TITLE: Development of Keap1-interactive small molecules that regulate Nrf2 transcriptional activity

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Abstract

There has been considerable progress recently in the discovery and development of Keap1-interactive compounds that enhance Nrf2 transcriptional activity. The compounds fall into two broad classes: electrophilic cysteine-reactive compounds and non-electrophilic Keap1-Nrf2 protein-protein interaction inhibitors. This short review highlights structures from both classes and discusses their development, biological properties and the future prospects for developing therapeutic agents. Molecules of both types have potential applications in areas including inflammatory conditions, chronic neurodegenerative diseases and possibly cancer chemoprevention.

Introduction

The activation of Nrf2 transcriptional activity has been highlighted as an attractive target for therapeutic intervention in a range of disease states spanning inflammatory and neurodegenerative conditions (including Parkinson's disease and Alzheimer's disease) and cancer chemoprevention [1]. Compounds that increase Nrf2 activity have therefore been sought as both chemical probes to evaluate the activity of Nrf2 in cells and as potential therapeutic agents. Most of the available compounds that increase Nrf2 activity do so by perturbing the behaviour of the ubiquitination facilitator protein Keap1 that targets Nrf2 for Cul3-mediated ubiquitination and destruction via a complex dynamic protein-protein interaction (Figure 1). By stalling the ubiquitination process, the compounds increase the concentration of *de novo* synthesised Nrf2 within the cell and facilitate the expression of genes with Nrf2-responsive anti-oxidant response elements (AREs) in their promoter regions. In this review we will focus upon the two main groups of Keap1-

interactive small molecules that up-regulate Nrf2 activity: those that modify cysteine residues and those that interact reversibly with the Keap1 *C*-terminal Kelch domain. We will also comment on the future potential of this therapeutic approach.

FIGURE 1.pptx

Figure 1. A and B. Schematic representation of the closed (A) and open (B) forms of the Keap1-Nrf2 complex. C and D. Electrophilic inducers result in a closed form of the complex (C) and protein-protein interaction inhibitors an open form (D). Under resting conditions (A and B) Nrf2 is ubiquitinated (in form A) and free concentrations are kept at a low level. In the case of forms B, C, and D ubiquitination is stalled and Nrf2 accumulates in the cell and translocates to the nucleus [2, 3].

Electrophilic inducers

Keap1 contains several cysteine residues in its BTB domain and intervening region that sense electrophilic and redox stress within the cell [4], and the first Nrf2 inducers to be identified were cysteine-interactive electrophilic or redox-active compounds with broad ranging biological activity [5]. By covalently modifying cysteine residues in Keap1, these compounds are able to stall the ubiquitination of Nrf2, apparently by inducing the formation of a closed non-functional form of the Keap1-Nrf2 complex (Figure 1C) [2, 6].

FIGURE 2.tiff

Figure 2: A. Selected examples of electrophilic and pro-electrophilic Keap1 inhibitors; B. Modification of Keap1 cysteine thiols by Michael acceptors; C. Reaction of SFN with Keap1 cysteine thiols.

Electrophilic inducers have been described that are of natural, semisynthetic or synthetic origin (Figure 2A). Many are Michael acceptors that contain α,β-unsaturated carbonyl functionality and undergo 1,4-addition with reactive Keap1 cysteine thiols (Figure 2B) [5]. Examples include the recently approved drug for multiple sclerosis, dimethyl fumarate **1** (Tecfidera®) [7], and the semisynthetic oleanolic acid derivative bardoxolone methyl **2** (CDDO-Me), which has been evaluated in clinical trials for the treatment of chronic kidney disease, pulmonary arterial hypertension and cancer [8]. Although both **1** and **2** have been shown to induce accumulation and nuclear translocation of Nrf2 and subsequent activation

of ARE-driven genes, their relative activities *in vitro* appear to differ considerably, with **2** being one of the most potent Nrf2 inducers identified to date [9].

