a multifunctional drug, including by preventing mitochondrial impairment. Thus, in the present study, we evaluated the protective effects of JM-20 against damage induced by glutamate, hydrogen peroxide or oxygen/glucose deprivation in PC-12 cells or rat primary cerebellar neurons, which well known in vitro models to be relevant to cerebral ischemia (Facci and Skaper, 2012; Im et al., 2010). We also evaluated the protective effects of JM-20 against Ca²⁺-induced mitochondrial impairment in rat liver mitochondria, a recognized model that has been used to study the mitochondrial mechanisms associated with neurodegeneration and to screen for neuroprotectors (Zhu et al., 2002; Zhang et al., 2008; Wang et al., 2009). We observed that JM-20 is protective against in vitro neurodamage induced by mediators of the ischemic cascade, and our results indicated that mitoprotection may play a role in the primary mechanism of action of JM-20.

2. Materials and methods

2.1. Compounds and reagents

All of the chemicals used were of the highest grade available and were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Stock solutions of JM-20 were prepared daily in absolute ethanol or dimethyl sulfoxide (DMSO) and added to the cell culture or mitochondrial reaction media, respectively, at 1/1000 (v/v) dilutions. Control experiments contained absolute ethanol or DMSO at a 1/1000 dilution. JM-20 was synthesized, purified and characterized as previously reported (Figueroed et al., 2014).

2.2. Experimental animals

For the mitochondrial assays, adult male Wistar rats (CENP-LAB, Havana, Cuba) weighing approximately 200 g were housed in a temperature-controlled room under a 12 h light/dark cycle with free access to food and water. The animals (n=15) were quarantined for a minimum of 7 days prior to experimentation. Primary cultures of cerebellar neurons were prepared from 7-day postnatal male Wistar rats (n=3) obtained from in-house breeding colonies at the UCTB Control Biológico (Drug Research and Development Center, CIDEM, Havana, Cuba). Animal housing, care, and the application of experimental procedures were in accordance with institutional guidelines and were conducted according to approved protocols (Animal Care Committee from CIDEM, Havana, Cuba).

2.3. PC-12 cell culture

Phaeochromocytoma (PC-12) cells were kindly supplied by Beatriz Caputto, Ph.D., from Cordoba National University, and Sandra Verstraeten, Ph.D., from Buenos Aires University, Argentina. Cells at passage seven were routinely maintained in Dulbecco’s Modified Essential Medium (DMEM) containing L-glutamine (Sigma, St. Louis, MO) and supplemented with 10% heat-inactivated equine serum (Gibco, Life Technologies, USA), 5% inactivated fetal bovine serum, 50 U/ml of penicillin and 10 mg/ml of streptomycin (all of which were obtained from Sigma, St. Louis, MO) in a humidified atmosphere containing 95% air/5% CO₂ at 37 °C. The growth medium was replaced every 3 days. At day seven, the cells were trypsinized (Sigma, St. Louis, MO) and seeded at a density of 1.5 × 10⁵ cells/ml in 96 well multiwell flat bottom plates (Corning Costar, Sigma) in 200 μl of culture medium at 37 °C and were flushed with 5% CO₂ in air for 24 h (Choi et al., 2011; Figueredo et al., 2011; Yoon et al., 2011).

2.4. Cerebellar cell cultures

Primary cultures of cerebellar cells were obtained by modifying previously outlined procedures (Whittemore et al., 1995). Briefly, whole brains from 7-day postnatal Wistar rats were maintained in calcium and magnesium-free Hank’s Balanced Salt Solution (HBSS, Gibco, Paisley, UK). The cerebellums were isolated and tissue pieces were trypsinized (1% trypsin/0.1% DNase; Gibco BRL), incubated for 20 min at 37 °C, and mechanically dissociated in DNase (0.05%) using flame polished Pasteur pipettes. The suspensions were centrifuged at 1800g for 3 min at 4 °C, and the resultant pellets were resuspended and passed through 30 μm Nytex filters to remove undissociated cells (Console-Bram et al., 1998). The cells were then reconstituted in growth medium containing 85% DMEM containing l-glutamine, 10% fetal bovine serum, 50 U/ml of penicillin and 10 mg/ml of streptomycin. The cells were plated on poly-l-lysine (Sigma) coated 96-multiwell flat bottom plates (Corning Costar) at a density of 5 × 10⁴ cells/ml in 200 μl of culture medium at 37 °C and were flushed with 5% CO₂ in air for 72 h.

