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Opinion

Advances and challenges in therapeutic targeting of NRF2

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Activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is emerging as an attractive therapeutic approach to counteract oxidative stress, inflammation, and metabolic imbalances. These processes underpin many chronic pathologies with unmet therapeutic needs, including neurodegenerative disorders and metabolic diseases. As the NRF2 field transitions into the clinical phase of its evolution, the need for an understanding of the factors influencing NRF2 pharmacology has never been greater. In this opinion article we describe the rationale for targeting NRF2, summarise the recent advances in drug development of NRF2 modulators, and reflect on the remaining challenges in realising the full clinical potential of NRF2 as a therapeutic target.

Why target NRF2?

Inducible transcription factor NRF2 (encoded by *NFE2L2*) is a member of the human cap'n'collar (CNC) basic-region leucine zipper transcription factor family. The protein products of its target genes perform versatile cytoprotective functions, including antioxidant, anti-inflammatory, metabolic, and drug-metabolising, and also have roles in the maintenance of protein homeostasis. Through its transcriptional targets, NRF2 activation orchestrates a comprehensive and long-lasting protection that allows adaptation and survival under diverse forms of cellular and organismal stress [1,2]. Pharmacological NRF2 activators have shown protective effects in numerous models of human disease and benefits in human intervention trials, and NRF2 is considered a drug target [3]. Several small-molecule NRF2 activators are currently in clinical trials, and one such compound, dimethyl fumarate, is in clinical practice for the treatment of **remitting-relapsing multiple sclerosis (RRMS)** (see Glossary) and psoriasis. This opinion article briefly summarises the protective role of NRF2 against non-neoplastic disease and its Janus face in cancer, and then focuses on the current state of drug development of NRF2 activators and inhibitors, and the challenges in going forward.

The protective role of NRF2 against non-neoplastic disease and its Janus face in cancer

Under unstressed conditions, NRF2 is an unstable, short-lived protein, the abundance of which is principally controlled by Kelch-like ECH-associated protein 1 (KEAP1), an E3 ubiquitin ligase substrate adaptor that targets NRF2 for ubiquitination and proteasomal degradation (Figure 1) [1,2]. Additionally, KEAP1 serves as a sensor for endogenous and exogenous **electrophiles** and oxidants due to it containing several highly reactive cysteine residues that, upon chemical modification, prevent its ability to target NRF2 for degradation, leading to NRF2 accumulation and upregulation of a large network of cytoprotective proteins. Both NRF2 and KEAP1 can undergo post-translational modifications and interact with other proteins that affect their functions [4,5]. Thus, in addition to NRF2, KEAP1 has multiple other binding partners, which in turn have roles in a plethora of cellular processes (Figure 2).

Highlights

The inducible transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) regulates the expression of several hundred genes encoding proteins with antioxidant, anti-inflammatory, drug metabolising, and other homeostatic functions. NRF2 activity is principally regulated by the redox sensor protein Kelch-like ECH-associated protein 1 (KEAP1).

Several small-molecule NRF2 activators are currently in clinical trials in different disease settings, and one such compound, dimethyl fumarate, is licensed for the treatment of remitting-relapsing multiple sclerosis (RRMS) and psoriasis.

Much progress has been made in understanding NRF2 pharmacology, although the true realisation of its value as a therapeutic target will depend on further advances in understanding the importance of target specificity, monitoring of pharmacodynamic responses and interindividual variability, safety considerations, and appropriate disease indications.

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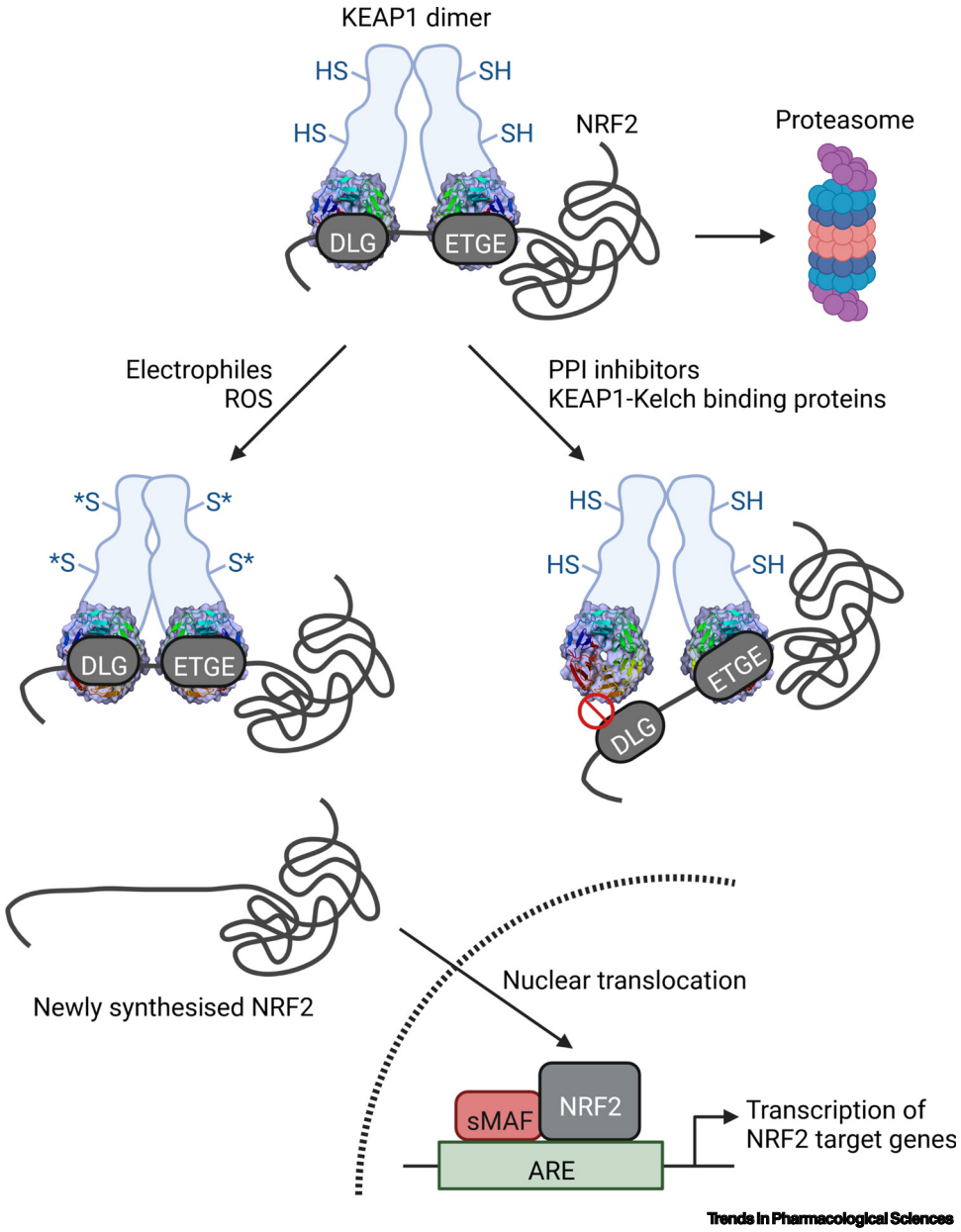

