Keap calm – and carry on covalently

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Abstract

The Nrf2-Keap1 system plays a major role in cellular defence against oxidative stress. Upon exposure to electrophiles, the cysteine-rich protein Keap1 is covalently modified, and it is this modification of Keap1 that allows the accumulation and subsequent nuclear translocation of Nrf2 where it induces the transcription of over one hundred protective genes. This mechanism can be exploited in drug discovery approaches to diseases such as chronic kidney disease (CKD), COPD, asthma, and neurodegenerative diseases like multiple sclerosis (MS) and Parkinson's utilizing the modification of Keap1 by electrophiles, compounds that would not normally be considered useful in drug discovery programmes. This Perspective discusses the development of potential therapies based on potent electrophiles, such as isothiocyanates and Michael acceptors, that far from being associated with toxic events, can actually initiate a range of beneficial protective pathways.

The Keap1-Nrf2 pathway

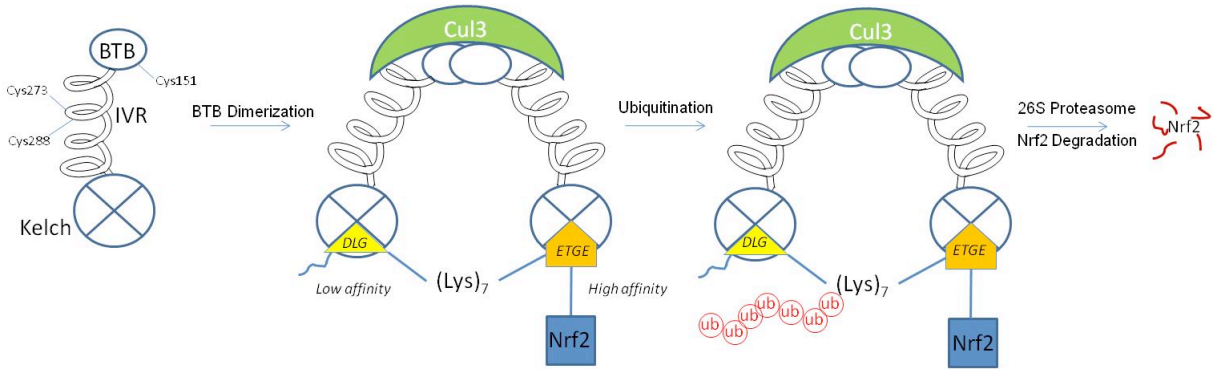
The Nrf2 (NF-E2-related factor 2) transcription factor plays a key role in cellular stress response mechanisms, and may be important in a number of disease processes. In response to both oxidative and electrophilic alkylative stress, Nrf2 up-regulates phase II gene products aimed at detoxification and cytoprotection including glutathione synthesis,[1] reactive oxygen species (ROS) scavenging, and drug metabolism and transport. Among the many genes and gene products upregulated by Nrf2, NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1) serve as common biomarkers. Heme oxygenase 1 (HO1) is also regulated by the Bach1-MafK repressor complex. Binding of heme to Bach1 releases the complex from DNA allowing Nrf2 mediated transcription. Thus release of the Bach1 complex from DNA is important for transcriptional activity of Nrf2. Importantly, Bach1 is induced by Nrf2 providing an inhibitory feedback mechanism.[2-4]

Nrf2 is a nuclear protein and is dynamically regulated. However, unlike many transcription factors the major path of activation is not controlled by a kinase. Rather, under basal conditions, Nrf2 is associated with the cysteine-rich Keap1 (Kelch-like ECH-associated protein 1) protein that is thought to shuttle Nrf2 from the nucleus to the cytoplasm.[5] Keap1 is central in orchestrating the ubiquitination of Nrf2 through its association with Cul3 (Cullin E3 ubiquitin ligase). Ubiquitination of Nrf2 and subsequent proteasomal degradation keeps Nrf2 at very low concentrations under non-stressed conditions. It is estimated that the half-life of Nrf2 under basal conditions is less than 20 minutes.[6]

Keap1, the scaffolding protein that organizes the ubiquitination event, is comprised primarily of five domains – (1) NTD (1-60 - N-terminal domain), (2) BTB (61-178 - Broad complex, Tramtrack, and Bric-a-Brac), (3) IVR (179-321 - intervening region), (4) DGR (322-608 - double glycine repeat or Kelch), and (5) CTR (609-625 - C-terminal region). A subset of the 27 cysteine

