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The Nrf2-ARE pathway: a valuable therapeutic target for the treatment of neurodegenerative diseases

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Abstract

Modulation of NF-E2 related factor 2 (Nrf2) has been shown in several neurodegenerative disorders. The overexpression of Nrf2 has become a potential therapeutic avenue for various neurodegenerative disorders such as Parkinson, Amyotrophic lateral sclerosis, and Alzheimer's disease. The expression of phase II detoxification enzymes is governed by the cis-acting regulatory element known as antioxidant response element (ARE). The transcription factor Nrf2 binds to ARE thereby transcribing multitude of antioxidant genes. Keap1, a culin 3-based E3 ligase that targets Nrf2 for degradation, sequesters Nrf2 in cytoplasm. Disruption of Keap1-Nrf2 interaction or genetic overexpression of Nrf2 can increase the endogenous antioxidant capacity of the brain thereby rendering protection against oxidative stress in neurodegenerative disorders. This review primarily focuses on targeted Nrf2 overexpression as a promising therapeutic strategy for the treatment of neurodegenerative disorders.

Introduction

Oxidative stress has been implicated in numerous disorders, including neurodegenerative disorders. The free radical-mediated oxidative stress is a widely accepted mechanism towards the formation of reactive oxygen species (ROS) (e.g., superoxide radical $O_2^{-\cdot}$ or hydrogen peroxide H_2O_2) and reactive nitrogen species (RNS) (e.g. nitric oxide radical; $NO^{\cdot}$, dinitrogen tetroxide; N_2O_4 or peroxynitrite; $ONOO^-$) that can damage biomolecules such as proteins and lipids [1]. The source of free radicals could be exogenous factors such as ionizing radiation, photochemical reactions, environmental toxins among others or endogenous biochemical and enzymatic processes. These reactive species are found intracellularly and extracellularly.

Various antioxidant systems are operational in the human body that inhibits the activity of ROS/RNS. Some examples of antioxidant systems are antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase), antioxidant proteins (e.g., thioredoxin, peroxyredoxin) and antioxidant molecules (e.g., lipoic acid, GSH, vitamin E). During disease conditions, the level of ROS/RNS may increase, antioxidant capacity may decrease or both may occur at the same time [1]. This condition is often described as oxidative stress.

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The brain is particularly vulnerable to free radical damage and oxidative stress because of high amounts of polyunsaturated fatty acids (PUFA) that are easily oxidizable by ROS, the presence of significant amount of trace metal ions such as iron (II) and copper (I) that can catalyze various redox reactions to produce ROS, the high consumption of oxygen compared to other organs, and the low antioxidant levels. These factors lead to increased lipid peroxidation, as well as protein, DNA and RNA oxidation causing neuronal dysfunction and/or death [2, 3]. Such changes have been mechanistically implicated in the pathogenesis and progression of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington disease (HD) [4]. Hence, studies focusing on molecules that modulate the cells endogenous antioxidant capacity are of vital significance and can form new therapies targeting the treatment of the neurodegenerative disorders.

Nrf2-ARE Pathway

One such way the cell regulates its endogenous antioxidant capacity is through activation of the transcription factor NF-E2-related factor 2 (Nrf2). The antioxidant response element (ARE) is a cis-acting regulatory element that governs the expression of phase II detoxification enzymes. Nrf2, a member of Cap 'n' collar/basic-leucine zipper family transcription factor, regulates the ARE containing genes. Nrf2 has six erythroid-derived CNC homology protein (ECH) domains. The cytoplasmic protein Keap1 is associated with Nrf2 via a Neh2 domain [5]. Keap1 functions as adaptor for the culin 3-based E3 ligase. Under normal unstressed condition, Nrf2 is ubiquitinated and rapidly degraded (half life ~ 20 min) by ubiquitin-proteasome system [6-8]. Under conditions of oxidative stress by either reactive electrophiles, toxins or ARE inducers, the interaction between Nrf2 and Keap1 is disrupted and Nrf2 translocates to the nucleus. In the nucleus, it binds to small Maf proteins that increase the transcription rate of ARE-driven genes (Fig 1).