Figure 1. The Kelch-like ECH-associated protein 1 (KEAP1)–nuclear factor erythroid 2-related factor 2 (NRF2) system. NRF2 binds to the Kelch domains of dimeric KEAP1 via the 'DLG' and 'ETGE' motifs in the Neh2 domain of the transcription factor. In turn, reduced KEAP1 serves as a E3 ubiquitin ligase substrate adaptor that targets NRF2 for ubiquitination and proteasomal degradation. Electrophiles and reactive oxygen species (ROS) chemically modify specific cysteine sensors in KEAP1, prompting a conformational change that impairs its substrate adaptor function without disrupting the KEAP1–NRF2 interaction. Small-molecule KEAP1–NRF2 protein–protein interaction (PPI) inhibitors, and proteins that bind to the Kelch domain of KEAP1, disrupt the DLG–KEAP1 interaction preferentially to the ETGE–KEAP1 interaction. In both cases, newly synthesised NRF2 accumulates, undergoes nuclear translocation, and as a heterodimer with a small musculoaponeurotic fibrosarcoma (sMAF) transcription factor, binds to specific sequences called antioxidant response elements (AREs) in the regulatory regions of NRF2-target genes, and activates their transcription. Abbreviations: SH, reduced cysteine; S*, modified cysteine.

Glossary

Electrophile: a chemical species which is attracted to an electron-rich centre. It is chemically reactive, and by accepting an electron pair it binds to a nucleophile.
Molecular glue: a small molecule that stabilises the interaction between two proteins, leading to changes in activity or stability, and in most cases to degradation of the target protein.
Oxeiptosis: a non-inflammatory cell-death pathway triggered by high levels of reactive oxygen species (ROS).
Oxidative stress: an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of cellular redox signalling and damage to proteins, lipids, and DNA.
Proteolysis-targeting chimera (PROTAC): a bifunctional small molecule which is capable of removing a protein of interest from the cell. It is composed of two covalently linked protein-binding molecules, one of which binds to the target protein and another, which recruits an E3 ubiquitin ligase, resulting in the ubiquitination and subsequent proteasomal degradation of the target protein.
Reactive oxygen species (ROS): an unstable oxygen-containing molecule that is formed by redox reactions or by electronic excitation.
Remitting-relapsing multiple sclerosis (RRMS): a form of multiple sclerosis in which patients have relapses and periods of stability in between relapses. Multiple sclerosis is a chronic inflammatory disease that can affect the brain, spinal cord, and the optic nerves, causing problems with vision, balance, and muscle control.

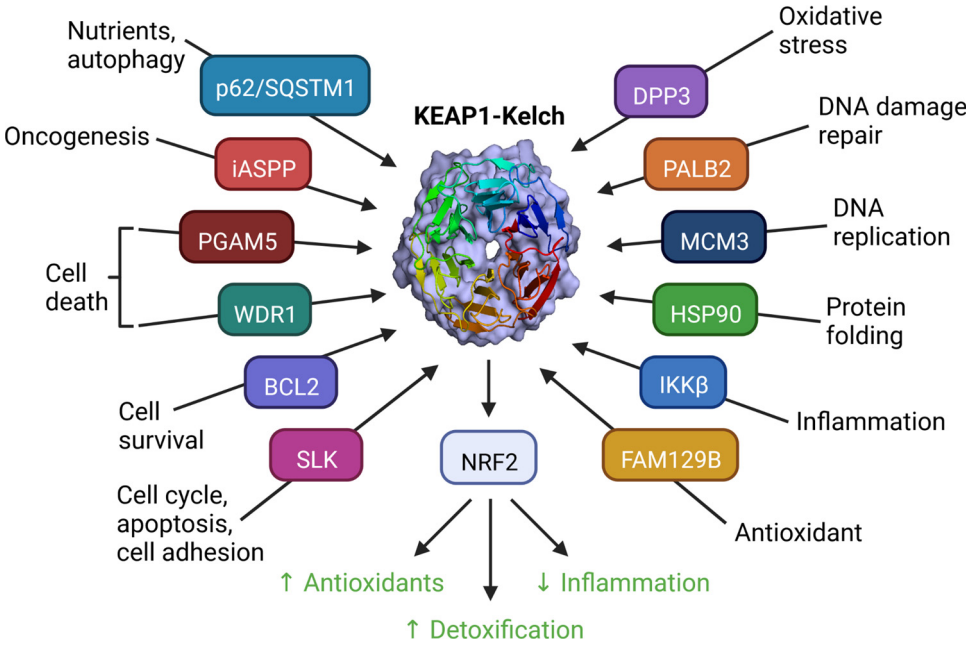

Figure 2. Examples of proteins that interact with Kelch-like ECH-associated protein 1 (KEAP1) and the cellular processes that they affect. The autophagy adaptor protein p62/SQSTM1 is phosphorylated by mammalian target of rapamycin complex 1 (mTORC1) in response to nutrients, and facilitates the autophagic degradation of KEAP1; p62 is also brought by KEAP1 to mitochondria to promote mitophagy. The interaction of dipeptidyl peptidase 3 (DPP3) with KEAP1 is increased by oxidative stress. KEAP1 mediates the ubiquitination of partner and localizer of BRCA2 (PALB2) and prevents its interaction with BRCA1, leading to suppression of homologous recombination in G_1 cells. KEAP1 also interacts with mini-chromosome maintenance protein 3 (MCM3), a protein involved in DNA replication, and the molecular chaperone heat shock protein 90 (HSP90). The interaction between KEAP1 and inhibitor of nuclear factor κB kinase β (IKKβ) promotes the degradation of the kinase, in turn affecting inflammatory processes. Binding to KEAP1, and the consequent nuclear factor erythroid 2-related factor 2 (NRF2) activation, mediates the antioxidant effects of FAM128B. KEAP1 also binds to SLK, which has roles in the cell cycle and apoptosis, as well as cell adhesion, although the consequences of KEAP1–SLK binding have not been investigated. The interaction of KEAP1 with BCL2 promotes cell survival, whereas inhibition of the interaction of KEAP1 with PGAM5 or WDR1 promotes cell death. Notably, ectopically expressed WDR1 and full-length KEAP1 have been shown to interact, and deletion of the ETGE motif of WDR1 abolished this interaction; curiously, however, no interaction was detected between WDR1 and KEAP1-Kelch. Inhibitor of apoptosis-stimulating protein of p53 (iASPP) is overexpressed during oncogenesis, and promotes NRF2 stabilisation, similarly to many but not all KEAP1-interacting partners. Stabilised NRF2 induces the expression of genes that increase the cellular antioxidant (e.g., HMOX1, SLC7A11, GCLC, GCLM, GPX2, PRDX1, SRXN1, TXN1, and TXNRD1) and detoxification (e.g., AKR1B10, AKR1C1, AKR1C2, AKR1C3, and NQO1) capacity and inhibits the expression of proinflammatory genes: for example, interleukin 6 (IL6) and IL1b.

