INTRODUCTION

There are significant evidence for dysregulation of brain iron metabolism in neurodegenerative disease of Parkinson’s disease (PD), Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS). Iron is thought to participate in oxidative stress initiated by the Fenton reaction [1] and monoamine oxidase generating hydrogen peroxide. Thus, the concept of iron chelation as a valuable therapeutic approach in neurological disorders led our group to develop multi target, nontoxic, lipophilic, brain permeable compounds with iron chelating-radical scavenging, monoamine oxidase inhibitory activity and anti-apoptotic properties for neurodegenerative diseases, such as PD, AD and ALS [2]. We incorporated the propargylamine moiety of rasagiline into the antioxidant-iron chelator moiety of an 8-hydroxyquinoline derivative of the iron chelating compound, VK28 [2, 3] to develop the multi target chelators M30 and HLA-20. N-propargyl functional group and its drug derivatives were shown in animal and cellular models of various neurodegenerative disorders with different insults that a series of propargyl derivatives exert significant neuroprotective and neurorescue activities [4-8]. The neuroprotection was ascribed mainly to a direct stabilization of the mitochondrial membrane potential and induction of anti-apoptotic pro-survival genes [8]. 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 [2, 9]. 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 [10, 11]. 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 [12]. Furthermore, M30 treatment provided clear benefits in G93A-SOD-1 ALS mice, significantly increasing their survival and delaying the onset of neurological dysfunction [13]. In vitro studies in SH-SY5Y neuroblastoma, motor neuron-like NSC-34 and primary cortical cells demonstrated that M30 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)-1 expression and HIF-1-target genes [13-17].

In vitro studies in SH-SY5Y neuroblastoma, motor neuron-like NSC-34 and primary cortical cells demonstrated that M30 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)-1 expression and HIF-1-target genes [13-17]. 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 [1]. More recently, an additional level of neuroprotection by iron chelators has been postulated to involve inhibition of the activity of iron-dependent HIF-prolylhydroxylase (PHD) enzymes, resulting in the stabilization/activation of HIF-1 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 [18-22]. Indeed, it was demonstrated that desferoxamine (DFO) can activate HIF-1 and prevent neuronal death in both in vitro and in vivo models of ischemia, likely via inhibition of PHDs [23-25]. PHD inhibitors prevent oxidative cell death and ischemic injury, via activation of HIF-1-pathway [21]. Considering the diverse pharmacological properties of the novel iron chelator M30, the aim of our 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.

MOLECULAR MECHANISM OF NEURORESTORATIVE ACTIVITY OF M30 AND MITOCHONDRIAL BIOGENESIS

Our previous studies have shown the novel multifunctional brain permeable iron, chelator M30 [5-(N-methyl-N-propar-gyaminomethyl)-8-hydroxyquinoline] and its piprezino derivative, HLA-20 possess neuroprotective, neurorescue and neurorestorative activities in vitro and in vivo, against several insults applicable to various neurodegenerative diseases, such as AD, PD, and ALS. We demonstrated that systemic chronic administration of M30 into mice resulted in up-regulation of hypoxia-inducible factor (HIF)-1 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-1-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 such as 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. Immunoblotting studies revealed that M30 induced a differentially enhanced phosphorylation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), protein kinase B (PKB/Akt), and glycogen synthase kinase-3 (GSK-3) (Fig. 1) [14].

Recently we have found 10 gene sets with previously unknown associations with the substantia nigra pars compacta of PD [26]. These gene sets pinpoint defects in mitochondrial electron transport, glucose utilization, and glucose sensing and reveal that they occur early in disease pathogenesis. Genes controlling cellular bioenergetics that are expressed in response to peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) are under expressed in patients with PD. Activation of PGC-1α results in increased expression of nuclear-encoded subunits of the mitochondrial respiratory chain and blocks the dopaminergic neuron loss induced by mutant α-synuclein or the pesticide rotenone in cellular disease models. Our systems biology analysis of PD has identified PGC-1α as a potential therapeutic target for early intervention since a defect in mitochondrial

Fig. 1. Molecular Mechanism of M30 as identified in cell culture and mouse brain.
complex I has been shown in PD. Indeed we have recently shown that M30 and HLA-20, which possess neurodifferentiating, neurorescue and neurorestorative properties in vitro and in vivo [13, 22] activate PGC-1α, SIRT1 (NAD-dependent protein deacetylase (neuroprotective), NFT (neurotrophic factor) and Tiam (mitochondrial transcription factor) in the hippocampal and cortical neurons in culture (Maoz et al, submitted for publication). These results indicate that the multi target M30 and HLA-20 initiate neuroprotection and neurorescue via biogenesis of mitochondria. Together, these results suggest that the multi target, 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 disease modifying therapeutic targets for the drug in the context of neurodegenerative disease therapy (Fig. 2) [13-15].