In addition, Keap1 may also be involved in accompanying Nrf2 out of the nucleus by shuffling itself from the cytoplasm to the nucleus [9-11]. The actual mechanism of disruption of the Keap1-Nrf2 interaction remains elusive but it is reported that ARE inducers may directly modify cysteine thiols groups in Keap1 [12] that leads to the release of Nrf2, thereby increasing Nrf2 activity [13-16]. Furthermore, activation of several kinases may participate in this process through phosphorylation of Nrf2 at serine and threonine residues that could mediate/modify translocation of Nrf2 to nucleus [17]. Finally, the major Nrf2 activation pathway is believed to be that discussed above, however, other data suggests that there may be exceptions. Members of the fibroblast growth factor family have been shown to transcriptionally regulate Nrf2 [18, 19], thereby increasing both Nrf2 mRNA and protein levels that could contribute to activation of Nrf2 and induction of ARE-driven genes.

Nrf2-ARE driven genes

A multitude of genes involved in redox status, anti-inflammation and detoxification are transcribed by Nrf2-ARE pathway activation. These genes are known to be involved in cytoprotection from various oxidative insult and cellular injuries in numerous different tissues and organs including brain. Antioxidant enzyme systems regulated by Nrf2 include, but not limited to, redox regulation [superoxide dismutase (SOD), catalase (CAT), sulfaredoxin (Srx), thioredoxin (Trx), peroxiredoxin (Prdx) system], glutathione synthesis and metabolism [glutathione peroxidase (Gpx), glutathione reductase (GR), γ-glutamine cysteine ligase (GCL) and synthase (GCS)], quinone recycling [NAD(P)H quinone oxidoreducase (Nqo1)] and iron homeostasis [heme oxygenase 1 (HO-1), Ferritin]. Some antioxidant genes have more active roles than others in brain depending on the disease condition, cellular environment or cell type.

Nqo1 is an antioxidant enzyme involved in two-electron reduction of endogenous quinones utilizing NADH or NADPH as a reducing co-factor [20]. Additionally, Nqo1 is also involved in α-tocopherol (vitamin E) metabolism and regeneration [21]. Small molecules such as phenolic and polycyclic aromatic hydrocarbons are known to induce Noq1 activity [22-24]. Various small molecules such as tertiary butyl hydroquinone (tBHQ) and sulforaphane, antioxidants, and H_2O_2 are known to activate Nqo1 mediated via the ARE [25-27]. Nqo1 is highly expressed in the central nervous system and lung epithelium and tissues that require high antioxidant protection [28]. In brain, astrocytes are known to show high level of Nqo1 compared to other cell type under normal condition. Gliosis is a common pathological hallmark in neurodegenerative disorder such as AD and PD. These reactive astrocytes show increased levels of Nqo1 that potentially indicate the presence of oxidative stress [29, 30].

Intracellular peroxidases are cleared by a group of enzymes that are transcribed by Nrf2-ARE pathway. The peroxisomal CAT catalyzes the conversion of H_2O_2 to water and molecular oxygen. However, the specific activity of CAT is much lower in brain than peripheral tissue [31]. Gpx is another enzyme that metabolizes H_2O_2 and depends on reduced glutathione (GSH). The oxidized GSH (GSSG) is recycled to GSH by GR. GSH, a tripeptide γ-glutamyl-cysteinyl-glycine, is the most abundant low molecuar weight thiol expressed ubiquitously. It is widely recognized as an endogenous non-enzymatic antioxidant and an oxyradical scavenger, and is thereby critical to maintaining a reducing environment in the cell and protect against oxidative damage by ROS [32-36]. GSH has been implicated in a wide range of metabolic processes, including cell division, DNA repair, regulation of enzyme activity, activation of transcription factors, modulation of anion and cation homeostasis, and protection against oxidative damage [37].

The consecutive action of two cytosolic enzymes, GCL and GCS, catalyze the synthesis of GSH. The first step involves ligation of γ-glutamate to cysteine to form γ-glutamylcysteine; a reaction catalyzed by GCL. This step is the rate-limiting step in GSH production and GCL is feedback inhibited by GSH itself. GSH is present in 1-3mM concentration throughout the brain [38], acting as a high capacity detoxification agent. GSH maintains the cellular redox balance depending upon the pH of the cellular compartment and is involved in various biosynthetic processes as well [35]. The level of GSH is reduced in specific regions of the central nervous system in various neurodegenerative disorders with concomitant increase in GSSG levels allowing for increased oxidative stress-mediated neuronal cell dysfunction and/or loss in these disorders [38, 39]. The basal and inducible levels of GCL, GCS and GR are regulated by Nrf2-ARE pathway [40-43]. GSH, in conjugation with GR, GPx, glutathione-S-transferase (GST) and NADPH provide protection against various toxic electrophiles and hydrogen peroxide.