In humans, the contributing role of NRF2 to susceptibility to non-neoplastic diseases is well documented. Indeed, a number of studies have revealed associations between functional genetic variations of *NFE2L2* and disease risk [3]. These gene-association studies are complemented by laboratory experiments generating a wealth of experimental evidence for the protective effect of NRF2 activation against lung, liver, eye, gastrointestinal, metabolic, neurodegenerative, and autoimmune diseases, most prominently in cases where **oxidative stress** and inflammation underlie disease pathogenesis [3,6]. Consequently, a number of clinical trials have been conducted. At this time, nearly 100 clinical trials with NRF2 activators – including pure compounds, dietary supplements, or plant extracts – are registered on Clinicaltrials.gov. Here we highlight three NRF2 activators that have progressed furthest in clinical development: the naturally occurring isothiocyanate sulforaphane, the semisynthetic cyanoenone triterpenoid RTA-408 (omaveloxolone),

and dimethyl fumarate (DMF, Tecfidera®), currently the only NRF2 activator in clinical practice (Box 1). The overall outcomes of the clinical trials with sulforaphane-rich preparations have strengthened the preclinical evidence that sulforaphane has the potential to prevent the toxic and neoplastic effects of environmental carcinogens, as well as to ameliorate a diversity of conditions characterised by chronic oxidative, metabolic, and inflammatory stress [7,8]. In a similar vein, a wealth of experimental evidence has provided a rationale for the use of semisynthetic cyanoenone triterpenoids for the prevention and treatment of chronic disease [6], leading to

Box 1. Examples of electrophilic NRF2 activators in clinical trials or clinical practice

The isothiocyanate sulforaphane (Figure I) is one of the first identified and most potent naturally occurring NRF2 activators known [7,64]. Broccoli and 3-day-old broccoli sprout extracts are rich sources of sulforaphane, and many clinical trials have employed such preparations in healthy or at-risk subjects [7,40,65]. In addition, two stabilised sulforaphane preparations have been developed – Prostaphane® and Sulforadex® (SFX-01) – and used in clinical trials in prostate cancer and subarachnoid haemorrhage patients, respectively.

The cyanoenone triterpenoids are the most potent NRF2 activators identified to date [6,66]. Clinical trials have been conducted with omaveloxolone (RTA-408) (Figure II), and the closely related bardoxolone methyl, for several disease indications, including chronic kidney disease, liver disease, pulmonary hypertension, pulmonary arterial hypertension, ocular inflammation, and radiation dermatitis [3].

Dimethyl fumarate (DMF, BG-12, Tecfidera®) (Figure III) is the only NRF2 activator currently in clinical practice. Curiously, DMF had been shown to induce the classical NRF2-transcriptional targets NQO1 and glutathione transferases in cells and in mice several years before the discovery of NRF2 [67]. Based on the clinical trials data, as well as its subsequent use, it is generally accepted that DMF is relatively well tolerated, with side effects that include flushing, gastrointestinal irritation, lymphocytopenia, and a rare (incidence of approximately one in 42 000) development of progressive multifocal leukoencephalopathy (PML); due to its seriousness, the latter is now included in the risk-management plans [11].

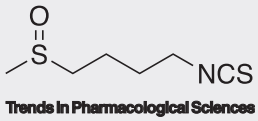

Figure I. Structure of sulforaphane.

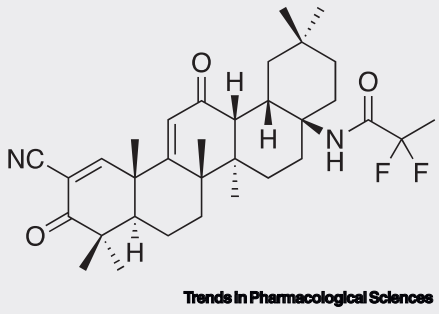

Figure II. Structure of omaveloxolone (RTA-408).

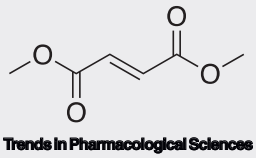

Figure III. Structure of dimethyl fumarate.

several clinical trials. A Phase 2 randomised, placebo-controlled, dose-escalation clinical trial (NCT02255435) assessing the safety, pharmacodynamics, and potential benefit of omaveloxolone in patients with Friedreich's ataxia (FRDA) reported that a dose of 160 mg/day for 12 weeks was well tolerated and improved neurological function [9]. Subsequently, a clinical trial conducted across 11 institutions in the USA, Europe, and Australia further confirmed that a once-daily 150 mg dose of omaveloxolone for 48 weeks significantly improved neurological function compared to placebo [10]. Based on extensive clinical trials data, which showed a reduction in relapse rates of approximately 50% compared with placebo, and a reduction in new lesion formation (assessed by magnetic resonance imaging, MRI), DMF was licensed as the first oral first-line therapy for RRMS in 2013 [11], and is also used for the treatment of moderate to severe plaque psoriasis [12].

In contrast to the non-neoplastic chronic diseases that are often associated with NRF2 impairment and the chemoprotective effects of NRF2 activation in the early stages of carcinogenesis, NRF2 is often hyperactive in established human tumours, where it contributes to the hallmarks of cancer [13]. Notably, persistent NRF2 activation in cancer cells, in cooperation with transcription factor CCAAT enhancer binding protein beta (CEBPB), generates enhancers at gene loci that are not regulated by transiently activated NRF2 under physiological conditions [14]. NRF2 activation in cancer creates metabolic imbalances, particularly within the cysteine and glutamate pools and the pentose phosphate pathway, and several strategies have been proposed to exploit these liabilities for therapeutic benefit, such as the use of inhibitors of glutaminase [15] or glucose 6-phosphate dehydrogenase [16]. A Phase 1 clinical trial with the glutaminase inhibitor CB-839 is under way in patients with advanced non-small-cell lung cancer (NSCLC), with a focus on those harbouring *NFE2L2* or *KEAP1* mutations [17]. These strategies are in parallel with ongoing efforts to develop NRF2 inhibitors using traditional high-throughput screening of large chemical libraries (and subsequent medicinal chemistry optimisation) and, more recently, approaches for inhibiting 'hard-to-drug proteins', such as fragment-based nuclear magnetic resonance spectroscopy screening against part of the DNA-binding domain of NRF2 that does not include the leucine zipper shared by other transcription factors; targeted protein degradation with **proteolysis-targeting chimeras (PROTACs)** and **molecular glues** are also being considered, although these strategies are currently limited by the paucity of small molecules that bind to NRF2 [18].

Challenges in the drug development of NRF2 activators

The advances in drug development of NRF2 modulators are accompanied by numerous challenges. In this section, we address the main challenges, including target specificity, monitoring target engagement/pharmacodynamic responses, short/long-term safety considerations, identification of the most appropriate disease indications, and understanding the extent and implications of variation in NRF2 activity (Figure 3, Key figure).