The Nrf2-inducing activity of **1** is dependent on the modification of Keap1 Cys151, while on the other hand structural analogues of **2** appear to maintain their activity in cells expressing mutant Cys151S Keap1 [10]. However, a co-crystal structure of **2** covalently bound to Keap1 Cys151 within the BTB domain of Keap1 was recently reported, raising the possibility of a functional interaction with this site [11].

Curcumin **3**, a *bis*-Michael acceptor, is a further example of a reactive Nrf2 inducer [5] that exhibits both *in vitro* and *in vivo* activity [12, 13]. Despite its poor bioavailability, diverse biological activities and relatively low Nrf2-inducing potential [14], **3** has shown promising results in clinical trials for a plethora of different diseases [15]. *tert*-Butylhydroquinone **4** is an example of a pro-Michael acceptor that is bio-activated to form *tert*-butyl-*p*-quinone [16], which can undergo Michael addition reactions with reactive thiol groups [17]. It interacts with Cys151 of Keap1 and in contrast to **2**, its Nrf2-inducing activity is greatly impaired in Cys151S Keap1-expressing cells compared to wild-type cells [10].

One of the most well-characterised Keap1 electrophilic inhibitors is the isothiocyanate natural product sulforaphane **5**, which is derived from its glucosinolate precursor glucoraphanin, found in cruciferous vegetables, including broccoli and Brussels sprouts [18]. Several Keap1 cysteine residues have been reported as targets of **5** (Figure 2C) [19], however, its activity is highly dependent on Cys151 both *in vitro* [20] and *in vivo* [21]. **5** is able to induce the nuclear accumulation of Nrf2 and subsequent transcription of its associated genes at mid-high nanomolar concentrations, exerting protective effects against inflammation and oxidative stress as well as reducing cancer cell proliferation and viability [18]. In addition, it has been evaluated in animal models and clinical trials for a plethora of diseases and degenerative conditions, including but not limited to several cancer types, diabetes and neurodegenerative conditions [18, 22-25].

Despite the interesting cellular activity of the various electrophilic inducers and their adoption as chemical probes to investigate Nrf2 in cellular processes and disease models [26], their reactive nature is problematic, resulting in a range of off-target effects [27]. **5** in particular has been shown to interact directly with a variety of redox-sensitive targets [28], leading to a complicated mode of action and several Nrf2-independent effects [29-32]. Molecules with a higher selectivity for Keap1 could exhibit lower toxicity and have a better therapeutic profile, while in addition they would be more attractive chemical tools to probe

the complex biology associated with the Keap1/Nrf2 pathway. In this respect the chemical properties of **2** are particularly interesting, because its cyano-enone moiety undergoes a reversible Michael-type reaction with cysteine residues. This raises the prospect of limiting off-target effects, because the reaction with cysteine residues is reversible, but promoting Keap1 interaction via complementary non-covalent interactions at the Keap1 binding site.

Direct (non-covalent) Nrf2 inducers

Despite the interesting biological activity of electrophilic Nrf2 inducers, direct non-covalent inhibition of the Keap1-Nrf2 protein-protein interaction (PPI) has been proposed as an alternative, more selective approach to increase Nrf2 activity [5, 27, 33]. Crystallographic (Figure 3B) and mutagenesis studies have shown that Nrf2 binds to Keap1 through relatively short sequences to a well-defined pocket within the Keap1 Kelch domain and this interaction is mediated mainly by the acidic residues of the ETGE and DLG Nrf2 motifs [34-36]. The interacting surface area between Keap1 and Nrf2 is relatively small and resembles a receptor-type interaction rather than a typical PPI, suggesting that the discovery of potent PPI inhibitors is possible [33].

A number of peptide ligands have been designed and evaluated [37-39]. Those based upon hybrid sequences derived from the high affinity Nrf2 (ETGE) binding sequence and the related p62 Keap1 interaction region yielded a high affinity 7mer peptide, Ac-DPETGEL-OH (IC_{50} = 116 nM) [38] a related 11mer peptide, Ac-CLDPETGEYLC-OH had a slightly higher affinity (K_d = 15.9 nM) [39]. The cell permeability of these acidic peptides is quite poor although efforts to improve their biological activity through fatty acid [40] or TAT peptide conjugation [41] have had some success, generally yielding molecules with micromolar activity in Nrf2-dependent enzyme expression assays (NQO1 and HO1 respectively).