2.5. Induction of glutamatergic damage in cerebellar and P-C12 cells

After the incubation period, the cells were rinsed with buffered saline solution and exposed to 50 mM glutamate (PC-12 cells),...
15 μM glutamate or 15 μM glutamate plus 40 mM pentyleneetetrazole (cerebellar granules) in the absence (control) or presence of JM-20 (0.001–10 μM). Diazepam and nifedipine, both at 1 and 10 μM, were used as reference drugs. The incubation medium contained a standard Tris-buffered salt solution (25 mM) with the following composition: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.4 (Pereira and Oliveira, 1997). The cells were incubated for 24 h in a humidified atmosphere containing 95% air and 5% CO₂.

2.6. Induction of H₂O₂-mediated oxidative damage in PC-12 cells

PC-12 cells were damaged with 200 μM H₂O₂ (Siddiqui et al., 2011) in the absence (control) or presence of JM-20 (0.01–10 μM). The incubation medium was the same as that used in the glutamateergic damage experiments. The cells were incubated for 18 h in a humidified atmosphere containing 95% air and 5% CO₂.

2.7. Induction of cyanide-induced hypoxia in PC-12 cells

For the cyanide-induced injury, the cells were exposed to 0.8 mM KCN in DMEM without glucose in the absence (control) or presence of JM-20 (0.001–10 μM) over the course of 1 h in a humidified atmosphere containing 95% air and 5% CO₂. Hypoxia was terminated by aspirating the poisoned medium and washing the cells with fresh media in all of the experimental groups, including the control culture. The reperfusion state was simulated by the addition of DMEM without KCN and with high glucose content for 24 h (Satpute et al., 2008). JM-20 was added to the incubation medium either (i) during the chemical hypoxia and reperfusion periods or (ii) only during reperfusion period.

2.8. PC-12 and cerebellar granule cell viability assays

Cell viability was measured by the levels of blue formazan products formed from the colorless 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are only active in viable cells. After the incubation period, the cells were gently washed with sterile dehydrogenases, which are only active in viable cells. After the incubation medium either (i) during the chemical hypoxia and reperfusion periods or (ii) only during reperfusion period.

2.8.1. Induction of H₂O₂-mediated oxidative damage in PC-12 cells

PC-12 cells were damaged with 200 μM H₂O₂ (Siddiqui et al., 2011) in the absence (control) or presence of JM-20 (0.01–10 μM). The incubation medium was the same as that used in the glutamateergic damage experiments. The cells were incubated for 18 h in a humidified atmosphere containing 95% air and 5% CO₂.

2.8.2. Induction of cyanide-induced hypoxia in PC-12 cells

For the cyanide-induced injury, the cells were exposed to 0.8 mM KCN in DMEM without glucose in the absence (control) or presence of JM-20 (0.001–10 μM) over the course of 1 h in a humidified atmosphere containing 95% air and 5% CO₂. Hypoxia was terminated by aspirating the poisoned medium and washing the cells with fresh media in all of the experimental groups, including the control culture. The reperfusion state was simulated by the addition of DMEM without KCN and with high glucose content for 24 h (Satpute et al., 2008). JM-20 was added to the incubation medium either (i) during the chemical hypoxia and reperfusion periods or (ii) only during reperfusion period.

2.8.3. Continuous-monitoring mitochondrial assays

The mitochondrial membrane potential was estimated spectrophotometrically using 10 μM Safranine O as a probe (Zanotti and Azzone, 1980) in a Model F-4500 Hitachi fluorescence spectrophotometer (Tokyo, Japan) at the 495/586 nm excitation/emission wavelength pair; these assays were performed in the presence of 0.1 mM EGTA and 2 mM K₂HPO₄. Ca²⁺ influx and efflux were monitored spectrophotometrically using 150 nM Calcium Green 5N (Molecular Probes, OR, USA) as a probe at the 506/531 nm excitation/emission wavelength pair (Rajdev and Reynolds, 1993). Mitochondrial swelling was estimated spectrophotometrically based on the decrease in the apparent absorbance at 540 nm using a Model U-2910 Hitachi spectrophotometer (Japan).