Target specificity

Many NRF2 activators are electrophiles that target cysteine 151 in KEAP1 [19–21]. However, depending on concentration, the electrophilicity of such molecules confers an ability to affect multiple cysteines within KEAP1 [20] and other proteins [6,22,23] (Figure 3A). Thus, whereas NRF2 activation represents an important component of the efficacy of these compounds, it may not be the only responsible factor. In addition to painting a complex picture, this 'promiscuity' suggests that, somewhat paradoxically, the lack of specificity might in fact be advantageous. Indeed, the anti-inflammatory effects of most electrophilic NRF2 activators are partly NRF2-independent [24,25] and include inhibition of other inflammatory mediators, such as the innate immune kinase interleukin-1 receptor-associated kinase 4 (IRAK4) [22] and cyclooxygenase-2 [26]. Notably, the DMF-mediated protection against acute inflammatory

Key Figure

Challenges in the drug development of nuclear factor erythroid 2-related factor 2 (NRF2) activators

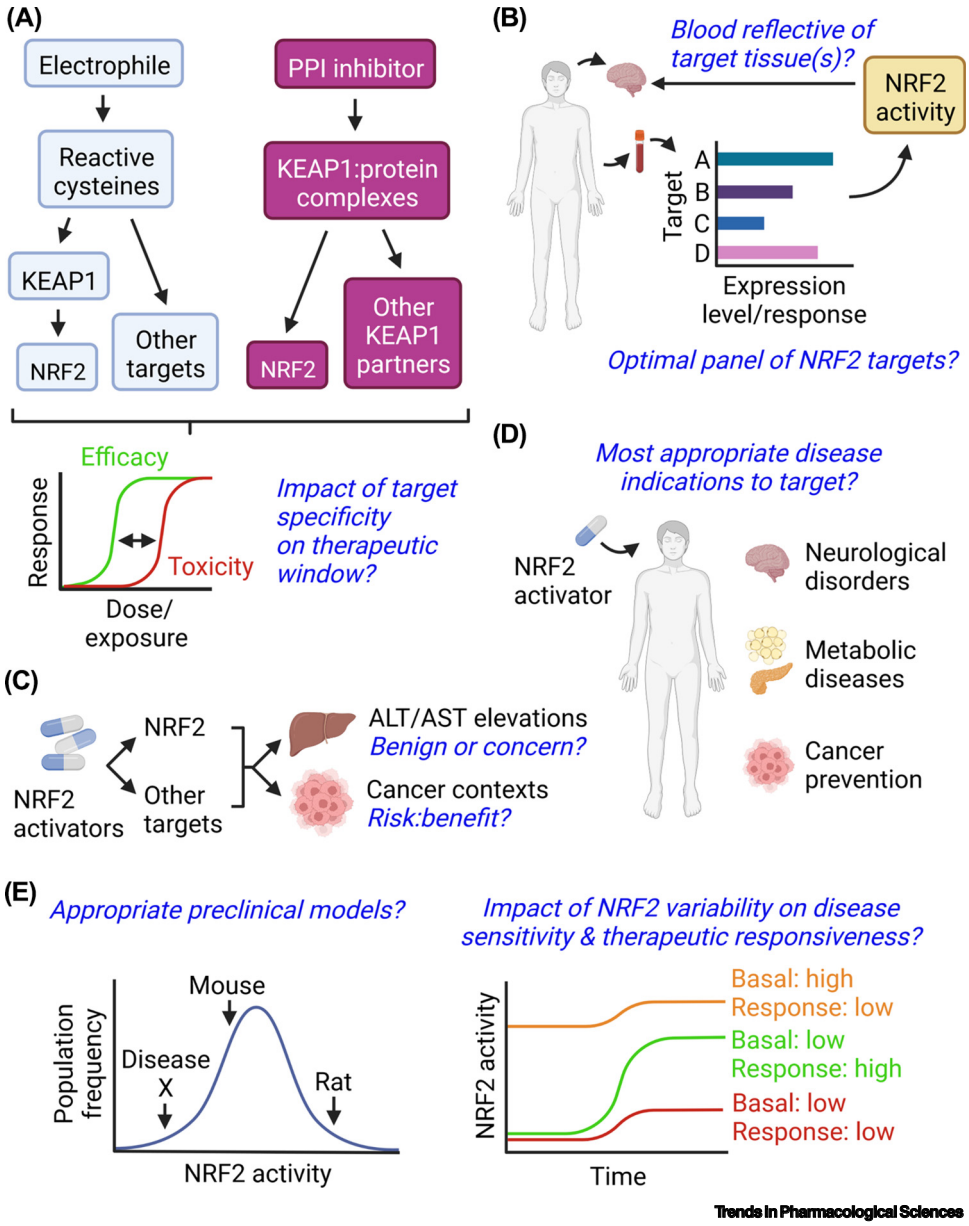

Figure 3. Overview of the challenges that need to be addressed in order to realise the full clinical potential of NRF2 as a therapeutic target. (A) It will be important to understand the role of target specificity in determining the balance between efficacy and safety for different classes of NRF2 activators. (B) A robust method for monitoring the pharmacological modulation of NRF2 in patients is needed. This will require the selection of an optimal panel of NRF2 target genes/proteins, and an understanding of the concordance between NRF2 activity/responses measured in blood and target

(Figure legend continued at the bottom of the next page.)

experimental autoimmune encephalomyelitis, a model of MS, is observed in both NRF2-proficient and NRF2-deficient animals [27]. The recent discovery that DMF disrupts the interaction between KEAP1 and WDR1, triggering apoptosis in neutrophils and macrophages [28], suggests that this on-target KEAP1 engagement by DMF is an important contributor to its NRF2-independent anti-inflammatory effects. The structurally related molecules monomethyl fumarate (Bafiartam®) and diroximel fumarate (Vemurity®) have recently been approved as therapies for RRMS. Yet, to our knowledge, NRF2 activators from other classes are not being developed clinically as candidate RRMS drugs.

Would a more specific NRF2 activator be better? The development of KEAP1–NRF2 protein–protein interaction (PPI) inhibitors that target the Kelch domain of KEAP1 represents, so far, the main approach to increasing specificity. Several peptide and small-molecule PPI inhibitors have been designed based on the crystal structure of KEAP1-Kelch, and in many cases, cocrystal structures are available [3]. A recent independent assessment questioned the characterisation of more than half of 19 reported KEAP1–NRF2 PPI inhibitors [29], highlighting the need for careful selection of such compounds for use in mechanistic and translational studies. Curiously, the recently described phenyl bis-sulfonamide PPI inhibitors bind to KEAP1-Kelch in a distinct 'peptidomimetic' conformation that resembles the KEAP1-Kelch : NRF2-ETGE peptide complex [30]. In addition, small-molecule PPI inhibitors with *in vitro* affinities for KEAP1 in the nanomolar range have been identified using structure-based virtual screening [31]. Despite their high affinities for KEAP1 *in vitro*, the majority of non-electrophilic PPI inhibitors are less potent in activating NRF2 in cellular systems than the electrophilic sulforaphane or triterpenoids. However, compounds with potencies similar to electrophilic NRF2 activators in cell-based assays are emerging, such as the benzotriazole KI-696 [32] and the isoquinoline PRL-295 [33] (Box 2). Notably, two recent studies employed KI-696 linked to a ligand for cereblon (CRBN)/Cullin 4/Rbx1 E3 ligase to create PROTACs that target KEAP1 for proteasomal degradation and thereby activate NRF2 [34,35].