DISCUSSION

The neuroprotective-neurorestorative mechanisms activated following M30 administration, are not completely understood. We have provided further insight into the various endogenous molecular mechanisms and prosurvival signaling pathways, activated in the brain following M30 systemic administration that might mediate neuroprotection. These include functional activation of HIF-1 signaling; regulation of a wide range of HIF-1-related protective genes, induction of mRNA expression levels of neurotrophic growth factors and antioxidant enzymes and upregulation of pro-survival signaling cascades. We have shown that the novel multifunctional compounds are strong chelators for iron and copper with higher selectivity for iron, and chelate iron (III) in a 3:1 M ratio, respectively [2, 27]. The fact that the new chelators have binding capability both for iron and copper, but with higher selectivity for iron may be important factors for the antioxidative-type drugs, since it is the excessive iron stores and iron-mediated generation of free radicals in the brain that are thought to be associated with neurodegenerative diseases [1, 2]. Therefore, the novel chelators with these properties would be expected to chelate iron instead of copper and hence would have potential use as drug candidates in neurodegenerative diseases. The current results demonstrate that M30 treatment produced a significant up-regulation of HIF-1 protein expression in the brain (e.g., cortex, striatum, and hippocampus and spinal cord). In addition, real time RT-PCR revealed that M30 differentially induced the transcription of a broad range of downstream HIF-1-related protective genes within the brain, such as those involved in erythropoiesis (EPO), angiogenesis (VEGF), glycolysis (Glu-1), and oxidative stress (HO-1), indicating a biological HIF-1 activation in the brain in response to M30 administration in vivo. This mechanism of HIF-1 up-regulation is consistent with previous studies demonstrating that iron chelators may function as hypoxia mimetic regulators; stabilizing and transactivating HIF-1, thus leading to the regulation of HIF-1-responsive genes [21, 24, 28, 29]. This may support adaptive mechanisms, which protect the brain from a hypoxic injury through regulation of cerebral metabolism and blood flow, promotion of angiogenesis, and induction of cytoprotection [20, 22, 30, 31]. Iron chelation by DFO enhanced HIF-1 activity and prevented neuronal death in both in vitro and in vivo models of ischemia via HIF-PHDS inhibition [12, 24, 29, 32, 33]. The protective effect of DFO against neuronal death after oxygen- and glucose-deprivation could be reversed by blockade of HIF-1 with antisense oligonucleotide transfection [12]. Thus, the activation of brain HIF-1 signal transduction pathway and consequent expression of HIF-1-target genes, possessing pro-survival properties, may implicate a link between M30-induced HIF-1-driven gene expression and neuroprotective capacities. Consequently, our in vitro findings demonstrated the ability of M30 to up-regulate HIF-1 and several HIF-1-target genes (e.g. enolase-1, VEGF, EPO, and p21) in cultured cortical neurons and NSC-34 cells, accompanied by protective effects against Aβ25-35- and mutant G93A-SOD-1-induced toxicity, respectively [13, 14, 34]. In vivo studies demonstrated that M30 significantly extended the survival of G93A-SOD-1 ALS mice and delayed the onset of the disease [13]. In addition, recent studies in APPswe/PSEN1 mouse model of AD have shown that M30 enhanced HIF-1 expression and reduced amyloid Aβ accumulation/plaque formation (mauscript in preparation). Activation of
HIF-1 signaling pathway by M30 was also achieved in the peripheral organs (e.g., liver and heart). For example, of the HIF-1 target genes we examined in the liver, VEGF, enolase-1, 1THR, iNOS, and GLUT-1 were significantly increased. Accordingly, activation of HIF-1 was recently shown to play a role in the effect of iron depletion by DFO on glucose metabolism in hepatocytes in vitro and in vivo [35]. In HepG2 cells, DFO stabilized HIF-1 and increased the expression of GLUT1 and insulin receptor. In addition, it was shown that DFO consistently increased the phosphorylation status of Akt/PKB and its targets FoxO1 and GSK-3, which mediate the effect of insulin on glucogenesis and glycogen synthesis, and up-regulated genes involved in glucose uptake and utilization. In vivo, iron depletion increased hepatic HIF-1 expression, GLUT-1 mRNA levels and Akt/PKB