2.8.4. Determination of the hydrolytic activity of F₁F₀-ATP synthase

The hydrolytic activity of F₁F₀-ATP synthase was assessed in submitochondrial particles. After incubation, the harvested mitochondria were resuspended in CellLytic™ (Sigma, USA) lysis buffer for 2 min on ice and centrifuged at 10,000 g for 5 min at 4 °C. Equal amounts of mitochondrial proteins (50 μg, quantified using the Biuret method) from the resulting supernatants were separated using SDS-PAGE (12%) and transferred to nitrocellulose membranes. After being blocked with 5% non-fat milk, the membranes were washed with TBST (TBS: Tris-buffered-saline, 50 mM Tris, 150 mM NaCl, pH 7.5, with 0.1% Tween 20) and incubated with a purified mouse anti-cytochrome c antibody from BD Biosciences Pharmingen (San Diego, CA, USA) at a 1:2000 dilution overnight. After being washed three times with TBST, a secondary anti-mouse antibody at a 1:10,000 dilution was applied for 2 h, followed by horseradish peroxidase-coupled detection. The blots were treated with ECL reagents for 5 min and exposed to CL-Xposure films. The blots were scanned and densitometric analyses of the scanned images were performed using Scion Image-Release Beta 4.02 software.

2.13. Determination of the hydrolytic activity of F₁F₀-ATP synthase

The hydrolytic activity of F₁F₀-ATP synthase was assessed in submitochondrial particles by measuring the amount of inorganic phosphate released by MgATP hydrolysis (Heinonen and Lahti, 1981) at 355 nm. The reaction was initiated by the addition of MgATP (10 μM) to 1 ml of medium (100 mM sucrose, 80 mM KCl and 50 mM HEPES–KOH, pH 7.4, at 37 °C) containing submitochondrial particles (20 μg of protein) in the absence or presence of JM-20 (5 μM), oligomycin (1 μM), or Triton X-100 0.1% (v/v) and was terminated by the addition of 0.5 ml of 30% trichloroacetic acid. The hydrolytic activity of F₁F₀-ATP synthase was also assessed in isolated mitochondria exposed to KCN (0.5 mM) in the presence...
of MgATP (1 mM) by measuring mitochondrial membrane potential in the absence or presence of JM-20 (5, 10 and 15 μM) or oligomycin (1 μM). The conditions were the same as those described for the mitochondrial membrane potential estimations.

2.14. Statistical analyses

The GraphPad Prism 5.0 (GraphPad Software Inc., USA) software program was used for the statistical analyses. The data were expressed as the means ± S.E.M. Comparisons among groups were performed using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparisons test. Differences were considered to be statistically significant at P < 0.05.

3. Results

3.1. Cytoprotective effects of JM-20 against glutamate-mediated damage in PC-12 cells

PC-12 cell viability, as assessed using the MTT reduction assay, was significantly reduced (by approximately 45%) after exposure to 50 mM glutamate for 24 h (Fig. 2). JM-20 treatment significantly prevented (P < 0.05) cell death in a concentration-dependent manner, with a very low half inhibitory concentration value (IC50 = 0.029 μM). Because the functionality of NMDA and GABA receptors in PC-12 cells has not been clearly defined (Hales and Tyndale, 1994; Edwards et al., 2007), it was not possible to evaluate the effect of the benzodiazepine fraction of JM-20 on the activation of the ionotropic receptors by glutamate stimulation in this cell line. We used a primary culture of cerebellar granule neurons, which are affected by excitotoxic stimuli mediated by the activation of the NMDA receptor via Ca2+–mediated processes, such as mitochondrial depolarization and ROS generation (Li et al., 2004).