Are PPI inhibitors necessarily more specific than some of the less promiscuous electrophiles? This is a valid question considering that: (i) all existing PPI inhibitors target KEAP1, and (ii) NRF2 is not the only binding partner of KEAP1. As mentioned earlier, several other proteins have been shown to bind to the Kelch domain of KEAP1 [36] (Figure 2), which in turn, engage in complex protein networks. Similar to NRF2, this binding facilitates protein degradation of some – such as for inhibitor of nuclear factor κB kinase β (IKKβ) – but not all KEAP1-interacting partners. Furthermore, depending on the protein function, binding to KEAP1 can have a range of cell fate consequences, from promoting cell survival by outcompeting NRF2 – for dipeptidyl peptidase 3 (DPP3) and p62 – and/or by antagonising the proapoptotic role of p53 (for inhibitor of apoptosis-stimulating protein of p53, iASPP), to causing premature cell senescence – for nitric oxide synthase (NOS)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – and even triggering a specific form of cell death termed **oxeiptosis** (for phosphoglycerate mutase 5, PGAM5, in conjunction with the proapoptotic factor apoptosis-inducing factor mitochondria associated 1, AIFM1). Furthermore, the interactions between KEAP1 and its other binding partners appear to

tissue(s). (C) Current and emerging safety concerns need to be addressed in a translational manner. (D) Whilst the modulation of NRF2 has been linked to beneficial effects in a large number of preclinical disease models, the most appropriate human diseases to target with NRF2 activators still requires clarification. (E) It will be important to understand the consequences of interindividual and interspecies variability for selection of translationally relevant preclinical models, sensitivity to disease, and responsiveness to NRF2 activator therapy. Abbreviations: ALT/AST, alanine aminotransferase/aspartate aminotransferase; KEAP1, Kelch-like ECH-associated protein 1; PPI, protein–protein interaction.

Box 2. Examples of KEAP1-NRF2 PPI inhibitors in development as NRF2 activators

The benzotriazole KEAP1 inhibitor KI-696 (Figure I) was identified by use of a fragment-based drug discovery approach [32]. This compound combines a high affinity for KEAP1 with drug-like physicochemical properties; it has high potency/efficacy in cell-based and animal models, is commercially available, and is increasingly being used as a tool to activate NRF2 in various contexts [68,69].

The isoquinoline PRL-295 [33] (Figure II) was developed with the aim to improve the safety and physicochemical properties of the earlier naphthalene- and isoquinoline-based KEAP1 inhibitors [70]. Compared to its predecessors, PRL-295 has greater lipophilicity and metabolic stability; its potency in cell-based assays is similar to that of sulforaphane, although its efficacy as an oral NRF2 activator in mice appears to be restricted to the liver [71]. In parallel with a closely related naphthalene compound, PRL-295 was recently used to test the 'hinge-and-latch' model of NRF2 activation, revealing that PPI inhibitors disrupt the DLG–KEAP1 interaction preferentially over the ETGE–KEAP1 interaction [72].

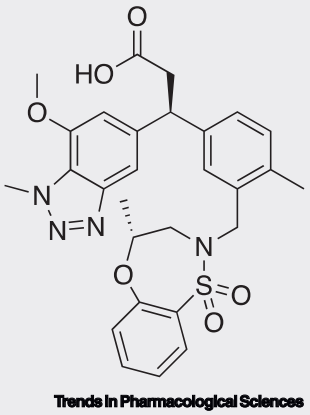

Figure I. Structure of KI-696.

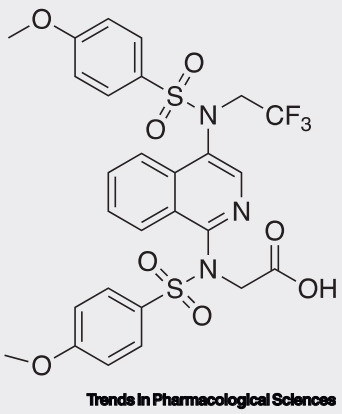

Figure II. Structure of PRL-295.

be cell-type- and context-dependent, and are likely to differ between rapidly proliferating and differentiated cells. Thus, binding of p62 to KEAP1 requires p62 phosphorylation at serine 349 [37], oxeiptosis occurs under conditions of high levels of **reactive oxygen species (ROS)**, such as H_2O_2 [38], whereas the complete absence of NRF2 in endothelial cells promotes formation of a complex of KEAP1 with NOS and GAPDH, in turn causing protein *S*-nitrosation and cell senescence [4]. The existing feedback regulatory loops should also be considered. In this context, by

activating NRF2, a selective KEAP1–NRF2 PPI inhibitor will increase the gene expression of p62, which in turn, upon phosphorylation, could cause further NRF2 activation by displacing it from KEAP1 as well as enhancing the autophagy-mediated KEAP1 turnover, decreasing its levels, and amplifying the initial effect. Thus, a PPI inhibitor which can both inhibit KEAP1-–NRF2 binding and KEAP1-phospho-p62 binding, or selectively inhibit binding of KEAP1 to phospho-p62, would break this positive-feedback loop. Indeed, the selective KEAP1-phospho-p62 PPI inhibitor K67 increases the levels of KEAP1, but decreases the levels of NRF2 [39]. Overall, the effects of the KEAP1-NRF2 PPI inhibitors reported to date on the interactions of KEAP1 with its other binding partners, and the ensuing biological consequences, are largely unknown and worthy of detailed investigation. This, together with a deeper understanding of the physicochemical properties that drive target specificity and membrane permeability of small-molecule NRF2 activators, will support the development and use of such compounds as novel medicines. Other means of stimulating NRF2 signalling, such as oligonucleotide-based therapeutic approaches, are also in early phases of exploration.

Monitoring target engagement/pharmacodynamic responses

Monitoring target engagement and pharmacodynamic responses in patients receiving NRF2 activators represents another challenge (Figure 3B), and very few clinical trials have addressed these so far. A study using peripheral blood mononuclear cells (PBMCs) isolated from boys (aged 6–12 years) with autism spectrum disorder (ASD) who received preparations delivering sulforaphane orally for 2 weeks showed a large interindividual variability in pharmacodynamic responses, but an overall significant increase in cytoprotective gene expression, including the NRF2 targets NAD(P)H quinone oxidoreductase 1 (NQO1) and aldo-keto reductase 1C1 (AKR1C1), and a decrease in proinflammatory markers [40]. In a subsequent study, the effects of sulforaphane on inflammatory biomarkers in PBMCs correlated with clinical improvements in a subset of ASD children (aged 3–12) [41]. Transcriptional analysis of peripheral immune cells from serial blood samples obtained from 43 RRMS patients detected an increase in NQO1 and AKR1C1 expression 4–6 weeks after DMF therapy initiation [42]. In agreement with lower NRF2 inducibility during ageing, NQO1 induction was most prominent in younger individuals, decreased with age, and correlated with the immune effects and clinical outcomes. It is not clear, however, whether NRF2 activity can be detected in healthy populations, or perhaps some type of a challenge test (e.g., the ‘equivalent’ of a glucose-tolerance test) might be needed to achieve this. Furthermore, it remains to be shown that measurement of NRF2 activation in blood samples can reflect the modulation of the pathway in target tissues, whilst the field has yet to reach a consensus on the best approach for monitoring NRF2 activation in humans, including selection of the optimal panel of gene/protein targets. Nonetheless, the above studies provide encouragement that target engagement and pharmacodynamic responses can be monitored in the context of chronic conditions, where NRF2 activation is likely to be most beneficial.