Small molecules

Following the success of peptide inhibitors of the Keap1-Nrf2 PPI *in vitro* several groups pursued small molecules that activate Nrf2 via this same mode of action (Figure 3A) [33]. Most of the initial hit compounds were identified using high-throughput screening approaches although rational design, fragment-based approaches and *in silico* methods have been used in recent discovery and lead optimisation studies.

FIGURE 3A.tiff

FIGURE 3B-D.pptx

Figure 3. A. Selected examples of small molecule Keap1-Nrf2 PPI inhibitors; B. Crystal structure of an Nrf2 ETGE peptide bound to Keap1 (Nrf2 ETGE residues displayed) (PDB: 2FLU); C. Crystal structure of 10 bound to Keap1 (PDB: 4XMB); D. Crystal structure of 13 bound to Keap1 (PDB: 5FNU). The Keap1 Kelch domain is shown as a surface representation, peptides and small molecules in stick representation.

The first small molecule inhibitor to be described was the tetrahydroisoquinoline LH601A **6** [42], a compound originally identified as an HTS hit from the MLPCN screening library (337,116 compounds). The (*S*,*R*,*S*) configuration of **6** was optimal for Keap1 binding (K_d = 1.0 µM, fluorescence polarisation assay) and it induced the nuclear translocation of Nrf2 in cells with an EC_{50} of 12 µM [42]. The compound up-regulated NQO1, HO-1 and TRX1 at mid-high micromolar concentrations in HEK293 cells but had limited effect on glutathione-related genes [43]. Interestingly, in the same study, sulforaphane **5** enhanced levels of GCLC (glutamate cysteine ligase, catalytic), GCLM (glutamate cysteine ligase, modifier) and GSR (glutathione reductase) in addition to NQO1, HO-1 and TRX1. Structural insights into the binding behaviour of **6** were obtained from a co-crystal structure with Keap1, but significant improvements in binding affinity were difficult to achieve [44]. An analysis of the DMPK properties of **6** showed that it was restricted to the peripheral compartment, due in part to it being a P-gp substrate. Attempts were made to obtain *in vivo* brain exposure, but binding affinity was lost [44].

Two sulphonamide compounds were identified through an HTS screen conducted using the Evotec Lead Discovery library (267,551 compounds) supplemented with 1,911 compounds, previously identified by virtual screening [45]. The benzenesulfonyl pyrimidone **7** and the symmetrical naphthalene *bis*-sulfonamide **8** had IC_{50} values of 118 µM and 2.7 µM respectively in a confocal fluorescence anisotropy assay. Compound **8** stabilised Nrf2 and up-regulated NQO1 expression at low-mid micromolar concentrations [45]. Attachment of acetate side chains to the sulfonamide nitrogen atoms of **8** had a profound effect on the overall activity of the compound series, yielding a symmetrical *bis*-acid **9** with an IC_{50} of 28.6 nM [46]. Molecular docking simulations demonstrated that the acetic acid moieties mimic the two glutamate side chains of the high-affinity ETGE motif of Nrf2 and interact with Arg483 and Arg415 in the Keap1 binding pocket. Co-crystallisation of a related *bis*-acetamide compound **10** confirmed the proposed binding

mode (Figure 3C) [47]. Despite the presence of two ionisable groups, **9** was able to activate the expression of Nrf2-dependent gene products at low μM concentrations [46]. A follow-up study revealed that a *p*-acetamido analogue of **11** had improved solubility and cellular activity in an Nrf2-dependent ARE luciferase assay at concentrations as low as 100 nM. *In vivo* a dose of 10 mg/kg was sufficient to reduce the levels of inflammatory markers in an LPS-induced inflammation model without exhibiting acute toxicity (Table 1) [48].