3.2. Cytoprotective effect of JM-20 against glutamate- or glutamate plus pentylenetetrazole-mediated damage to primary cerebellar cell cultures

Fig. 3A shows that exposure of primary cultures of cerebellar granule neurons to 15 μM glutamate significantly reduced cell viability (by approximately 50%). JM-20 treatment increased cell survival in a concentration-dependent manner, with a very low IC50 value (0.011 μM). This molecule reached its maximal protective effect at 0.1 μM, a concentration that is approximately 100 times lower than the concentrations of diazepam (DZP) and nifedipine (Nif) that are required to see a similar effect. Fig. 3B shows that the combined presence of glutamate (Glut) and pentylenetetrazole (PTZ) in the incubation medium was more cytotoxic than either compound individually, as expected. PTZ is a well-known GABA_A receptor antagonist (Pomés et al., 1993; Qu et al., 2005) that inhibits regulatory GABAergic mechanisms in cells. Under such conditions, JM-20 elicited a concentration-dependent cytoprotective effect, with an IC50 value of 0.09 μM. As expected, DZP was unable to confer substantial cytoprotection to cells exposed to both neurotoxicants. Interestingly, nifedipine, a well-known L-type Ca2+ channel blocker (Nguego et al., 2013), provided almost full protection to the cells at a concentration of 1 μM, which was similar to JM-20.

3.3. Cytoprotective effects of JM-20 against H2O2-induced oxidative damage in PC-12 cell cultures

The effects of JM-20 against oxidative insults in PC-12 cell cultures were investigated. Exposure of the cells to H2O2 for 18 h significantly reduced cell viability (by approximately 50%) compared to undamaged control cells (Fig. 4). JM-20 exerted significant protective effects (P < 0.05) against H2O2-induced cytotoxicity at concentrations greater than or equal to 1 μM (Fig. 4). The estimated IC50 value was 6.92 μM, a value that is substantially higher than that observed when measuring the cytoprotective effects of JM-20 against glutamatergic damage.

3.4. Protective effects of JM-20 against KCN-mediated damage in PC-12 cell cultures

Incubation of PC-12 cells with KCN (800 μM) in glucose-free medium for 1 h, followed by 24 h reperfusion (fresh growth medium without KCN and with a high glucose content), resulted in approximately 50% cell death. Fig. 5A shows a strong cytoprotective effect of JM-20 when it was added during hypoxia and the reperfusion period (the lower cytoprotective concentration was 0.001 μM), with a very low IC50 value (0.008 μM). Fig. 5B also shows a cytoprotective effect of JM-20 when it was added only during the reperfusion state (the lower cytoprotective concentration was 0.01 μM). The estimated IC50 value under this condition was 0.056 μM, a value that is seven times higher than that obtained when JM-20 was incubated from the beginning of the chemical-induced ischemic insult (0.008 μM). Given the fact that the mitochondria play a critical role in ischemia/reperfusion-induced neuronal cell injury (Sanderson et al., 2013), particularly during glutamate- or oxidant-induced cell death (Pereira and Oliveira, 2000; Hwang and Yen, 2008), we assessed the effects of JM-20 in isolated rat liver mitochondria, which are a classical model for studies of mitochondrial mechanisms (Zhu et al., 2002; Zhang et al., 2008; Wang et al., 2009).

3.5. JM-20 protects the mitochondria from Ca2+ plus inorganic phosphate (Pi)-mediated mitochondrial swelling and membrane potential dissipation

A key pathogenic event during stroke is the opening of pores in the inner mitochondrial membrane, the mitochondrial permeability transition pores (mPTP) (Hirsch et al., 1998). In the next set of experiments, we assessed the effects of JM-20 on Ca2+ and Pi-induced opening of mPTP, as estimated by swelling of succinate-energized rat liver mitochondria. This classic swelling technique monitors the net influx of the osmotic support associated with a non-specific increase in membrane permeability. Fig. 6A shows that 50 μM Ca2+ plus 2 mM Pi induced mitochondrial swelling, as
revealed by the large decrease in the turbidity of the mitochondrial suspension at 540 nm (trace a). This swelling was associated with a faster mitochondrial membrane potential dissipation (Panel B, trace a). JM-20 completely inhibited both swelling and mitochondrial membrane potential dissipation (trace e, and Panels A and B, respectively) in a similar manner to the protection elicited by the classic mPTP inhibitor, cyclosporine A (CsA, line f).