Short-/long-term safety considerations

Although at first glance not unique to NRF2 activators, the ability of NRF2 to impinge on multiple aspects of biology poses special considerations (Figure 3C). Increases in the serum levels of the liver injury markers alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase have been observed at 4 weeks after intervention with bardoxolone methyl in a placebo-controlled Phase 3 clinical trial in patients with type 2 diabetes mellitus (T2D) and stage 4 chronic kidney disease [43]. Subsequent studies in cultured cells and mice attributed the ALT increase to NRF2 activation, although the modest upregulation of gene expression observed may not fully account for the increases in serum ALT/AST of up to 20-fold that were detected in a small number of patients following commencement of drug treatment [44]. Similar

effects on circulating ALT/AST levels have been reported in clinical trials of omaveloxolone [10] and DMF [45], but not in studies of sulforaphane. Hence, it remains to be clarified whether this is an expected transcriptional response to NRF2 stimulation in humans, an asymptomatic off-target effect caused by certain classes of NRF2 activators, or a true reflection of hepatotoxicity risk.

The dual role of NRF2 in cancer presents additional complexity in assessing long-term benefits versus risks. In mice, chronic pharmacological NRF2 activation promotes progression of pre-existing lung tumours [46] and enhances metastasis of liver tumours [47]. On the other hand, it is encouraging that: (i) genetic activation of NRF2 by KEAP1 knockdown in mice does not lead to spontaneous tumour development, (ii) genetic (by KEAP1 knockdown) or chronic pharmacological (by the tricyclic cyanoenone TBE-31) activation of NRF2 does not affect adenoma development in a genetic mouse model of colorectal cancer [48], (iii) chronic treatment with sulforaphane post-initiation suppresses tumour development in a mouse model of UV-radiation-mediated cutaneous squamous-cell carcinoma [49], (iv) high consumption of cruciferous vegetables, a source of isothiocyanates such as sulforaphane, is associated with a lower frequency of bulky DNA lesions, particularly in former smokers [50], and a decreased cancer risk [51], and (v) there was no evidence for a difference in cancer rate between placebo- and DMF-treated groups in a meta-analysis of a Phase 3 clinical trial [52]. However, it is not known whether in humans, pharmacological activation of NRF2, in the context of specific oncogenic drivers such as phosphoinositide 3-kinase (PI3K) and/or Kirsten rat sarcoma viral gene (KRAS), may promote tumour progression as seen in mice [53]. Furthermore, it is currently unclear whether chronic pharmacological NRF2 stimulation could alter the risk of tumour formation in conditions predisposing to cancer, possibly influenced by the timing of the intervention relative to the state of disease progression. In addition to the oncogenic context, the overall outcome will likely depend on compound selectivity; for example, electrophiles such as sulforaphane may suppress tumour development independently of NRF2 activation.

The frequency of dosing with an NRF2 activator should also be considered. Mouse studies suggest that even after a single dose of an NRF2 activator, increased levels of the actual protectors (i.e., the downstream transcriptional targets of NRF2) persist over long periods of time (days), exceeding the half-life (hours) of the drug [54]. Indeed, chronic treatment with an NRF2 activator two or three times a week is sufficient for protection in animal models of non-neoplastic diseases [55]. It is thus possible that a reduced dosing frequency may overcome some of the challenges and potential risks whilst still maintaining the benefits. Establishing this possibility requires further investigations in a context-specific manner, such as in the presence of oncogenic drivers.

Identifying the most appropriate disease indications to target with NRF2 activators

Whilst chronic conditions with underlying oxidative stress and inflammation are most likely to benefit from NRF2 activation, the precise disease context is not entirely clear. Moreover, in certain contexts, the role of NRF2 is complex and cell-type-specific, for example in mouse models of atherosclerosis. Thus, whereas loss of NRF2 in bone-marrow-derived cells exacerbates atherogenesis [56], loss of NRF2 globally increases plaque inflammation and vulnerability [57], but decreases lesion development [58]. Considering that NRF2 activation functions to restore the cellular redox and protein homeostasis, preserve mitochondrial function, and inhibit inflammation, perhaps the most logical disease areas are neurological conditions where all of these processes contribute to the survival of neurons and astrocytes, as well as metabolic disease and cancer prevention (Figure 3D).

Understanding the extent and implications of variation in NRF2 activity

Whilst a key function of NRF2 is to maintain cellular homeostasis, the activity of the pathway varies between individuals [59] (Figure 3E). Age and genetic polymorphisms are contributing factors,

and NRF2 activity can also be influenced by disease status. For example, in addition to suppression of NRF2 activity in neurological disorders [60], downregulation of NRF2 signalling is associated with the progression of non-alcoholic fatty liver disease and steatohepatitis in mice and humans [61]. However, relatively little is known about the full extent of variation in NRF2 activity across human populations, including healthy individuals. There are multiple factors that can theoretically influence NRF2 activity, even in healthy individuals, including exercise and consumption of natural foodstuffs enriched with NRF2-activating phytochemicals. It is unclear whether such variation in NRF2 activity could impact the effectiveness of NRF2-activating therapies. For example, are individuals with low basal NRF2 activity likely to benefit more from these interventions, and is the efficacy of NRF2 activators greater in such individuals, given the potential for a larger dynamic range of pharmacological responses compared with those exhibiting high basal NRF2 activity? The greater health benefits of sulforaphane observed in some human intervention studies – for example, in obese individuals with T2D in comparison with their non-obese counterparts [62] – suggest this possibility. Is NRF2 activity low in some individuals due to factors that cannot be surmounted by small-molecule activators? Can NRF2 be stimulated to a meaningful extent in individuals with high basal NRF2 activity, or is the pathway already close to an upper limit in these cases? Is it even necessary to do this, or should the focus be on restoring impaired NRF2 signalling? Greater knowledge in these areas will help guide the optimal use of therapeutic NRF2 activators, and may highlight a need to determine an individual's 'NRF2 status' prior to/during treatment with such drugs.

Highly related to the issue of variation of NRF2 in human populations is our burgeoning appreciation of differences in the basal and inducible activity of the pathway in animals (Figure 3E). This is particularly relevant in preclinical species that are commonly used in academic research and for the evaluation of drug candidates by the pharmaceutical industry. Recently [63], rats were shown to exhibit a higher hepatic basal NRF2 activity compared with mice, along with a stronger activation of the pathway when the two species were challenged with pharmacokinetically equivalent doses of the hepatotoxin acetaminophen. By extending the analysis of the basal activity of NRF2 to normal liver samples from patients, it was found that mice better reflect the typical NRF2 activity of humans. Broadening of this work will reveal the comparative activity of NRF2 in other preclinical species, enhancing our understanding of where a given species maps on to the human spectrum of NRF2 activity. For example, does the use of rats represent only a portion of the human population that exhibit relatively high NRF2 activity? If so, what are the implications for the use of rats in preclinical safety testing of new medicines, particularly those that intentionally suppress or unintentionally cause chemical or oxidative stress, in terms of predicting clinical efficacy or toxicity? Answering these questions will inform the selection of appropriate preclinical species in these scenarios, and thus improve the efficiency of translational research and drug development.

