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NOVEL MOLECULAR TARGETS OF THE NEUROPROTECTIVE/NEURORESCUE MULTIMODAL IRON CHELATING DRUG M30 IN THE MOUSE BRAIN

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Abstract—The novel multifunctional brain permeable iron, chelator M30 [5-(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline] was shown to possess neuroprotective activities *in vitro* and *in vivo*, against several insults applicable to various neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. In the present study, we demonstrate that systemic chronic administration of M30 resulted in up-regulation of hypoxia-inducible factor (HIF)-1α protein levels in various brain regions (e.g. cortex, striatum, and hippocampus) and spinal cord of adult mice. Real-time RT-PCR revealed that M30 differentially induced HIF-1α-dependent target genes, including vascular endothelial growth factor (VEGF), erythropoietin (EPO), enolase-1, transferrin receptor (TfR), heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), and glucose transporter (GLUT)-1. In addition, mRNA expression levels of the growth factors, brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) and three antioxidant enzymes (catalase, superoxide dismutase (SOD)-1, and glutathione peroxidase (GPx)) were up-regulated by M30 treatment in a brain-region-dependent manner. Signal transduction immunoblotting studies revealed that M30 induced a differential enhanced phosphorylation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), protein kinase B (PKB/Akt), and glycogen synthase kinase-3β (GSK-3β). Together, these results suggest that the multifunctional iron chelator M30 can up-regulate a number of neuroprotective-adaptive mechanisms and pro-survival signaling pathways in the brain that might function as important therapeutic targets for the drug in the context of neurodegenerative disease therapy.

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Abbreviations: AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; DFO, deferoxamine; dNTP, deoxynucleosidetriphosphate; EPO, erythropoietin; GDNF, glial cell-derived neurotrophic factor; GLUT-1, glucose transporter-1; GPx, glutathione peroxidase; GSK-3β, glycogen synthase kinase-3β; HIF-1α, hypoxia-inducible; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; M30, 5-(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline; PCR, polymerase chain reaction; PHD, prolyl hydroxylase; PKC, protein kinase C; RT, reverse transcription; SOD-1, superoxide dismutase-1; TBS, tris buffered saline; TfR, transferrin receptor; VEGF, vascular endothelial growth factor.

Key words: multifunctional drug, iron chelation, hypoxia-inducible factor, brain-derived neurotrophic factor, pro-survival signaling pathways, systemic chronic administration.

The concept of chelation therapy as a valuable therapeutic approach in neurological disorders led our group to develop multifunctional, nontoxic, lipophilic, brain-permeable compounds with iron chelation and anti-apoptotic properties for neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Zheng et al., 2005a). Based on a multimodal drug design paradigm, we incorporated the propargylamine moiety of the anti-Parkinsonian drug, rasagiline (Azilect®, Teva Pharmaceutical Netanya Industries, Ltd., Israel) into the antioxidant-iron chelator moiety of an 8-hydroxyquinoline derivative of the iron chelating compound, VK28 (Shachar et al., 2004; Zheng et al., 2005a). Regarding the N-propargyl functional group, it was shown in animal and cellular models of various neurodegenerative disorders with different insults that a series of propargyl derivatives (e.g. rasagiline and ladostigil) exert significant neuroprotective/neurorescue activities (Maruyama et al., 2002; Bar-Am et al., 2004b; Weinreb et al., 2004; Youdim and Buccafusco, 2005; Naoi and Maruyama, 2009). Studies on the structure-activity relationship reported that the neuroprotection was ascribed mainly to a direct stabilization of the mitochondrial membrane and induction of anti-apoptotic pro-survival genes (Naoi and Maruyama, 2009). In accordance, the novel multifunctional iron chelator, M30 was found to confer potential neuroprotective effects in preclinical neurodegenerative models with distinct etiologies, exerting selective iron chelation potency (compared with zinc and copper), radical scavenging, and inhibition of iron-induced membrane lipid peroxidation (Zheng et al., 2005a; Amit et al., 2008). M30 was shown to possess a significant neuroprotective, as well as neurorescue activities against the Parkinsonism-inducing neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice (Gal et al., 2005, 2010). In addition, both M30 and VK28 were found to significantly improve behavioral performances and attenuate dopaminergic neuronal loss, proteasomal inhibition, iron accumulation, and microglial activation in the substantia nigra of mice injured with the proteasome inhibitor, lactacystin (Zhu et al., 2007). Recently, we reported that M30 treatment provided clear benefits in G93A-SOD-1 ALS mice, significantly increasing their survival and delaying the onset of neurological dysfunction (Kupershmidt et al., 2009). *In vitro* studies in SH-SY5Y neuroblastoma, motoneuron-like NSC-34 and primary cortical cells demonstrated that M30

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possesses multiple pharmacological activities, including improvement of neuronal survival in various neurotoxic models, induction of neuronal differentiation and up-regulation of hypoxia-inducible factor (HIF)-1α expression and HIF-1-target genes (Avramovich-Tirosh et al., 2007a,b, 2010; Kupershmidt et al., 2009).