activity [35]. The specific activation of HIF-1 signaling and upregulation of HIF-1-related genes in the liver may be also associated with hepatic cytoprotection, as it was shown in various models of injury that stimulation of HIF system can protect the liver against apoptosis [36]. Another interesting finding in this study is the differential up-regulation of BDNF and GDNF in the CNS following M30 treatment. These data complement previous observations showing the ability of M30 and another multifunctional iron chelator drug, HLA20, to induce mRNA levels of BDNF in NSC-34 cells and cortical neurons [13, 34]. These drugs were also shown to promote neuronal differentiation, including cell body elongation, stimulation of neurite outgrowth and triggering cell cycle arrest in G0/G1 phase [16]. It was demonstrated that motor neuron differentiation, induced by M30 was modulated by the signaling inhibitors, PD98059 and GF109203X, indicating the involvement of MAPK and PKC pathways [13]. Additionally, in the current study we showed that M30 induced mRNA expression levels of the major antioxidant defense system, comprised of the antioxidant enzymes, catalase, SOD-1, and GPx, in various brain regions. These effects on transcriptional up-regulation of neuronal growth factors and antioxidant enzymes are presumably associated with the propargyl moiety, embedded in M30 molecule. Indeed, previous studies reported that several propargyl derivatives up-regulated mRNA expression of BDNF and NGF and increased protein levels of BDNF [37], suggesting that the stimulation of these neuronal survival pathways may provide an important step in their neuroprotective activity. In line with this, it was previously shown that propargylamines possess an antioxidant action and suppress the formation of free radicals by increasing the activity of the antioxidant enzymes, SOD and catalase in rat brain dopaminergic regions [22, 37]. By inducing antioxidant enzymes and decreasing the formation of reactive oxygen species, propargylamine-containing drugs may combat an oxidative challenge, implicated as a common causative factor in neurodegenerative diseases. Finally, M30 treatment induced a significant increase in brain expression of phosphorylated PKC, ERK1/2, AKT, and GSK-3. Regarding the role of these signal pathways in the regulation of neuroprotection, it has been reported in a number of studies that MEK/ERK and PI3K/AKT/GSK-3 pathways can promote cell survival, especially neuronal survival by both enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic ones [38-40]. In addition, these signaling pathways are well documented to play a key role in the regulation of HIF-1 [22] and thus, might be involved in the increased expression of HIF-1 following M30 treatment. Also, it cannot be ruled out that these signaling cascades are activated in the brain of M30-treated mice as a secondary phenomenon by an HIF-1-dependent gene product. Thus, considering the mechanism of action of M30, it can be assumed that the neuroprotective effects of the drug may be also associated with the activation of these pro-survival signaling cascades. Indeed, N-propargylamine and rasagiline confer neuroprotection and neurorescue effect via activation of PKC and MAPK pathways, coupled to pro-survival Bcl-2 family members and mitochondrial members stabilization [6, 37]. Although misregulation of the HIF pathway is only one component of a spectrum of reactions occurring in neurodegeneration, HIF-1 is a ‘master switch’ being an important physiological response mechanism, likely resulting in several reproducible neuroprotective effects [41, 42]. Given the wide range and diversity of cellular functions regulated by the whole spectrum of HIF-1-target genes, it is suggested that this compensatory pathway mediated neuroprotection and is crucially involved in many physiological processes within the brain. Thus, in conclusion the novel therapeutic approach of pluripotential multitarget iron chelating compounds, such as M30 and HLA-20 that targets a number of pharmacological sites involved in neurodegenerative processes and activates HIF pathway, mitochondrial biogenesis and downstream neuroprotective and neurotrophic genes, will broaden the current strategies for the treatment of neurological disorders such as PD and AD, and overall will open a new window for future development of drugs possessing a profound impact on neuron preservation, restoration and function.

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