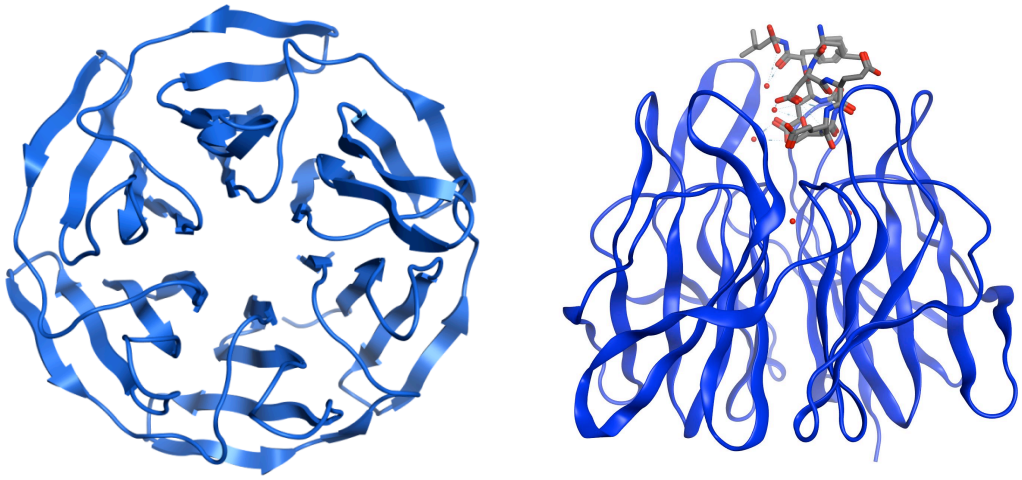
residues found in human Keap1 has been found to be more reactive to electrophiles and act as sensors regulating the levels of Nrf2. Evidence for this hypothesis was first reported by Dinkova-Kostova *et al*. where they highlighted Cys257, Cys 273, Cys288 and Cys297,[7] while a subsequent publication implicated Cys151, Cys273, and Cys288 as important for stress sensing and Nrf2 activation.[8] Though the role of the cysteine residues in Keap1 has yet to be fully elucidated, chemoselective reactivity of various agents for different Keap1 cysteine residues has been demonstrated.[9, 10]

Keap1 dimerizes through the N-terminal BTB domain.[11] While an X-ray crystal structure of full length Keap1 has not been solved, a 20 Å EM structure has elucidated a cherry-bob like structure.[12] The BTB region provides an interaction surface for Cul3, the E3-ligase that targets Nrf2 for ubiquitination.[13, 14] At the C-terminus of Keap1, the DGR or Kelch domain interacts with Nrf2 through a high (Nrf2 ETGE) and low (Nrf2 DLG) affinity interaction resulting in a complex with 1:2:1 (Nrf2:Keap1:Cul3) stoichiometry.[15, 16] The stoichiometry of the protein complex has recently been challenged by a model built from crystallographic analysis that suggests a 1:2:2 ratio of Nrf2:Keap1:Cul3.[14] An α-helical structure spans the ETGE and DLG regions and contains seven lysine residues, the targets of Cul3 (Scheme 1).[17]



Scheme 1. Mechanism of Nrf2 degradation.

Though an X-ray structure of full length Keap1 has not been reported, the DGR or Kelch domain of mouse and human Keap1 has been crystallized and high resolution structures have been determined (Figure 1).[18] These Kelch repeats, common in protein-protein interactions, form a familiar β-propeller tertiary structure. In the context of Keap1, the Kelch domain contains arginine residues that form an ionic interaction with the acidic ETGE and DLG motifs in Nrf2 with the affinity of the ETGE motif being ~100 fold greater than the DLG motif.[15, 16]