GSTs are key detoxification enzymes that catalyze the conjugation of various electrophiles, reactive alkenals, and numerous other xenobiotic to GSH. These GSH-S-conjugates are removed from cells by the multidrug resistant protein-1 (MRP-1), an ATP binding cassette (ABC) family protein [44, 45]. MRP-1 is an integral plasma membrane protein that exports glutathione-S conjugates out of the cell in an ATP-dependent manner [46, 47]. Studies have shown reduced GST activity in brain and ventricular fluids in AD [48]. Increased expression of GST leads to increased resistance towards oxidative stress in neuroblastoma cells and provides protection against HNE-mediated toxicity in neuronal cell culture [48]. Several members of the GST family and MRP1 expression levels are regulated by Nrf2 [49, 50].

The chaperone protein HO-1, in concert with cytochrome p450, catalyzes degradation of heme to biliverdin that is subsequently converted to bilirubin. Both biliverdin and bilirubin have shown antioxidant and immunomodulatory properties [51, 52]. Increased expression of

HO-1 has been reported in various neurodegenerative disorders such as AD, PD, ALS and multiple sclerosis [19, 53-55], which may be linked to an attempt by HO-1 to restore redox state or attenuate inflammation in these conditions [reviewed in, [56]]. Several line of evidence show that Nrf2-mediated regulation of HO-1 protect cells from toxic and oxidative injuries [57, 58].

Nrf2-mediated neuroprotection in neurodegenerative disorders

Aging is common risk factor in neurodegenerative disorders. Increased protein and lipid oxidation and decreased antioxidant defense has been implicated in neurodegenerative conditions. In consequence, a considerable importance has been given to Nrf2-ARE pathway as a potential therapeutic target towards prevention of these disorders [59-61].

In CNS, the Nrf2-ARE dependent gene expression is preferentially less activated in neurons compared to astrocytes [57, 62, 63]. Additionally, astrocytes have higher GSH content than neurons [64, 65]. Hence, neurons often depend on astrocytes for protection against oxidative stress. Several lines of evidence show that neurons are more resistant to the oxidative stress in presence of astrocytes [66-68]. Most of the studies are targeted at either astrocytic Nrf2 overexpression-mediated protection of neurons or modulating endogenous neuronal antioxidant capacity by small molecule Nrf2 activators or cell specific overexpression. In the following sections, we will review disease specific studies involving Nrf2 activation/ overexpression as a therapeutic strategy to modulate the progression of major neurodegenerative disorders.

Alzheimer's disease

AD is an age-associated progressive neurodegenerative disorder characterized by memory loss, cognitive dysfunction and is the most common form of dementia in the elderly population effecting more than 5 million Americans. Pathological hallmarks of AD includes brain atrophy due to neuronal and synapse loss, senile plaques predominantly consisting of fibrillar amyloid β-peptide and neurofibrillary tangles (NFT) made up of hyperphosphorylated tau, a cytoskeletal protein [69]. Some of the major risk factors for AD are unhealthy aging in sporadic AD cases, the presence of ApoE-4 alleles in both sporadic and familial AD [70] and genetic factors, such as mutation in amyloid precursor protein (APP) and presenilin-1 (PS1) in familial AD [71] among others. AD brain is characterized by mitochondrial dysfunction, reactive gliosis and oxidative damage to lipids and proteins [72-76].

Growing evidence demonstrates that the AD brain is under tremendous oxidative stress. A significantly increased HO-1 expression was reported in post-mortem AD temporal cortex and hippocampus compared to aged-matched control [73]. Additionally, an increased Nqo1 activity and expression was found in astrocytes and neurons of AD brain [30, 77] and Nrf2 was predominantly localized in cytoplasm in AD hippocampal neurons [78]. Furthermore, there is increased protein oxidation [reviewed in [79, 80]] and lipid peroxidation [81-83] in AD brain when compared to aged matched controls. Recent studies in aged APP/PS1 AD mouse models showed reduced Nrf2, Nqo1, GCL catalytic subunit (GCLC) and GCL modifier subunit (GCLM) mRNA and Nrf2 protein levels [84]. Additionally, in a triple transgenic AD mouse, the GSH/GSSG ratio was reported to be reduced [85].