3.6. JM-20 prevented mitochondrial cytochrome c release in isolated rat liver mitochondria

Cytochrome c release from the mitochondria plays an important role in cell death (Li et al., 1997). Because we demonstrated that JM-20 prevented neuronal cell death in different paradigms associated with the ischemic process (Figs. 2–5), we next assessed the potential involvement of inhibition of cytochrome c release; Western blotting was used to detect the levels of free cytochrome c in the supernatant fraction after mitochondrial swelling experiments (Fig. 7). Ca2+/Pi overload induced a substantial release of cytochrome c from rat liver mitochondria, an effect that was almost completely abolished by CsA and EGTA. JM-20 prevented this release at low micromolar concentrations, in a concentration-dependent manner.

3.7. JM-20 inhibits the hydrolytic activity of F1F0-ATP synthase

The hydrolytic activity of F1F0-ATP synthase is one of the largest ATP consumers under ischemic conditions. This activity consumes any available ATP and favors Ca2+ accumulation due to ATP-dependent mitochondrial membrane potential recovery, potentiating mitochondrial damage (Christophe and Nicolas, 2006). Therefore, we evaluated the effects of 5 μM JM-20 on the hydrolytic activity of F1F0-ATP synthase for both the membrane-bound enzyme and its Triton X-100 solubilized form (Fig. 8A). The inhibitory effects of JM-20 on the enzyme activity followed a similar pattern to that of oligomycin, a classical F1F0-ATP synthase inhibitor, showing a marked...
inhibition of the membrane-bound but not the solubilized form of the enzyme. In intact mitochondria exposed to the cytochrome c oxidase inhibitor, KCN, JM-20 also inhibited the hydrolytic activity of F1F0-ATP synthase (Fig. 8B), as evidenced by its ability to prevent the ATP hydrolysis-mediated recovery of the mitochondrial membrane potential after ATP addition.

3.8. JM-20 inhibits mitochondrial Ca2+ influx

JM-20 inhibited Ca2+ influx in succinate-energized rat liver mitochondria (Fig. 9). JM-20 at a concentration of 15 µM elicited 64% inhibition (line c) in relation to the inhibition elicited by the mitochondrial Ca2+ uniporter blocker, ruthenium red (line d).

4. Discussion

Neuroprotection for ischemic stroke refers to strategies applied either individually or in combination that antagonize the injurious biochemical and molecular events occurring in irreversible ischemic injury (Ginsberg, 2008). The complexity of the ischemic cascade, which is in contrast to the mono-ligand approaches followed by the majority of investigations, has contributed to the failures to improve clinical translation (Minnerup and Schäbitz, 2009). In this study, we demonstrated a strong cytoprotective effect of JM-20, a new benzodiazepine-dihydropyridine hybrid molecule, which was likely mediated through its multimodal
action not only at the neural level but also at the mitochondrial level. We used two cellular models of exposure to either glutamate or H₂O₂, which have been associated with neuronal injury during ischemia. In such cell death paradigms, JM-20 was able to protect cells at very low micromolar concentrations, with IC₅₀ values of 0.029, 0.011, 0.09, 0.008 and 0.056 μM, respectively. Oligomycin at a concentration of 1 μM was used as a control for enzyme inhibition (trace e). RLM, KCN, MgATP and CCCP (1 μM) were added when indicated by the arrows. The results represent three experiments that were conducted using different mitochondrial preparations. *P < 0.05 versus Control with no triton.

