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INVITED REVIEW

The status of Nrf2-based therapeutics: current perspectives and future prospects

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Abstract

This mini-review presents the authors' vision on the current status and future trends in the development of neuroprotective agents working *via* activation of nuclear factor erythroid 2-related factor 2 (Nrf2), and in particular, *via* disruption of Nrf2-Keap1 interaction. There are two opposite "chemical" mechanisms underlying such activation: the first one is a non-specific covalent modification of Keap1 thiols, resulting in side effects of varied severity, and the second one is the shift of the Nrf2-Kelch-like ECH associated protein-1 (Keap1) binding equilibrium in the presence of a competitive and chemically benign displacement agent. At this point, no displacement activators exhibit sufficient biological activity in comparison with common Nrf2 activators working *via* Keap1 thiol modification. Hence, the hope in therapeutics is now linked to the FDA approved dimethylfumarate, whose derivative, monomethylfumarate, as we demonstrated recently, is much less toxic but equally biologically potent and an ideal candidate for clinical trials right now. A newly emerging player is a nuclear inhibitor of Nrf2, BTB domain and CNC homolog 1 (Bach1). The commercially developed Bach1 inhibitors are currently under investigation in our laboratory showing promising results. In our viewpoint, the perfect future drug will present the combination of a displacement activator and Bach1 inhibitor to insure safety and efficiency of Nrf2 activation.

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Nrf2 based Therapeutics

A promising strategy for the treatment of neurodegenerative diseases involves pharmacologically induced activation of a coordinated antioxidant genetic program to shift the intracellular redox balance towards a homeostatic state by means of expression of detoxifying proteins and antioxidant enzymes. A key transcription factor orchestrating antioxidant defense is nuclear factor erythroid 2–related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper transcription factors. In addition to its role in protection from oxidative stress, Nrf2 triggers expression of genes responsible for drug detoxification, iron metabolism, excretion transporters, immunomodulation, calcium homeostasis, growth factors (such as brain derived neurotrophic factor and nerve growth factor-γ), intracellular signaling (including neuronal guanine nucleotide exchange factor), and neurotransmitter receptors (Moi et al., 1994). In addition a recent report on identification and validation of 12 antioxidant response element (ARE)-sequences targeted by Nrf2 in 9 autophagy genes (Pajares et al., 2016) points to additional role of Nrf2 in combating proteinopathies. The breadth of this endogenous response suggests that its activation might counterbalance many of the large number of etiological pathways implicated in the pathogenesis of majority if not all neurodegenerative diseases (Crunkhorn, 2012; Joshi and Johnson, 2012; Gan and Johnson, 2014).

Numerous proof-of-principle studies involving gain and loss of function of Nrf2 have suggested that its induction can ameliorate neurodegeneration, whereas its deficiency can exacerbate neurodegeneration (Joshi and Johnson, 2012; Gan and Johnson, 2014). Despite the well-known fact of Nrf2 activation at late stages of all neurodegenerative diseases, there is insufficient nuclear staining of Nrf2 in the brains of Alzheimer's (AD) (Ramsey et al., 2007), as well as in aging (senescent animals) (Shenvi et al., 2012) and an increased nuclear staining of Nrf2 in surviving neurons of postmortem Parkinson's disease (PD) patients (Ramsey et al., 2007). Pre-treatment of pharmacological activators of Nrf2 has been shown to be protective in animal models of numerous neurological conditions such as Huntington's disease, amyotrophic lateral sclerosis, PD, AD, and cerebral ischemia (Gan and Johnson 2014; Tu et al., 2015). Post-treatment with Nrf2 activators such as sulforaphane and tert-butylhydroquinone has been shown to be beneficial mainly

for acute injury models such as traumatic brain injury and hemorrhagic stroke, with a very narrow therapeutic window of 30 minutes to 2 hours (Saykally, 2009). We have shown that pre-treatment of Nrf2 activators demonstrate therapeutic effects in animal models of PD. Recently we demonstrated that L-3,4-dihydroxyphenylalanine (L-DOPA) bearing a catechol motif as a weak Nrf2 activator, and nordihydroguaiaretic acid, a potent natural di-catechol activator of Nrf2, is neuroprotective against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Smirnova et al., 2016). Another recent work of ours that used the FDA approved dimethylfumarate (DMF) and its bioactive metabolite monomethylfumarate (MMF) also showed neuroprotective effects against MPTP-induced PD in mice (Ahuja et al., 2016).