Antioxidant therapy in human AD clinical trials has meet with limited to no success. A α-tocopherol (vitamin E) treatment in AD patients delayed progression compared to placebo treated individuals [86], suggesting an increase in antioxidant capacity can alter AD pathogenesis. However, the majority of antioxidant trials have not shown similar positive outcomes [86, 87]. These failures could be due to many factors including bioavailability,

distribution, metabolism or blood-brain-barrier penetration. Alternatively, the clinical trial design is not necessarily optimized to actually determine effectiveness of such treatments. The trial length and stage of disease may be to short and to late, respectively, for antioxidant treatments to have a positive outcome leading to multiple failed trials. Manipulation of the cells own endogenous antioxidant pathways via Nrf2 activation/overexpression does show significant protection against Aβ-mediated toxicity, *in vitro* [84, 88]. In addition, stereotactic injection of lentiviral-Nrf2 into the hippocampus of the APP/PS1 AD mouse model improved spatial learning, but did not alter Aβ levels compared to wild type littermate controls [89]. Synthetic triterpenoid compounds have been shown to activate Nrf2-ARE pathway in various rodent models [90-94]. Recently in transgenic mice carrying two human APP mutations (Tg19959 mouse), synthetic triterpenoids attenuated inflammation and oxidative stress. Additionally, these mice also showed improved spatial memory retention and reduced Aβ plaque load [95]. These results and several other *in vitro* studies suggest that the Nrf2-ARE pathway is a viable drug target for the treatment of AD.

Parkinson Disease

PD is a progressive neurodegenerative disorder characterized by motor symptoms such as tremor, bradykinesia, posture instability and rigidity and non-motor neuropsychiatric problems such as mood, cognition, behavior and sometimes dementia [reviewed in [96]]. The main pathological hallmark of PD is loss or dopaminergic neurons in substantia nigra pars compacta that project to striatum and lewy body inclusions primarily composed of abnormal accumulation of α-synuclein bound to ubiquitin [97] as well as increased gliosis characterized by both astrogliosis and microgliosis. Familial PD, although accounting for only 15% of total cases [98] involves mutations in specific genes that code for α-synuclein (SNCA), parkin (PRKN), leucinerich repeat kinase 2 (LRRK2 or dardarin), PTEN-induced putative kinase 1 (PINK1) and DJ1 [99]. Oxidative stress has been implicated in PD and this is consistent with the predominantly nuclear distribution of Nrf2 [78] along with mitochondrial dysfunction in the dopaminergic neuron [100-103]. In the postmortem PD brain, an increased HO-1 and Nqo1 expression was observed in reactive glial cells [29, 53].

Our laboratory and others have showed differential sensitivity of Nrf2 deficient mice towards 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical model of PD [104, 105]. It has also been reported that there is a greater loss of dopamine transporter levels in striatum of Nrf2 knockout mice post MPTP administration compared to wildtype mice [106]. Moreover, Nrf2 activation protects dopaminergic neurons from 6-hydroxydopamine (dopamine analog) toxicity and MPP+ toxicity, *in vitro* [107, 108] and *in vivo* [107]. Additional *in vivo* evidence for Nrf2-mediated neuroprotection was demonstrated by transplantation of astrocytes overexpressing Nrf2 in striatum [107]. Mice receiving the Nrf2 overexpressing astrocytes were increasingly resistant to 6-hydroxydopamine-induced toxicity. Finally, in a separate study, our laboratory showed that transgenic mice with astrocyte-specific Nrf2 overexpression (GFAP-Nrf2) completely reversed dopaminergic neuronal loss in substantia niagra and loss of terminal in striatum [104]. These study strongly support the concept that modulation of the Nrf2-ARE pathway in astrocyte is sufficient to confer protection to neurons in mouse models of PD.

Huntington Disease

HD is a progressive autosomal dominant neurodegenerative disorder that is caused by a CAG repeats expansion in the HD gene resulting in an expansion of polyglutamines at the N-terminus of the huntingtin protein and accumulation of the mutant protein as cytoplasmic and nuclear aggregate inclusions [109]. HD is pathologically characterized by degeneration in neostriatal (caudate and putamen) and cerebral cortex that is believed to be the underlying