Excitotoxicity is one of the main mechanisms underlying neuronal death during stroke, which is triggered by a massive extracellular release of the excitatory transmitter glutamate, leading to intracellular Ca²⁺ overload, mitochondrial dysfunction, and eventually cell death (Dirnagl et al., 1999). In addition, the generation of ROS, such as hydroxyl radicals, superoxide anions and nitric oxide, can cause lipid peroxidation and membrane damage (Love, 1999; Warner et al., 2004), and may eventually lead to neuronal apoptosis or necrosis, depending on the intensity of the initial insult and the extent of energy recovery (Sims and Muysderman, 2010). Given the chemical structure of JM-20, its benzodiazepine moiety and/or to act as an inhibitor of glutamate-mediated Ca²⁺ entry in the neurons (dihydropyridine portion). We recently demonstrated GABAergic effects of JM-20 in different relevant animal models (Figueroedo et al., 2014); therefore, we presumed that this mechanism may contribute, at least in part, to the anti-glutamatergic and neuroprotective effects elicited by JM-20 in cells. The anti-Ca²⁺ effect was indirectly demonstrated in the experiment in which cerebellar granule cells were exposed to PTZ plus glutamate. PTZ is a chemical convulsant frequently used in the study of seizures. It is generally accepted that PTZ exerts its effects by binding to the picrotoxin-binding site of the postsynaptic GABA₆ receptor. PTZ reduces GABA-mediated responses in a concentration-dependent manner (Elloqlayli et al., 2002). Cells treated with PTZ plus glutamate showed a lower percentage of survival than those exposed to each cytotoxic compound individually. One of the mechanisms implicated in the neuroprotective effects of GABAergic transmission is inactivation of the excitatory glutamate receptors (Herrero et al., 1999; Green et al., 2000; Ouyang et al., 2007). Thus, the increase in cell death after cell exposure to glutamate plus PTZ may be due to the blockade of the endogenous GABAergic response by PTZ, which may leave cells more susceptible to the toxicity of NMDA receptor-mediated Ca²⁺ overload. In this context, JM-20 maintained its neuroprotective properties and protected cerebellar granule neurons with a very low IC₅₀ value, suggesting that JM-20 has a neuroprotective effect that is mediated by mechanisms that are not simply associated with the GABAergic response. Indeed, the lack of DZP effect and the strong nifedipine protection that took place under this experimental condition suggest that JM-20 may be acting as a Ca²⁺ antagonist or that JM-20 may be more potent than DZP in acting on GABA receptors. Because we recently observed that DZP was almost twice as potent as JM-20 when acting as an anxiolytic in vivo (Nuñez-Figueroedo et al., 2014), the former mechanistic assumption most likely contributes more to the neuroprotection elicited by JM-20 under the conditions outlined above. Moreover, when cells were injured only with glutamate, JM-20 reached its maximal neuroprotective effect at 0.1 μM (IC₅₀ value = 0.011 μM), a concentration that was 100 times lower than that by which nifedipine or DZP elicited full cell protection (10 μM). These results suggest that the strong neuroprotective action of JM-20 may involve both anti-excitotoxic and anti-Ca²⁺ effects, rendering JM-20 a potential neuroprotective agent. Accordingly, due to the importance of Ca²⁺ overload for cell death, dual Ca²⁺ channel and NMDA receptor modulators are considered to be useful neuroprotective drugs in neurodegenerative diseases (Van der Schyf, 2011).

Oxidative stress is another key mediator of the ischemic process, particularly after reperfusion (Endres et al., 2008). In this sense, it is not surprising that several antioxidants are under preclinical or clinical investigation as neuroprotective agents during
ischemic stroke (Lau and Tymianski, 2010). JM-20 prevented H$_2$O$_2$-induced PC-12 cell death at relatively high concentrations that were one hundred times higher than those required for JM-20-mediated neuroprotection against glutamate or KCN-induced cell damage, suggesting that the antioxidant mechanism is indirectly involved in the neuroprotective action of JM-20. In fact, although very low, the IC$_{50}$ value for PC-12 cell protection against glucose and chemical-induced anoxia was seven times higher when JM-20 was added during the reperfusion stage than when it was added from the beginning of the experiment. Because mitochondrial ROS generation seems to be secondary to Ca$^{2+}$ influx via NMDA receptors (Lau and Tymianski, 2010) and because mitochondrial Ca$^{2+}$ accumulation seems to play a key role in glutamate excitotoxicity (Nicholls et al., 2003), the neuroprotection elicited by JM-20 against oxidant-induced cell damage or during ischemia/reperfusion suggests that it could be acting either upstream of the mitochondria or at the organelle level. In this regard, mitoprotection could be another important mechanism contributing to the overall neuroprotective action of JM-20 because diazepam and nimodipine, both of which exhibit structural similarities to JM-20, have been reported to protect neuronal cells in different experimental models of brain ischemia via mitochondrial mechanisms (Taya et al., 2000; Sarnowska et al., 2009). Therefore, in the present study, we also addressed the idea that the mitochondria are potential pharmacological targets for the neuroprotective action of JM-20.