Concluding remarks

Following the discovery of NRF2 and KEAP1 in the 1990s, the past two decades have witnessed immense progress in our understanding of the composition, regulation, and function of the KEAP1–NRF2 pathway and its interaction with other signalling networks. These advances have supported the evolution of the field into the clinic, leading to a heightened interest in NRF2 pharmacology. In this article we have highlighted some of the remaining challenges (also see Outstanding questions) that need to be addressed in order to realise the full clinical potential of NRF2 as a therapeutic target. These challenges encompass issues related to both efficacy and safety, which, as for any new medicine, must be optimally balanced to maximise patient benefit. The ultimate measure of success in addressing these challenges will be the approval of more NRF2 activators as novel therapies over the coming years.

Outstanding questions

In which disease contexts can NRF2 be modulated safely for therapeutic benefit?

How important is target specificity in determining the efficacy and safety of NRF2 activators?

Can we find a consensus approach to monitoring NRF2 activity/responses in humans?

Which preclinical species best reflect the basal/inducible activity of NRF2 in patient populations?

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Declaration of interests

A.T.D.K. is a member of the Scientific Advisory Board of Evgen Pharma, and collaborates with GlaxoSmithKline and Reata Pharmaceuticals. I.M.C. collaborates with AstraZeneca, Evgen Pharma, GlaxoSmithKline, and Merck & Co, and provides consultancy services to Korro Bio.

References

1. Yamamoto, M. *et al.* (2018) The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98, 1169–1203
2. Hayes, J.D. and Dinkova-Kostova, A.T. (2014) The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* 39, 199–218
3. Cuadrado, A. *et al.* (2019) Therapeutic targeting of the NRF2 and KEAP1 partnership in chronic diseases. *Nat. Rev. Drug Discov.* 18, 295–317
4. Kopacz, A. *et al.* (2020) Beyond repression of Nrf2: an update on Keap1. *Free Radic. Biol. Med.* 157, 63–74
5. Matzinger, M. *et al.* (2020) AMPK leads to phosphorylation of the transcription factor Nrf2, tuning transactivation of selected target genes. *Redox Biol.* 29, 101393
6. Liby, K.T. and Sporn, M.B. (2012) Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease. *Pharmacol. Rev.* 64, 972–1003
7. Yagishita, Y. *et al.* (2019) Broccoli or sulforaphane: is it the source or dose that matters? *Molecules* 24, 3593
8. Yagishita, Y. *et al.* (2020) Current landscape of NRF2 biomarkers in clinical trials. *Antioxidants (Basel)* 9, 716
9. Lynch, D.R. *et al.* (2019) Safety, pharmacodynamics, and potential benefit of omaveloxolone in Friedreich ataxia. *Ann. Clin. Transl. Neurol.* 6, 15–26
10. Lynch, D.R. *et al.* (2021) Safety and efficacy of omaveloxolone in Friedreich ataxia (MOXIe Study). *Ann. Neurol.* 89, 212–225
11. Linker, R.A. and Haghikia, A. (2016) Dimethyl fumarate in multiple sclerosis: latest developments, evidence and place in therapy. *Ther. Adv. Chronic Dis.* 7, 198–207
12. Pellacani, G. *et al.* (2022) Efficacy and safety of dimethyl fumarate in patients with moderate-to-severe plaque psoriasis: DIMESKIN-2, a multicentre single-arm Phase IIIb study. *J. Clin. Med.* 11, 4778
13. Rojo de la Vega, M. *et al.* (2018) NRF2 and the hallmarks of cancer. *Cancer Cell* 34, 21–24
14. Okazaki, K. *et al.* (2020) Enhancer remodeling promotes tumor-initiating activity in NRF2-activated non-small cell lung cancers. *Nat. Commun.* 11, 5911
15. Sayin, V.I. *et al.* (2017) Activation of the NRF2 antioxidant program generates an imbalance in central carbon metabolism in cancer. *eLife* 6, e28083
16. Ghergurovich, J.M. *et al.* (2020) A small molecule G6PD inhibitor reveals immune dependence on pentose phosphate pathway. *Nat. Chem. Biol.* 16, 731–739
17. Riess, J.W. *et al.* (2021) Phase 1 trial of MLN0128 (Sapanisertib) and CB-839 HCl (Telaglenastat) in patients with advanced NSCLC (NCI 10327): rationale and study design. *Clin. Lung Cancer* 22, 67–70
18. Srivastava, R. *et al.* (2022) The current status and future prospects for therapeutic targeting of KEAP1-NRF2 and beta-TrCP-NRF2 interactions in cancer chemoresistance. *Free Radic. Biol. Med.* 192, 246–260
19. Zhang, D.D. and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol.* 23, 8137–8151
20. Dayalan Naidu, S. *et al.* (2018) C151 in KEAP1 is the main cysteine sensor for the cyanoenone class of NRF2 activators, irrespective of molecular size or shape. *Sci. Rep.* 8, 8037
21. Suzuki, T. and Yamamoto, M. (2015) Molecular basis of the Keap1-Nrf2 system. *Free Radic. Biol. Med.* 88, 93–100
22. Zaro, B.W. *et al.* (2019) Dimethyl fumarate disrupts human innate immune signaling by targeting the IRAK4-MyD88 complex. *J. Immunol.* 202, 2737–2746
23. Sauerland, M.B. and Davies, M.J. (2022) Electrophile versus oxidant modification of cysteine residues: kinetics as a key driver of protein modification. *Arch. Biochem. Biophys.* 727, 109344
24. Liu, H. *et al.* (2008) Coordinate regulation of enzyme markers for inflammation and for protection against oxidants and electrophiles. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15926–15931
25. Ryan, D.G. *et al.* (2022) Nrf2 activation reprograms macrophage intermediary metabolism and suppresses the type I interferon response. *iScience* 25, 103827
26. Diskin, C. *et al.* (2021) 4-Octyl-itaconate and dimethyl fumarate inhibit COX2 expression and prostaglandin production in macrophages. *J. Immunol.* 207, 2561–2569
27. Schulze-Topphoff, U. *et al.* (2016) Dimethyl fumarate treatment induces adaptive and innate immune modulation independent of Nrf2. *Proc. Natl. Acad. Sci. U. S. A.* 113, 4777–4782
28. Poganik, J.R. *et al.* (2021) Wdr1 and cofilin are necessary mediators of immune-cell-specific apoptosis triggered by Tecfidera. *Nat. Commun.* 12, 5736
29. Tran, K.T. *et al.* (2019) A comparative assessment study of known small-molecule Keap1-Nrf2 protein–protein interaction inhibitors: chemical synthesis, binding properties, and cellular activity. *J. Med. Chem.* 62, 8028–8052
30. Georgakopoulos, N. *et al.* (2022) Phenyl bis-sulfonamide Keap1-Nrf2 protein-protein interaction inhibitors with an alternative binding mode. *J. Med. Chem.* 65, 7380–7398
31. Gorgulla, C. *et al.* (2020) An open-source drug discovery platform enables ultra-large virtual screens. *Nature* 580, 663–668
32. Davies, T.G. *et al.* (2016) Monoacidic inhibitors of the Kelch-like ECH-associated protein 1: nuclear factor erythroid 2-related factor 2 (KEAP1:NRF2) protein–protein interaction with high cell potency identified by fragment-based discovery. *J. Med. Chem.* 59, 3991–4006
33. Lazzara, P.R. *et al.* (2020) Isoquinoline Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 (KEAP1-NRF2) inhibitors with high metabolic stability. *J. Med. Chem.* 63, 6547–6560
34. Chen, H. *et al.* (2022) Design and characterization of a heterobifunctional degrader of KEAP1. *Redox Biol.* 59, 102552
35. Du, G. *et al.* (2022) Exploring the target scope of KEAP1 E3 ligase-based PROTACs. *Cell Chem. Biol.* 29, 1470–1481
36. Hast, B.E. *et al.* (2013) Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. *Cancer Res.* 73, 2199–2210
37. Ichimura, Y. *et al.* (2013) Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Mol. Cell* 51, 618–631