contributor of motor impairment, cognitive decline and psychiatric symptoms that worsen as the disease progresses. Oxidative stress due to mitochondrial dysfunction has been implicated in human patients and HD animal models [110]. Defects in mitochondrial complex II, III and IV were observed in striatum of post mortem HD brain [111, 112]. 3-Nitropropionic acid (3NP) and malonate are mitochondrial complex II inhibitors that produce striatal medium spiny neuron degeneration, a characteristic feature observed in HD [113-116]. An increase in ROS production due to disruption of electron transport chain by these inhibitors is also observed. Our laboratory and others have demonstrated that mice lacking Nrf2 are more susceptible to mitochondrial complex II inhibitors compared to wild type mice and grafting of astrocytes overexpressing Nrf2 or chemical activation of Nrf2 protected from 3NP- and malonate-induced lesioning in striatum respectively [58, 117]. In a separate study, mice overexpressing Nrf2 in astrocytes (GFAP-Nrf2) were resistant to malonate-induced lesioning *in vivo*. In the same study, the mouse neuroprogenetor cells (NPCs) infected with either adeno-Nrf2 or adeno-GFP virus were grafted in straital region and were subjected to malonate-induced lesioning. The Nrf2 overexpressing NPCs showed significant protection against malonate toxicity [117]. Additionally, cystamine, an Nrf2-ARE activator, conferred protection in both *in vitro* and *in vivo* model of 3NP toxicity [118]. More recently, synthetic triterpenoids were shown to activate Nrf2 and rescued rodents against 3NP-mediated striatal lesioning. Synthetic triterpinoids also rendered protection against 3NP-mediated increased DNA and protein oxidation, lipid peroxidation, and disrupted glutathione homeostasis [93]. A transgenic mouse model of HD (N171-82Q mice) also showed improved motor function and improved longevity when synthetic triterpinoids were fed in diet. A reduction in oxidative stress marker and straital atrophy was also observed [119]. Most recently, dimethyl fumarate (DMF), an Nrf2-ARE activator, was given orally to the R6/2 and YAC 128 mouse model of HD. Mouse receiving DMF showed increased neuronal Nrf2 and a significant improvement in motor function and preservation of neurons in motor cortex and striatum [120]. These studies strongly suggest that small molecule intervention or ex-vivo manipulation of Nrf2-ARE pathway is a promising therapeutic strategy got treatment of HD.

Amyotrophic Lateral Sclerosis

ALS is caused by the progressive degeneration of motor neurons in the spinal cord, brainstem, and motor cortex [121] characterized by progressive weakness in muscle, spasticity and muscle atrophy. Pathological contributors to ALS are oxidative damage, neuroinflammation, and mitochondrial dysfunction. Although the etiology of sporadic ALS remains unclear, approximately 5-10% of ALS is familial and about 20% of the familial ALS cases are associated a toxic gain-of-function mutation in Cu/Zn-superoxide dismutase (SOD1) [122]. The overexpression of mutant hSOD1 in rodent models has been shown to cause an ALS-like phenotype [123, 124]. A strong gliosis surrounding degenerating motor neurons and increase oxidative stress markers were observed in ALS patients and rodent models of ALS [125].

Oxidative stress has been implicated in ALS and most likely affects the course of the disease [126, 127]. Modulation in Nrf2-ARE pathway in response or as a consequence of oxidative stress has been well documented. The neurons from primary motor cortex and spinal cord from postmortem ALS tissue and primary embryonic motor neurons from ALS rat model showed reduced Nrf2 mRNA and protein expression [128, 129]. Our laboratory showed increase in ARE-driven gene in spinal cord and muscle of ALS mice crossed with ARE-hPAP (human placental alkaline phosphatase) reporter mice prior to disease onset [130]. Others also show a strong increase in HO-1 was found at onset of disease symptoms in an ALS rat model [19].

Our laboratory and others have demonstrated a toxic effects of astrocytes isolated from the hSOD1^{G93A} rat [131] or mouse on co-cultured motor neurons [132, 133]. Studies have shown that activation or overexpression of Nrf2 in the hSOD1 mutant astrocytes completely reversed their toxicity toward motor neurons the co-culture system. These observations translated to the in vivo situation since crossing the GFAP-Nrf2 mice with multiple mouse models of ALS lead to a delay onset of disease and increased life span in the ALS mice [134]. These observations were first *in vivo* evidence that Nrf2 activation in astrocytes can be beneficial to protect neurons in chronic neurodegenerative models suggesting that Nrf2 activation should be a suitable therapeutic target in ALS. Recently, triterpenoids that are potent activators of Nrf2-ARE pathway showed significant attenuation in weight loss, enhanced motor function and extended life span in hSOD1^{G93A} mice when treated at pre-symptomatic age. Furthermore, when treatment of these mice was initiated at symptom onset, there was also significant neuroprotection and slowed disease progression. These data provide further evidence that compounds activating the Nrf2-ARE pathway can be potential therapeutic agents in treating ALS [135].