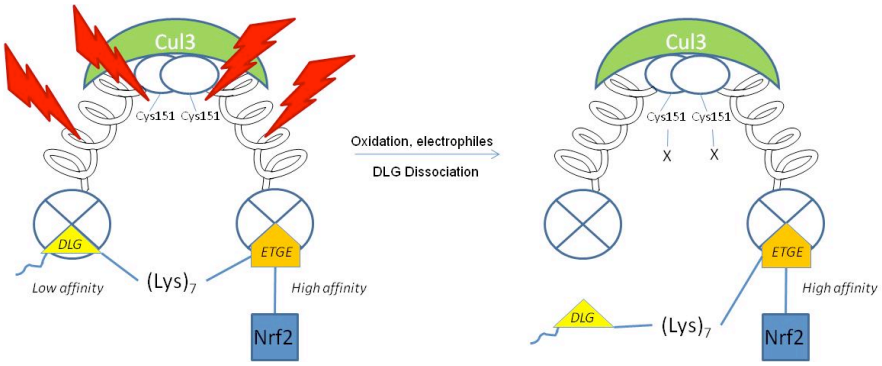
A **B**

Figure 1. **A.** X-ray crystal structure (PDB 1u6d) of human Keap1 DGR or Kelch Domain;[19] **B**. X-ray crystal structure (PDB 1x2r) of human Keap1 with LDEETGEFL.[18]

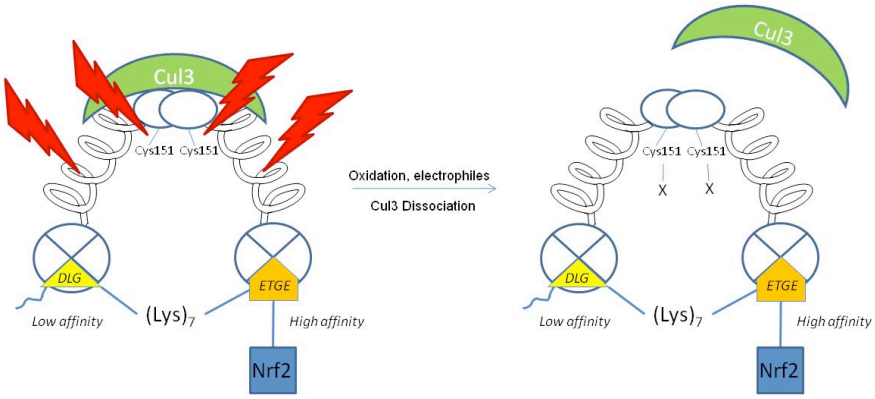
The different affinity of the ETGE and DLG motifs of Nrf2 with Keap1 give rise to a hinge-latch mechanism (Scheme 2A) with the ETGE forming the hinge and the DLG the latch.[16] In this mechanistic hypothesis, interruption of DLG binding after reaction of electrophiles with the Keap1 protein is sufficient to block ubiquitination. While Nrf2 may remain anchored to Keap1

through the ETGE motif, newly synthesized Nrf2 is transcriptionally active. A second and distinct mechanistic hypothesis involves dissociation or alteration of the Cul3-Keap1 binding after reaction with electrophiles (Scheme 2B). This effectively inhibits the ubiquitination and subsequent degradation of Nrf2 allowing it to accumulate. Support for this scheme comes from an examination of Cul3 binding affinity for a series of Keap1-Cys151 mutants where partial molar volume plays an important role.[20]

A



B



Scheme 2. A, Hinge and latch mechanism; **B**, dissociation or alteration of Cul3 binding mechanism.

Electrophiles as drugs

The fact that Nrf2 can be activated by electrophiles that form a covalent bond to a cysteine residue in the target Keap1 protein prompts a wider discussion of the use of electrophiles as drug candidates. Traditional 'wisdom' advocates that electrophilic compounds that act as irreversible enzyme inhibitors make unsuitable drugs because the covalent binding might lead to general toxicity through off target effects. In addition their electrophilic nature renders them highly susceptible to attack by cellular nucleophiles such as glutathione, and hence the compounds are often metabolically unstable with very short half lives. Therefore molecules that contain electrophilic centers are often discarded from medicinal chemistry discovery programs, and the electrophilic motifs are referred to as structural alerts or toxicophores.[21] Some well known examples of such structural alerts are shown in Figure 2. However, it is noted that such electrophiles need not necessarily act irreversibly, since it is highly mechanism dependent, Michael (conjugate) addition, for example, being well known to be highly reversible. This begs the question as to whether a fully reversible covalent modifier is actually any different to a non-covalent inhibitor.[22, 23]