Indeed, due to the important role of biometals in neurodegenerative diseases, considerable focus has been previously placed on developing therapeutic approaches involving regulation of metal-protein interaction (Cherny et al., 2001; Lee et al., 2004). The iron chelator, deferoxamine (DFO) was reported to alter the rate of cognitive decline in AD patients, although it is unknown whether this was due to chelation of Fe or modulation of other metals. More recently, the metal ligand clioquinol was reported to inhibit Aβ accumulation in an AD transgenic mouse model through its actions as a metal chelator (Cherny et al., 2001). A second generation, 8-hydroxyquinoline derivative of clioquinol (PBT2) was also found to restore cognition in Alzheimer transgenic mice, in association with decreased interstitial Aβ (Adlard et al., 2008). The mechanism of action of clioquinol was suggested to be through metal sequestration, resulting in Aβ dissolution. However, clioquinol could also act by modulation of cellular biometal metabolism and expression/processing of the Alzheimer's amyloid precursor protein (APP) (Treiber et al., 2004). Alternatively, it was proposed that clioquinol induced neuroprotection by enhancing intracellular Cu2+ and Zn2+ uptake thereby acting as an ionophore that favors the clearance of Cu/Zn from the amyloid plaques and synaptic space (White et al., 2006). Clinical studies demonstrated that both clioquinol and PBT2 can be safely used in AD patients and can attenuate some of the AD-associated cognitive deficits (Cherny et al., 1999; Ritchie et al., 2003; Bush, 2008; Lannfelt et al., 2008; Zatta et al., 2009).

Neuroprotection by iron chelating agents has been widely attributed to their ability to prevent the iron from redox cycling and thereby, inhibit hydroxyl formation by the Fenton or Haber-Weiss reaction (Zecca et al., 2004). More recently, an additional level of neuroprotection by iron chelators has been postulated to involve inhibition of the activity of iron-dependent HIF-prolyl hydroxylase (PHD) enzymes, resulting in the stabilization/activation of HIF-1α and the consequent activation of a broad set of HIF-1-target genes that may contribute to cell survival, iron regulation, and energy metabolism in the nervous system (Jaakkola et al., 2001; Yu et al., 2001; Siddiq et al., 2005, 2007; Weinreb et al., 2010b). Indeed, it was demonstrated that DFO can activate HIF-1α and prevent neuronal death in both *in vitro* and *in vivo* models of ischemia, likely via inhibition of PHDs (Hurn et al., 1995; Zaman et al., 1999; Hamrick et al., 2005). PHD inhibitors have been also shown to prevent oxidative cell death and ischemic injury via HIF-1-pathway activation (Siddiq et al., 2005; Harten et al., 2010).

Considering the diverse pharmacological properties of the novel iron chelator M30, the aim of the present study was to identify distinct regulatory molecular mechanisms in the brain, that might be associated with the neuroprotective activity of the drug, including activation of HIF-1 signaling pathway and up-regulation of specific HIF-regulated target genes, expression of neurotrophic factors and antioxidant enzymes and induction of pro-survival cell signaling cascades.

EXPERIMENTAL PROCEDURES

Materials

Rabbit polyclonal antibodies against phospho-protein kinase C (PKC-pan) (1:500), p44/42 mitogen-activated protein (MAP)/ERK kinase (1:500), phospho-protein kinase B (AKT) (ser 473) (1:500), phospho-glycogen synthase kinase (GSK)-3β (ser 9) (1:500) were from Cell Signaling (Beverly, MA, USA). Monoclonal β-actin antibody (1:15,000) was from Sigma (St Louis, MO, USA). Polyclonal HIF-1α antibody (1:350) was from Upstate (New York, NY, USA). Electrophoresis reagents were obtained from Invitrogen (Carlsbad, CA, USA). Other chemicals and reagents were of the highest analytical grade and were purchased from local commercial sources. The iron chelator M30 [5-(N-methyl-N-propargylaminomethyl)-8-hydroxyquinoline] (MW 299.3) was synthesized (Zheng et al., 2005b) and kindly provided by Varinel Inc. (Philadelphia, PA, USA). M30 possesses solubility and selective iron-chelation properties (compared with zinc and copper). M30 is not cytotoxic, as shown by the genotoxicity assay, performed in several cell lines, inhibition of cytochrome p450 isozymes and voltage-dependent potassium channel-blocking test (Weinreb, et al., 2010b).

Animals

All procedures were carried out in accordance with the National Institutes of Health Guide for care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of the Technion, Haifa, Israel. Male C57BL mice were kept on a 12 h light/dark cycle. Food and water were available *ad libitum*. Before all experiments, mice were randomly assigned to vehicle (n=11) or M30 (n=12). M30 (10 mg/kg/d) and vehicle were administered for 30 days by oral gavage method, started at 30 days of age. The dosage of M30 was selected according to previous studies who described the *in vivo* neuropharmacological properties of M30 (10 mg/kg, P.O.) administered chronically to C57/BL mice (Gal et al., 2005). In addition, M30 (10 mg/kg, P.O.) was recently found to improve spatial learning and memory in the streptozotocin-induced model of AD in rats (Salkovic-Petrisic et al., 2011; unpublished observations). Mice were weighed once a week. The animals were sacrificed by cervical dislocation 1 day following the last administration. Blood samples were collected from mice by a cardiac puncture. Cortices, hippocampi, striata, spinal cords, livers, and hearts were dissected out and frozen at −80 °C for further analysis.

Liver iron content

Liver nonheme iron was measured by the bathophenanthroline method as described by Torrance and Bothwell (Torrance and Bothwell, 1980). Liver samples were dried at 65 °C for 12 h, crushed, and weighed. Subsequently, each sample was incubated with 3 M HCl and 10% trichloroacetic acid solution for 12 h at 65 °C. After digestion, the samples were centrifuged at 1000×g for 5 min, and then, 20 μl of the supernatant was removed from each sample and added to 80 μl of the chromogen bathopenanthroline (10.2 g sodium acetate, 0.134 g bathopenanthroline disulfonic salt in a total volume of 50 ml). The optical density of each sample was measured at 535 nm and serum iron concentration of each sample was calculated using an iron standard curve.

Serum iron assay

50 μl of each serum sample was mixed with 50 μl of an acid-precipitating/reducing solution (4.9 g trichloroacetic acid, 2.28 g