Recent Patents on Nrf2 Modulation

The manipulation of the Nrf2-ARE pathway at the genetic level is being studied through the use of siRNA or antisense oligonucleotides against Keap1 to activate/overexpress Nrf2. Antisense drugs are being researched to study neurodegenerative disorders, cancer, metabolic disorders and disorders with inflammatory components among others. Antisense drug fomiversen, marketed as Vitravene, has been approved by the US food and drug administration (FDA) for treatment of cytomegalovirus retinitis. Since then numerous antisense therapies have been tested but have not produced significant clinical result. This hasn't diminished the potential of gene therapies. Antisense oligonucleotide can bind to the target RNA and disrupt RNA splicing, transcription, translation and replication thereby modulating gene expression. Our laboratory recently showed that siRNA-mediated knockdown of Keap1 activated Nrf2-ARE pathway in mouse cortical astrocytes and provide partial protection against MPTP-mediated toxicity in mouse, *in vivo* [136]. The overexpression of target gene can also be achieved by viral-mediated gene transduction but it is too early to conclude on efficacy of viral-mediated gene therapy in human neurodegenerative disorder cases. Nrf2 modulation in various neurodegenerative disorders has been previously described in this review. Hence using the antisense oligonucleotide against Keap1, lenti-viral-mediated Nrf2 overexpression or siRNA against keap1-mediated overexpression of Nrf2 treatment can prove beneficial in neurodegenerative disorders. Among recent patents, Curna, Inc. filed patent for use of antisense for treatment of Nrf2-related disorders. The initial study published under international application for the patent cooperation treaty (PCT) showed that antisense CUR-0330 and CUR 0332 showed 2 to 3 fold increase in Nrf2 mRNA expression compared to control (PCT/US2010/027394). The invention is targeted at inhibition of natural antisense transcript to Nrf2 as a strategy towards modulation of Nrf2 expression in disease models.

The modulation of Nrf2 expression by using several other pharmacological interventions to inhibit Keap1 and Nrf2 interaction are under investigation. Table 1 lists some of the other patents on small molecule that interact at the specific region of Keap1 that binds to Nrf2. Disruption of these Keap1-Nrf2 interacting region activates Nrf2. A detailed description of chemicals and small molecules that are targeted towards disruption of Keap1-Nrf2 interaction towards increasing the biological activity of Nrf2 as a strategy towards protection against neurodegenerative disorder is discussed in following section.

Targeting the Keap1-Nrf2 Interaction

Keap1 has four discrete domains. The BTB (Broad complex, Tramtrack and Bric-à-Brac) domain important for stress sensing and homodimerization of Keap1 protein [137], the intervening region (IVR) domain, a double glycine repeat (DGR) and C-terminal Kelch domain contains six conserved Kelch repeat sequences that binds to the Neh2 domain of Nrf2 [138, 139]. Keap1 not only sequesters Nrf2 in cytoplasm but also plays a role in its subsequent ubiquitination and proteasomal degradation [6, 7, 140, 141]. Keap1 represses the Nrf2 activation, which is a master regulator of antioxidant genes, hence making it a potential drug target towards treatment of oxidative stress related disorders, including neurodegenerative disorders. Increasing Nrf2 expression or biological activity by small molecules such quinone based compounds, flavonoids, polyphenols, α, β-unsaturated esters, reactive electrophiles among others can be achieved in various cell types, however, they are limited by bioavailability and blood-brain-barrier permeability. Chemical modification of certain base molecules that can interrupt Keap1-Nrf2 interaction can achieve the desirable increased expression or biological activity of Nrf2 in brain.

Among the 27 cysteine residues on Keap1, C273 and C288 in the linker region and C151 in the BTB region were identified as key sites that chemicals bind to leading to Nrf2 release and Nrf2 activation [13, 14, 16, 142, 143]. The reactive electrophiles potentially alkylate the cysteine residues thereby releasing and stabilizing Nrf2 [142, 144]. Electrophiles that can S-alkylate are classified as α, β-unconjugated enones such as curcumin and neurite-outgrowth promoting prostaglandin (NEPP) or polyphenols such as catechol-like carnosic acid, and resveratrol among others. Polyphenols, although not electrophilic, can cross the hydrophobic lipid membrane and get oxidized intracellularly to form quinone-like electrophiles. Hence, these catechol-like molecules can function as pro-drugs. Mechanisms of protection by these electrophiles differ and have been described previously [62, 145-147]. Carnosic acid, a catechol-like electrophile has been shown to protect neurons against glutamate-mediated toxicity *in vitro* and brain against ischemia reperfusion injury *in vivo* via s-alkylation of Keap1 and activation of Nrf2-ARE pathway [148]. Among other non-flavanoid polyphenols, Curcumin and resveratrol have shown to activate Nrf2 leading to subsequent protection of neural cells from oxidative insult and toxins in *in vitro* and *in vivo* models of AD and PD. Flavonoid polyphenols such as epigallocatechin 3-gallate (EGCG) and quercetine, and organo-sulphur based compounds such as sulforaphane are potent activator of Nrf2 that have been shown to be neuroprotective against oxidative stress *in vitro*. The chemical analogues of these base molecules (Fig 2) have been patented (PCT/US2007/021748, Lipton and Satoh 2007) and are under investigation.

