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

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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 F_1F_0-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.

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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).

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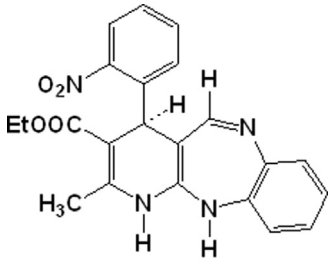

JM-20

Fig. 1. Chemical structure of JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5]benzodiazepine).

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 (Figueredo et al., 2014). Its dihydropyridine moiety does not appear to interfere with the GABAergic activity associated with its benzodiazepine portion but may confer Ca^{2+} channel blocking properties, indicating that JM-20 may act as a potential neuroprotectant. 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 Ca^{2+}-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* neural damage 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 (Figueredo et al., 2014).

2.2. Experimental animals

For the mitochondrial assays, adult male Wistar rats (CENPALAB, 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

Pheochromocytoma (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% CO_2 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 1.5×10^5 cells/ml in 96 multiwell flat bottom plates (Corning Costar, Sigma) in 200 µl of culture medium at 37 °C and were flushed with 5% CO_2 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 1800*g* 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 5×10^5 cells/ml in 200 µl of culture medium at 37 °C and were flushed with 5% CO_2 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),

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