The problem with pharmacologic activation of Nrf2 for the treatment of chronic neurodegenerative disorders originates from the fact that most common Nrf2-based pharmacophores activate Nrf2 by increasing oxidative stress and are harmful chemicals (for *e.g.*, sulforaphane, curcumin, fumarate, triterpenoids, etc.). Increasing oxidative load on already unhealthy cells may significantly counterbalance the benefits of activation of the antioxidant genetic program, making the unambiguous interpretation of the posttreatment results difficult. This is especially true for the MPTP mouse model of PD, where only pre-treatment, but not post-treatment with Nrf2 activators are protective. There is a desperate need in Nrf2 activating chemical tools that are non-electrophilic, non-toxic, and sufficiently stable in the body to unequivocally demonstrate benefits of Nrf2 activation for chronic neurodegenerative diseases. One of the alternative strategies to activate Nrf2 pathway is to bypass the Keap1-Nrf2 interaction by inhibiting BTB domain and CNC homolog 1 (Bach1), which is an Nrf2 inhibitor at the step of transcription complex formation (see **Figure 1**). Bach1 inhibition will complement Nrf2 stabilization achieved *via* disruption of its complex with Keap1. One may speculate that a combinatorial approach [a) to inhibit Bach1, and b) disrupt Keap1-Nrf2 interaction] is the best way to activate this neuroprotective pathway. The recent investigation on the cause of a significantly lower level of glutamate cysteine ligase (GCLC) pointed to Nrf2 binding from an active ARE to an alternative ARE element, which is not adequate to maintain basal expression of hepatic GCLC in old rats, provides a potential mechanism for the age-related loss of glutathione synthetic and other phase II enzymes. Moreover, the activity at this ARE locus is diminished during aging because of the presence of Bach1 and the absence of CREB-binding protein (CBP), a transcriptional repressor and co-activator, respectively (Shenvi et al., 2012).

There are many Nrf2 activators known from herbal medicines that work *via* the alkylation/covalent modification mechanism, *e.g.*, curcumin (turmeric), sulforaphane (broccoli), flavonoids (quercetin, fisetin), nordihydroguaiaretic acid (chapparal). There are multiple herbal supplements on the market based on curcumin (turmeric) in combination with other herbs such as Protandim, Nrf2 Activator (Xymogen), Nrf2 Optimizer (Nuley), Nrf2 Antiox (Progressive Nutracare), and Ultimate Protector, however, there is only one FDA-approved Nrf2 activator – Tecfidera (dimethylfumarate) for the treatment of multiple sclerosis. All currently reported stabilizers of Nrf2 protein work *via* covalent modification (alkylation) of Keap1 active cysteines. For example, bardoxolone (oral formulation of a triterpenoid CDDO-Im) is the most potent Nrf2 activator described to date, working in the nanomolar range, likely having a specific binding site in the Keap1 intervening region (IVR) near Cys-298 and Cys-226, as we described (Kaidery et al., 2013). However, it becomes highly toxic in the sub-micromolar range presumably due to covalent and indiscriminate alkylation of multiple proteins required for normal cellular homeostasis (**Figure 2**).

Both Bardoxolone and Tecfidera (dimethylfumarate) are electrophiles and potent alkylating agents, meaning that they can non-specifically and covalently modify nucleophilic groups in proteins such as cysteine residues. Clinical trials of bardoxolone were discontinued because of patient death http://reatapharma.com/companystatement-termination-of-the-beacon-trial/). Dimethylfumarate is also an alkylating agent, similar to the classic Nrf2 activator sulforaphane, and has been approved by the FDA in 2013 for the treatment of multiple sclerosis (Fox et al., 2012), despite its common side effect of a 30% decline in the lymphocyte count (Sweetser et al., 2013). It has been recently repurposed as a therapeutic against a mouse model of α-synuclein-induced PD (Lastres-Becker et al., 2016). Our recent study demonstrates similar therapeutic benefits for DMF and its bioactive metabolite MMF in an *in vivo* rodent model of PD (Ahuja et al., 2016). Despite these similarities in *in vivo* efficacy, our *in vitro* studies revealed that MMF is a less potent Nrf2 activator and has much lower toxicity due to the orders of magnitude lesser non-specific alkylating capacity than DMF itself. Our discovery of therapeutic effects of MMF in an experimental PD model without inducing significant alkylating properties compared to DMF suggests that MMF could be a promising candidate for PD therapeutics. Monoethylfumarate (MEF) can also be considered as a therapeutic agent, since its alkylating capacity is very low and is similar to that of MMF. Despite the fact that fumarates are also alkylating agents, their monosubstituted variants such as MMF and MEF will be less toxic than the FDA approved DMF, and may be ideal candidates for future clinical trials in PD. It is obvious that the magic bullet could be a non-alkylating Nrf2 activator, but in the absence of one, MMF and MEF could be the best candidates that can be used as therapeutic agents for PD.