Another class of Nrf2 activators is triterpenoid-based compounds (Fig 2). Synthetic triterpenoids are derived from 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO). CDDO and it's derivatives have been tested for their antioxidant and anti-inflammatory properties and are known to activate the Nrf2-ARE pathway in *in vitro* and *in vivo* in animal models of various disorders [90-94]. As mentioned earlier, in the neurodegenerative disorder paradigm, CDDO-methyl amine (CDDO-MA), an analogue of CDDO, showed improved memory and reduced Aβ plaques and oxidized proteins in AD transgenic mouse model [95], as well as showed neuroprotective properties against acute and chronic toxicity models of PD and HD and in transgenic mouse model of HD [93, 119], identifying these compounds as potential therapeutics for neurodegenerative disorders. The synthetic CDDO based analogues have been patented (PCT/US2007/071933) for testing against inflammatory and oxidative stress-mediated neurological disorders.

The ester of fumaric acid is a class of electrophiles that is known to be neuroprotective in mouse of models of multiple sclerosis (MS) and HD by activating the Nrf2-ARE pathway

[120, 149-152]. In the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, reduced oxidative stress and subsequent preservation of nerve fiber myelenation was observed post DMF administration and the protection was lost in Nrf2 knockout mouse [150]. The Nrf2-dependent genes were upregulated and motor function was improved in EAE mice upon DMF treatment. As discussed earlier, DMF also preserved neurons from motor cortex and striatum, reduced behavioral deficits, and improved life span in transgenic HD mice [120]. DMF increased Nqo1 level and was shown to protect rat cortical neurons against peroxide-mediated toxicity *in vitro*. Furthermore, oral administration of DMF or its metabolite mono-methyl fumarate (MMF) to mice was shown to increase Nrf2 levels [152]. These studies confirmed that Nrf2 activation by DMF and its metabolites could protect the CNS from oxidative insult in mouse models of neurodegeneration.

Conclusion

In past decade, Nrf2-ARE pathway activation has shown promising results for the treatment of many disorders including neurodegenerative disease. Several of these Nrf2 activators or their brain accessible synthetically modified compounds have passed phase II and III clinical trials. BG-12, an oral formulation of DMF (Biogen Idec, Inc.) is in phase III clinical trials for the treatment of MS. Bardoxolone methyl, an oral formulation of CDDO-MA (Reata Pharmaceuticals, Inc.) is currently in phase III clinical trials for chronic kidney disease in type II diabetes mellitus patients, but there are no existing clinical trails in pipeline for neurodegenerative disorders. EGCG, resveratrol and curcumin are in various phases of clinical trial for treatment and efficacy in neurodegenerative disorders such as AD, PD and ALS. The knowledge gained from these studies will further help in identifying clinically relevant approaches for activation of Nrf2 in CNS and potentially lead to finding treatments for these devastating neurological disorders.

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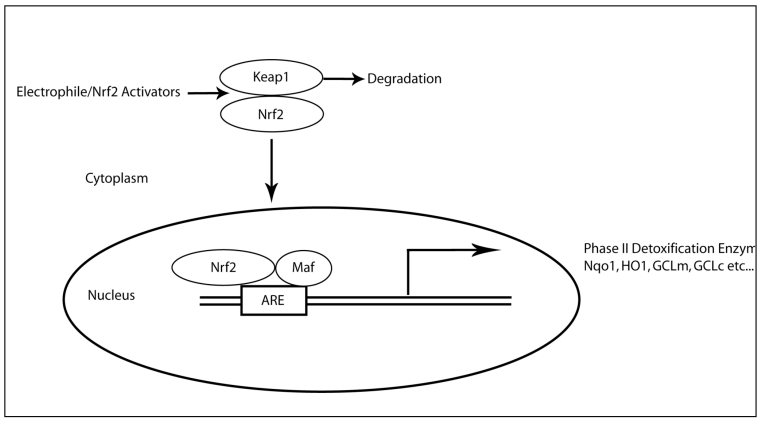

Figure 1.