38. Holze, C. *et al.* (2018) Oxeiptosis, a ROS-induced caspase-independent apoptosis-like cell-death pathway. *Nat. Immunol.* 19, 130–140
39. Saito, T. *et al.* (2016) p62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. *Nat. Commun.* 7, 12030
40. Liu, H. *et al.* (2020) Biomarker exploration in human peripheral blood mononuclear cells for monitoring sulforaphane treatment responses in autism spectrum disorder. *Sci. Rep.* 10, 5822
41. Zimmerman, A.W. *et al.* (2021) Randomized controlled trial of sulforaphane and metabolite discovery in children with autism spectrum disorder. *Mol. Autism* 12, 38
42. Hammer, A. *et al.* (2018) The NRF2 pathway as potential biomarker for dimethyl fumarate treatment in multiple sclerosis. *Ann. Clin. Transl. Neurol.* 5, 668–676
43. Lewis, J.H. *et al.* (2021) Effects of bardoxolone methyl on hepatic enzymes in patients with type 2 diabetes mellitus and stage 4 CKD. *Clin. Transl. Sci.* 14, 299–309
44. Church, R.J. and Watkins, P.B. (2021) The challenge of interpreting alanine aminotransferase elevations in clinical trials of new drug candidates. *Clin. Transl. Sci.* 14, 434–436
45. Munoz, M.A. *et al.* (2017) Liver injury associated with dimethyl fumarate in multiple sclerosis patients. *Mult. Scler.* 23, 1947–1949
46. Tao, S. *et al.* (2018) The effects of NRF2 modulation on the initiation and progression of chemically and genetically induced lung cancer. *Mol. Carcinog.* 57, 182–192
47. Wang, H. *et al.* (2016) NRF2 activation by antioxidant antidiabetic agents accelerates tumor metastasis. *Sci. Transl. Med.* 8, 334ra351
48. Knatko, E.V. *et al.* (2021) Nrf2 activation does not affect adenoma development in a mouse model of colorectal cancer. *Commun. Biol.* 4, 1081
49. Dinkova-Kostova, A.T. *et al.* (2006) Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. *Cancer Lett.* 240, 243–252
50. Peluso, M. *et al.* (2022) Cruciferous vegetable intake and bulky DNA damage within non-smokers and former smokers in the Gen-Air Study (EPIC Cohort). *Nutrients* 14, 2477
51. Morrison, M.E.W. *et al.* (2021) Cruciferous vegetable consumption and pancreatic cancer: a case–control study. *Cancer Epidemiol.* 72, 101924
52. Pakpoor, J. *et al.* (2015) No evidence for higher risk of cancer in patients with multiple sclerosis taking cladribine. *Neurol. Neuroimmunol. Neuroinflamm.* 2, e158
53. Best, S.A. *et al.* (2018) Synergy between the KEAP1/NRF2 and PI3K pathways drives non-small-cell lung cancer with an altered immune microenvironment. *Cell Metab.* 27, 935–943 e934
54. Knatko, E.V. *et al.* (2015) Nrf2 activation protects against solar-simulated ultraviolet radiation in mice and humans. *Cancer Prev. Res. (Phila.)* 8, 475–486
55. Sharma, R.S. *et al.* (2018) Experimental nonalcoholic steatohepatitis and liver fibrosis are ameliorated by pharmacologic activation of Nrf2 (NF-E2 p45-Related Factor 2). *Cell Mol. Gastroenterol. Hepatol.* 5, 367–398
56. Ruotsalainen, A.K. *et al.* (2013) The absence of macrophage Nrf2 promotes early atherogenesis. *Cardiovasc. Res.* 98, 107–115
57. Ruotsalainen, A.K. *et al.* (2019) Nuclear factor E2-related factor 2 deficiency impairs atherosclerotic lesion development but promotes features of plaque instability in hypercholesterolaemic mice. *Cardiovasc. Res.* 115, 243–254
58. Sussan, T.E. *et al.* (2008) Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PLoS One* 3, e3791
59. Liu, J. *et al.* (2021) Sex-, age-, and race/ethnicity-dependent variations in drug-processing and NRF2-regulated genes in human livers. *Drug Metab. Dispos.* 49, 111–119
60. Quinti, L. *et al.* (2017) KEAP1-modifying small molecule reveals muted NRF2 signaling responses in neural stem cells from Huntington's disease patients. *Proc. Natl. Acad. Sci. U. S. A.* 114, E4676–E4685
61. Bathish, B. *et al.* (2022) Nonalcoholic steatohepatitis and mechanisms by which it is ameliorated by activation of the CNC-bZIP transcription factor Nrf2. *Free Radic. Biol. Med.* 188, 221–261
62. Axelsson, A.S. *et al.* (2017) Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes. *Sci. Transl. Med.* 9, eaah4477
63. Russomanno, G. *et al.* (2022) A systems approach reveals species differences in hepatic stress response capacity. *bioRxiv* Published online August 15, 2022. https://doi.org/10.1101/2022.04.13.488145
64. Zhang, Y. *et al.* (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U. S. A.* 89, 2399–2403
65. Egner, P.A. *et al.* (2014) Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: results of a randomized clinical trial in China. *Cancer Prev. Res.* 7, 813–823
66. Dinkova-Kostova, A.T. *et al.* (2005) Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc. Natl. Acad. Sci. U. S. A.* 102, 4584–4589
67. Spencer, S.R. *et al.* (1990) Induction of glutathione transferases and NAD(P)H:quinone reductase by fumaric acid derivatives in rodent cells and tissues. *Cancer Res.* 50, 7871–7875
68. Kang, Y.P. *et al.* (2021) Non-canonical glutamate-cysteine ligase activity protects against ferroptosis. *Cell Metab.* 33, 174–189
69. Ding, H. *et al.* (2021) Activation of the NRF2 antioxidant program sensitizes tumors to G6PD inhibition. *Sci. Adv.* 7, eabk1023
70. Richardson, B.G. *et al.* (2018) Replacement of a naphthalene scaffold in Kelch-like ECH-associated protein 1 (KEAP1)/nuclear factor (erythroid-derived 2)-like 2 (NRF2) inhibitors. *J. Med. Chem.* 61, 8029–8047
71. Dayalan Naidu, S. *et al.* (2022) The isoquinoline PRL-295 increases the thermostability of Keap1 and disrupts its interaction with Nrf2. *iScience* 25, 103703
72. Horie, Y. *et al.* (2021) Molecular basis for the disruption of Keap1-Nrf2 interaction via hinge & latch mechanism. *Commun. Biol.* 4, 576