The recently developed Bach1 inhibitor by vTv Therapeutics (High Point NC, USA) represents a novel class of heme-oxygenase 1-inducing compounds. These Bach1 inhibitors are not reactive electrophiles, are not suppressed by N-acetyl cysteine, and do not perturb either ROS or cellular glutathione and are activators of the antioxidant response through the modulation of Bach1 binding to the ARE binding site of target genes (Attucks et al., 2014). Our preliminary results validate Bach1 inhibition as a very effective target against MPTP-induced dopaminergic neurodegeneration by virtue of its ability to activate neuroprotective Nrf2/ARE genetic program (Ahuja et al., 2016). However, more mechanistic and preclinical validation studies in genetic models of PD are required to determine Bach1 inhibition as a safe strategy

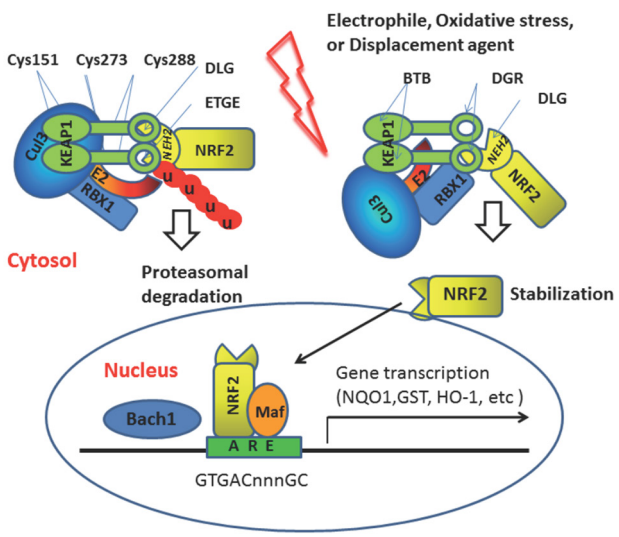

Figure 1 Current model of Keap1/Nrf2/ARE pathway activation.
Nuclear factor erythroid 2-related factor 2 (Nrf2) is constitutively produced in the cell, however, in the absence of environmental stress, Nrf2 is sequestered in the cytoplasm by binding to an inhibitory protein, Kelch-like ECH associated protein-1 (Keap1), which promotes continuous ubiquitinylation. Keap1 serves as a bridge between Nrf2 and the Cul3-Rbx1 E3 ubiquitin ligase. Electrophilic stress leads to modification of reactive cysteines within Keap1 that induces conformational changes resulting in Nrf2 stabilization. The Nrf2 protein then translocates into the nucleus. There, it forms heterodimers with other transcription regulators, such as small Maf proteins. This binding of the Nrf2-Maf complex to the (antioxidant response elements) antioxidant response elements (AREs) of the ARE-containing genes occurs following the nuclear exit of the Nrf2 repressor BTB domain and CNC homolog 1 (Bach1) to subsequently induce Nrf2-dependent gene expressions. DGR: Double glycine repeat; GST: glutathione S-transferase; HO-1: heme oxygenase 1; NQO1: NAD(P)H:quinone oxidoreductase.

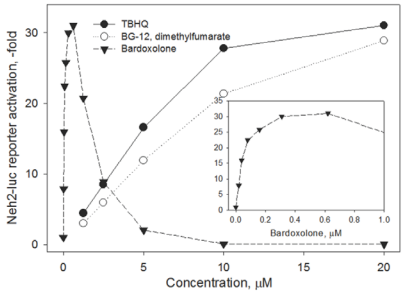

Figure 2 Activation of Neh2-luc reporter– a perfect screening tool for nuclear factor erythroid 2-related factor 2 (Nrf2) activators working *via* stabilization of Nrf2 protein (Smirnova et al., 2011) – shows a very narrow safe window for bardoxolone at 3 hour incubation in contrast to a wide range of safe biologically active concentrations of dimethylfumarate (DMF).
BG-12: Dimethyl fumarate; TBHQ: tertiary butylhydroquinone.

to activate the Nrf2 pathway.