Schematic representation of Keap1-Nrf2 interaction and activation. Under basal conditions Keap1 continuously degrades Nrf2 following ubiquitination. Electrophiles and oxidants directly modify reactive cysteine residues on Keap1 thereby disrupting Keap1-Nrf2 interaction. Nrf2 translocates to nucleus and binds to small Maf protein and transcribes ARE-driven genes.

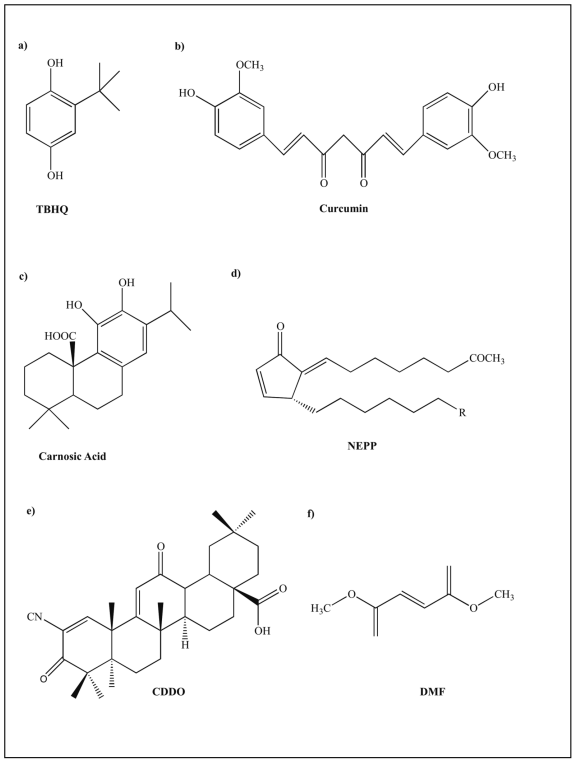

Figure 2.
Example of some of the base compounds that activate Nrf2-ARE Pathway.

Table 1

Recent patents in Nrf2-ARE pathway activators for treatment of central nervous system disorders.

S.No	Patent	Application Number	Title	Applicant	PubDate
1	WO/2011/156889	PCT/CA2011/000649	NOVEL MODULATORS OF NRF2 AND USES THEREOF	TRT Pharma Inc.	12/22/11
2	WO/2010/107733	PCT/US2010/027394	TREATMENT OF NUCLEAR FACTOR (ERYTHROID-DERIVED 2)-LIKE 2 (NRF2) RELATED DISEASES BY INHIBITION OF NATURAL ANTISENSE TRANSCRIPT TO NRF2	OPKO CURNA, LLC.	9/23/10
3	WO/2010/036711	PCT/US2009/058050	METHODS OF MODULATING PROTEIN HOMEOSTASIS, METABOLIC SYNDROME, HEAVY METAL INTOXICATION AND NRF2 TRANSCRIPTION FACTORS	Bach Pharma Inc.	4/1/10
4	WO/2009/036204	PCT/US2008/076064	PHASE II DETOXIFICATION AND ANTIOXIDANT ACTIVITY	Joslin Diabetes center Inc.	3/19/09
5	WO/2008/136838	PCT/US2007/071933	NOVEL AMIDE DERIVATIVES OF CDDO AND METHODS OF USE THEREOF	Trustees of Dartmouth College	11/13/08
6	WO/2008/108825	PCT/US2007/021748	NEUROPROTECTIVE COMPOSITIONS AND METHODS	Burnham Institute for medical research	9/12/08
7	WO/2007/008652	PCT/US2006/026503	METHODS AND COMPOSITIONS DIRECTED TO DJ-1 AS REGULATOR OF THE ANTI-OXIDANT TRANSCRIPTION FACTOR NRF2	the University of North Carolina at Chapel Hill	1/18/07
8	WO/2007/005879	PCT/US2006/026056	COMPOSITIONS AND METHODS FOR THE TREATMENT OR PREVENTION OF DISORDERS RELATING TO OXIDATIVE STRESS	The Johns Hopkins University	1/11/07
9	WO/2008/097596	PCT/US2008/001602	NRF2 SCREENING ASSAYS AND RELATED METHODS AND COMPOSITIONS	Biogen Idec Inc.	8/14/08