The *bis*-sulfonamide structure has been de-symmetrised in several studies [47, 49], one of which resulted in RA839, **12** which inhibited the Keap1-Nrf2 PPI with an FP IC_{50} of 140 nM and an ITC K_d of 6 μM. The compound had promising cell-based activity, but was found to be subject to oxidative metabolism, which hampered *in vivo* evaluation [49].

Cpd	Keap1 K_D (ITC)	Disease model	Treatment	Disease relevance	Outcome	Ref
6	39.8 nM	LPS-induced model of inflammation (mouse)	10 mg/kg/day or 80 mg/kg/day for 3 days before LPS challenge	Inflammatory conditions	Prophylactic treatment relieves LPS-induced inflammation: ↓ cytokine levels (TNF-α, IFN-γ, IL-6, IL-12, IL-17)	[48]
6	39.8 nM	DSS-induced model of UC (mouse)	10 mg/kg or 40 mg/kg (oral) simultaneous administration with 3% w/v DSS 8 days on 8 days off (x4 cycles)	UC, Crohn's disease, inflammatory bowel disease, colorectal cancer	Protects against DSS-induced damage: elimination of inflammatory cells, preserved colonic structure, prevented of lamina propria expansion, ↑ colonic GSH:GSSG ratio	[50]
8	1.3 nM	Ozone (O_3)-induced rat model of pulmonary inflammation	16.5 mg/kg (iv) for 6 hours 24 hours before ozone injury: 1 ppm for 3 hours.	COPD	Attenuation of pulmonary inflammation: ↓ levels of inflammatory cells in the bronchoalveolar fluid, preserved levels of GSH, ↓ oxidative stress	[51]
10	NA	CDDA rat model of NASH	6 weeks pre-feeding of CDAA diet followed by 4 weeks 60 mg/kg/day administration	NASH	Treatment prevented progression of established fibrosis in NASH model rats liver: ↓ fibrosis score, ↓ % fibrosis area	[52]

Table 1: *In vivo* disease model studies with Keap1-Nrf2 protein-protein interaction inhibitors. Notes: NA – data not available; LPS – lipopolysaccharide; DSS – dextran sodium sulfate; UC – ulcerative colitis; COPD – chronic obstructive pulmonary disease; CDDA – choline deficient, L-amino acid defined; NASH – non-alcoholic steatohepatitis.

Recently, Astex and GSK identified a high affinity lead compound through a crystallographic fragment-based screen. Linkage of three fragments with orthogonal Keap1 binding behaviour followed by SAR-based optimisation yielded the mono acid **13** (K_d 1.3 nM) (Figure 3D). The compound induced the expression of a range of Nrf2 target genes (NQO1, HO1, glutamate-cysteine ligase, thioredoxin reductase-1) at low nanomolar concentrations. Subsequently the compound was shown to exert protective effects in an *in vivo* COPD model (Table 1) [51].

A further compound to emerge from Toray Industries was the urea derivative **14** [53]. Although its binding affinity for Keap1 has not been described, it has been co-crystallised with the Keap1 Kelch domain. The related pyridine derivative **15**, although a relatively weak Keap1 ligand (Keap1-Nrf2 PPI inhibition > 50 μM), exerts protective effects in a rat model of non-alcoholic steatohepatitis (Table 1) [52].

Amongst the non-electrophilic Nrf2 inducers a class of 1,4-diphenyl-1,2,3-triazoles has been studied in detail with regard to their effects on cellular functions associated with mitochondrial autophagy (mitophagy) and Parkinson's disease. Prototype compounds such as **16** and **17** inhibit the Keap1-Nrf2 interaction in live cells and increase the expression of Nrf2 target genes [3]. A direct comparison between **17** and sulforaphane **5** indicated differences in cellular effects between the two types of inducer. In particular, **17** was able to promote mitophagy without an apparent effect on mitochondrial membrane potential or function, an activity that was partially independent of the PINK1/Parkin mitophagy pathway [54, 55]. **5** was less able to induce these effects, suggesting that there may be different therapeutic applications for non-electrophilic ligands, particularly in the area of Parkinson's disease where PINK1 and Parkin play a role in hereditary and early onset forms of the condition [56].