Nrf2 activation without covalent modification of Keap1 cysteines can be achieved *via* Nrf2 protein displacement from its complex with Keap1 either by a small molecule, or Nrf2 peptide (Steel et al., 2012). Academic laboratories, small and big pharmaceutical companies are hunting for small molecule Nrf2 activators working *via* this mechanism. Five small molecules have been reported so far, but only two validated (**Figure 3**). Consistent with this mechanism, more

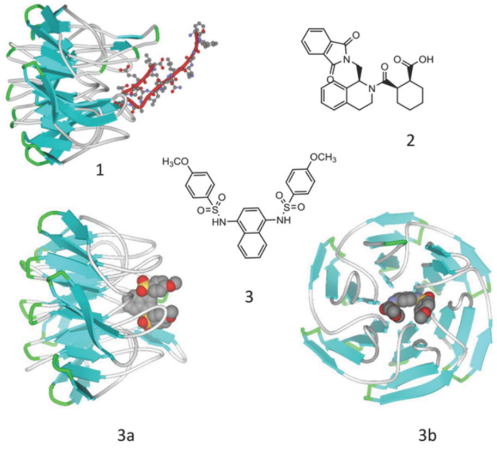

Figure 3 Displacement activators of nuclear factor erythroid 2-related factor 2 (Nrf2).
Validated small molecules identified in fluorescence polarization assay based on labeled Nrf2-peptide displacement from recombinant Kelch domain of Kelch-like ECH associated protein-1 (Keap1). 1: Crystal structure of Nrf2 peptide in Kelch domain of Keap1; 2: molecule discovered by Lonquin Hu's group (Compound 1) (Hu et al., 2013); 3: deposited crystal structure of compound 2 with Keap1 side view (3a), top view (3b).

recently a peptide activator of the Nrf2 pathway that functions by disrupting Nrf2-Keap1 interaction was shown to exhibit robust therapeutic effects in a global cerebral ischemia model in rats when the peptide was administered systemically in a post-injury paradigm (Tu et al., 2015). While these findings are highly significant in ameliorating disease pathology in an acute preclinical model, similar peptide-based approaches should be also utilized as a therapeutic strategy for chronic neurodegenerative diseases.

Compound 1 has been identified in the *in vitro* homogeneous assay, re-synthesized, 4 enantiomers separated from each other (compound 1 has 3 chiral centers), and only one enantiomer showed the biological activity: a binding constant of 1 μM to recombinant Keap1, and activation in the *in vitro* cellular assay only above 10 μM (Hu et al., 2013). Compound 2 has been identified in a joint effort by Biogen Idec, Merrimack Pharmaceuticals, Celgene Corporation (USA), Evotec AG (Germany), and NoValix (France) (Marcotte et al., 2013). It binds Keap1 in the *in vitro* assay in the low micromolar range, however, in the *in vitro* cellular assay it works only above 30 μM. There is a shift in potency by at least one order of magnitude when one compares the *in vitro* binding constant to Keap1 with the actual performance of the Nrf2 activator in a cellular assay. This offset in potency when one switches from a test-tube to a cell line illustrates the fact that competing with a protein for binding another protein in the real life is far more challenging than competing with a peptide for a recombinant protein in the tube. On the top of their actual low potency, the molecules identified have little chance of crossing blood-brain barrier because of the complexity of the structure bearing multiple cycles and negatively charged groups, predicted easiness of oxidative decomposition in the liver to form active intermediates, and capable of inducing Nrf2 by Keap 1 covalent modification.

One of the future developments could be a combination of

Nrf2 displacement activity with mild alkylation potency: in this way the concentration of an alkylating agent will be enriched around Nrf2-Keap1 complex to enhance the specificity of alkylation. The very recent discovery of Nrf2 activators in the low micromolar range among NAD-dependent deacetylase sirtuin-2 (SIRT2) triazole inhibitors (Quinti et al., 2016) may reflect their specific action on Keap1, since in addition to a pro-alkylating motif, they have a stereostructure similar to compound 3 in **Figure 3**. However, in the absence of a crystal structure, this is just a speculation.

Conclusion

Activation of the Nrf2 signaling pathway is an established mechanism for reducing neurodegeneration due to oxidative stress, and represents a promising therapeutic approach for several neurodegenerative diseases. Unfortunately, Nrf2-based therapeutics has relied on electrophilic pharmacophores. There is indeed a desperate need of safe, potent, blood-brain barrier permeable displacement-type Nrf2 activators as therapeutic agents for chronic neurodegenerative diseases. Given the very low potency of existing displacement-type Nrf2 activators and their complex chemical structures that are not ideal for them to cross the blood-brain barrier, it is going to be an arduous task to develop such compounds as therapeutic agents. At this point MMF, which upon hydrolysis gives a natural metabolite – fumaric acid, has a low alkylating potency and thus, low toxicity, could be the best option to treat chronic neurodegenerative disease such as PD. More studies should be carried out to test the therapeutic effects of peptidebased inhibitors of Nrf2-Keap1 interaction as therapeutic agents in preclinical models of chronic neurodegenerative diseases. Furthermore, efforts should also focus on validating Bach1 inhibition as an alternate strategy for safe and efficient activation of the Nrf2 pathway as a novel therapeutic target for PD and related neurodegenerative diseases.

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