Under normal conditions, the mitochondria are primarily active during energy metabolism and as high-capacity Ca$^{2+}$ sinks (Giacomello et al., 2007). However, the excessive mitochondrial Ca$^{2+}$ loading following an ischemic insult may result in the opening of mitochondrial permeability transition pores (mPTP) (Sullivan et al., 2005). This mPTP opening will in turn result in a massive leak of protons and complete and immediate dissipation of the mitochondrial membrane potential, indicating a commitment to death. The release of cytochrome c from the intermembrane space of the mitochondria to the cytosol is one of the critical events that occurs during apoptotic neuronal cell death (Lau and Tymianski, 2010; Fiskum, 2000). In some cases of apoptosis, mPTP opening is involved (Kroemer, 1997). Thus, mPTP has been proposed to be integral to the apoptotic mechanism implicated in ischemia-triggered mitochondrial dysfunction and neuronal cell death (Mazzeo et al., 2009). Accordingly, agents that can block mPTP opening have produced neuroprotective effects, as demonstrated in several studies (Osman et al., 2011; Zhu et al., 2002; Zhang et al., 2008; Wang et al., 2009). In the present study, we demonstrated the protective effect of JM-20 against Ca$^{2+}$-induced mPTP opening and mitochondrial membrane potential dissipation, together with the inhibition of Ca$^{2+}$-induced cytochrome c release from mitochondria. These effects may be associated with the ability of JM-20 to prevent neuronal cell death induced by glutamate or H$_2$O$_2$, which are well known inducers of cytochrome c-mediated neuronal cell death (Pereira and Oliveira, 2000; Hwang and Yen, 2008).

JM-20 significantly prevented mitochondrial Ca$^{2+}$ influx, which may have been responsible for the Ca$^{2+}$-induced mitochondrial swelling. In this regard, abnormal Ca$^{2+}$ accumulation by neuronal mitochondria in response to excitotoxic levels of excitatory neurotransmitters, such as glutamate, is an important mediator of mitochondrial dysfunction and delayed cell death (Fiskum, 2000; Drago et al., 2011). Thus, JM-20-mediated protection against Ca$^{2+}$-induced mitochondrial impairment and its neuroprotective effects may include its ability to prevent mitochondrial Ca$^{2+}$ influx.

JM-20 does more than just prevent Ca$^{2+}$-mediated mitochondrial damage. It also inhibits the hydrolytic activity of F$_{1}$F$_{0}$-ATP synthase in both submitochondrial particles and intact mitochondria at low micromolar concentrations, suggesting that JM-20 crosses the mitochondrial membrane and targets the enzyme, thus preserving the existing ATP during ischemia and promoting neuronal cell survival. F$_{1}$F$_{0}$-ATP synthase is responsible for mitochondrial ATP synthesis in mammalian cells. However, it can also hydrolyze ATP during cellular anoxia, so the direction of the reaction has a profound effect on cellular metabolism (Jennings et al., 1990). During anoxia, the electrochemical gradient collapses and F$_{1}$F$_{0}$-ATP synthase switches to its hydrolytic state, causing undesirable ATP hydrolysis, leading to ATP depletion (Grover et al., 2008). Thus, preserving ATP through the inhibition of F$_{1}$F$_{0}$-ATP synthase hydrolytic activity may represent a protective strategy for neuronal cells exposed to ischemic damage (Christophe and Nicolas, 2006). Therefore, our results suggest that JM-20 may also prevent neuronal cell death during ischemia by maintaining the cellular energy balance, which in turn may drive neuronal recovery processes.

5. Conclusion

The results of the present study support a pleiotropic mechanism underlying the neuroprotective action of JM-20, which may involve both anti-excitotoxic and anti-Ca$^{2+}$ effects at the neuronal level as well as mitoprotective effects against Ca$^{2+}$-induced impairment in association with ATP preservation. The neuroprotective activity of JM-20 in vitro, as evidenced in this study, prompted us to extend our research to in vivo experimental models of brain damage, which are now underway.

Acknowledgments

This work was partially supported by CAPES-Brazil/ MES-Cuba projects 140/11 and 092/10 and by the Non-Governmental Organization MEDICUBA-SPAIN. We are grateful to Ana C. Morselli-Polizelli for technical assistance.

References