Future perspectives

There has been considerable recent progress in the development of compounds that induce Nrf2 activity for potential therapeutic applications. The high-potency bardoxolone methyl-type inducers are examples of promising electrophilic compounds with an intriguing reversible covalent binding mode. They have shown therapeutic efficacy in a range of disease settings although their therapeutic window and toxicities need further research after their recent failure in a phase 3 clinical study for chronic kidney disease

[57]. Reversible inhibitors of the Keap1-Nrf2 PPI have been developed recently. Despite the difficulty of targeting protein-protein interactions, several structural classes of inhibitors have been identified that bind to Keap1 with high affinity. The potential for these compounds to exert different effects of Nrf2, both in magnitude and timescale, and different pharmacological effects on treated cells suggests that they may have a different range of therapeutic applications, particularly in the area of neurodegenerative diseases. However, in order to progress to *in vivo* studies for CNS applications the current pool of lead compounds will require further optimisation in order to achieve the physicochemical properties required for crossing the blood-brain barrier.

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Figure 1

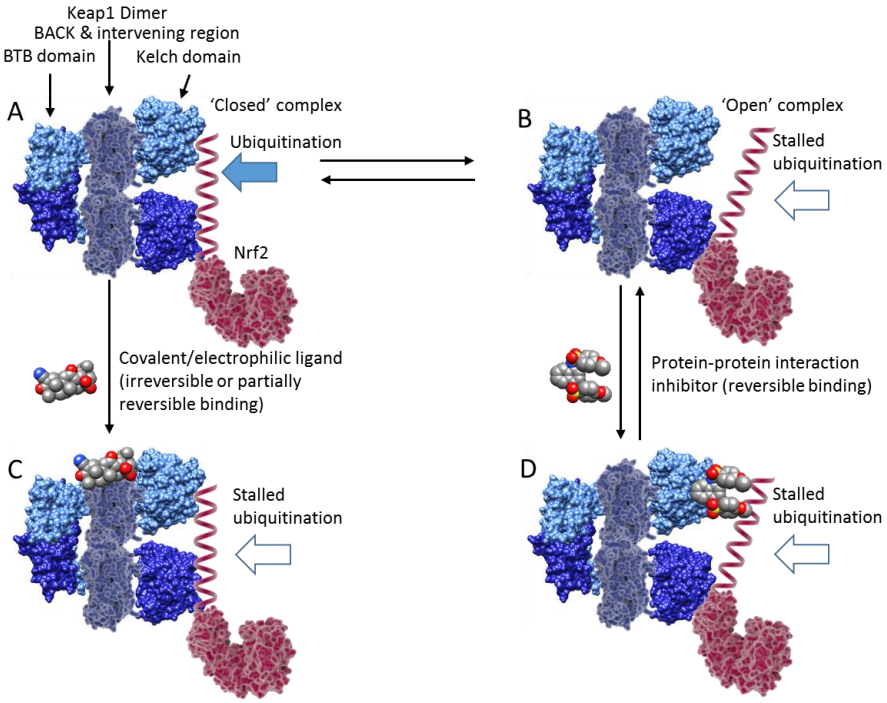


Figure 2

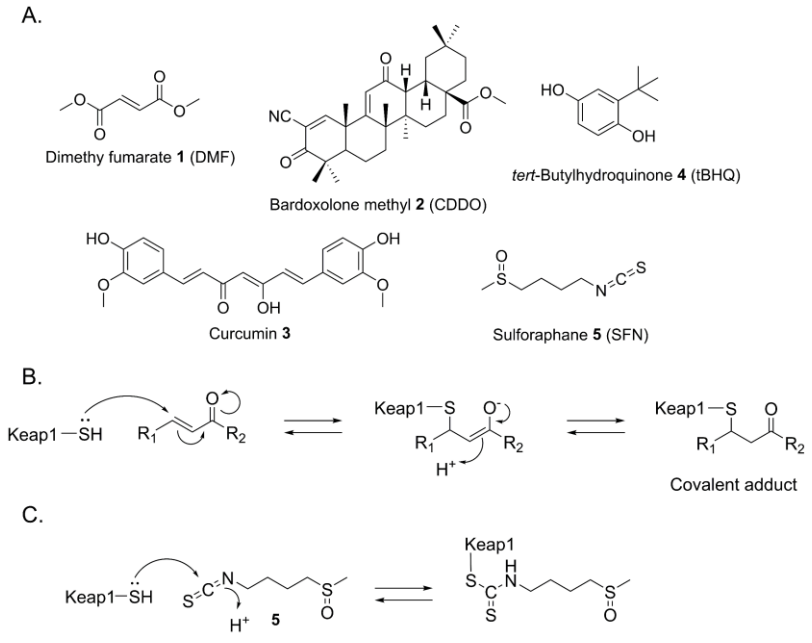

A.
Dimethy fumarate 1 (DMF)
Bardoxolone methyl 2 (CDDO)
tert-Butylhydroquinone 4 (tBHQ)
Curcumin 3
Sulforaphane 5 (SFN)
B.
Keap1—SH
Keap1—S
Covalent adduct
C.
Keap1—SH
H+
5
Keap1

Figure 3A

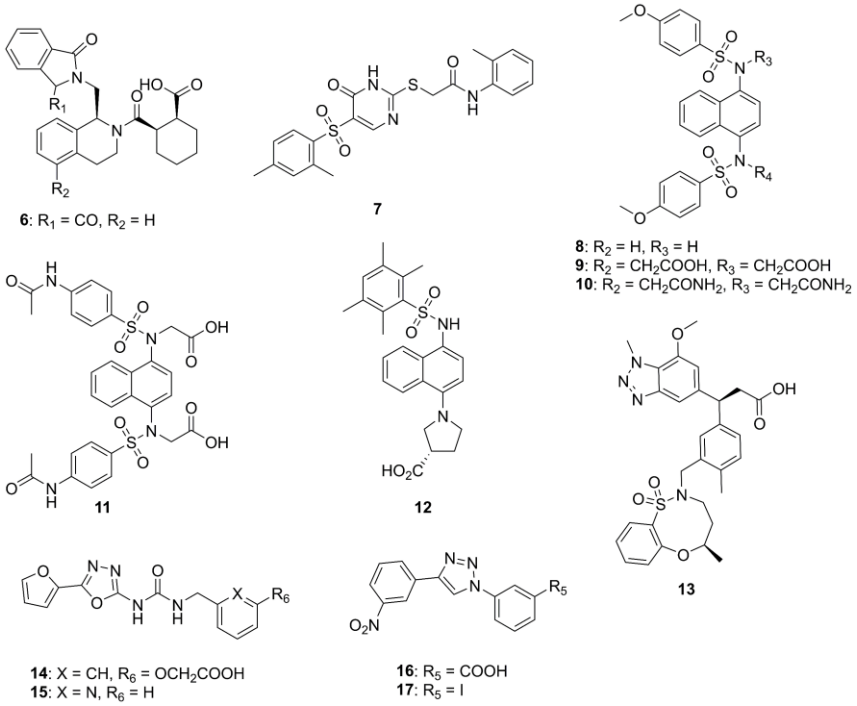
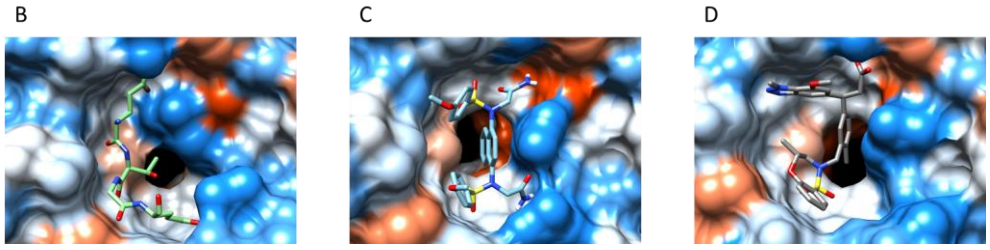


Figure 3B,C,D



Graphical Abstract