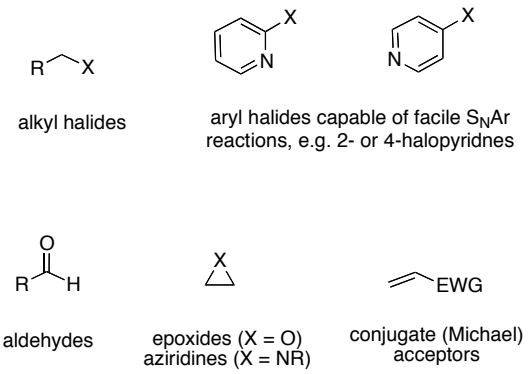
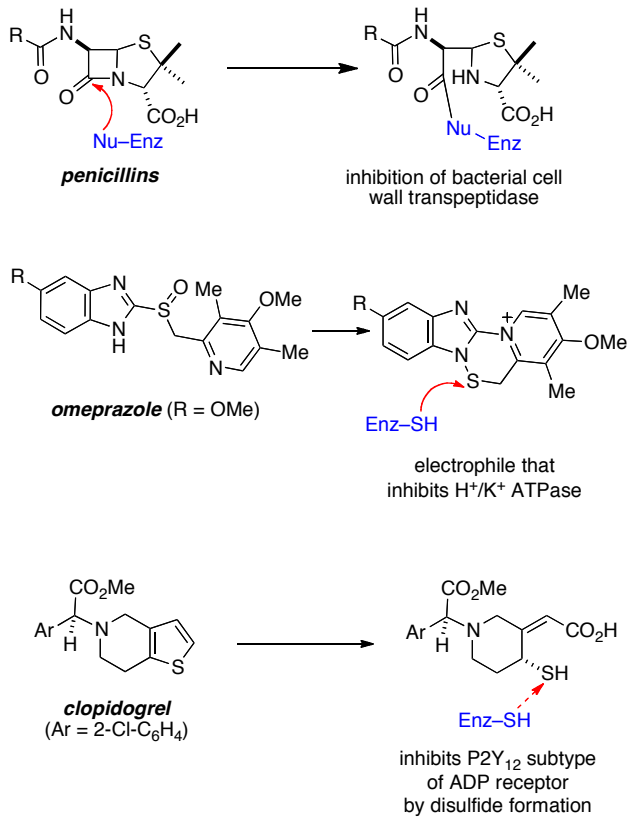
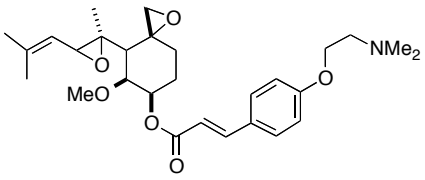


Figure 2. Structural alerts for electrophilic reactivity.

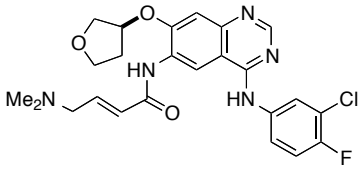
Drugs that function by a covalent mechanism are already widely used,[24, 25] many based on electrophilic natural products.[26] Some of these clinically used drugs are inherently electrophilic such as β-lactam antibiotics, or can generate electrophilic intermediates through metabolism. More modern 'targeted covalent inhibitors' are somewhat different as outlined in an excellent recent review,[27] and a recent Perspective in this *Journal* discusses irreversible kinase inhibitors.[28] Three well known examples of clinically used drugs that operate by a covalent mechanism are shown in Scheme 3, whilst some recent compounds containing electrophilic centers that are currently in clinical trials are shown in Figure 3.



Scheme 3. Clinically used drugs that operate by a covalent mechanism by being inherently electrophilic (β-lactams) or by generating an electrophile upon metabolism.



beloranib
inhibitor of methionine aminopeptidase 2
(obesity)



afatanib
inhibitor of EGFR kinase
(cancer)

Figure 3. Two examples of covalent drugs in clinical trial. Electrophilic centers are the epoxides and α,β-unsaturated carbonyl groups.

Electrophilic Modifiers of the Keap1 pathway

The Keap1-Nrf2 pathway has been described as an *in vivo* sensor for electrophiles,[29] and although the pathway also responds to exposure to hydrogen peroxide and certain heavy metals such as cadmium chloride, electrophilic groups are often found on molecules which modulate this pathway. Thus dimethyl fumarate (DMF) **1** and monomethyl fumarate (MMF) **2** are both activators of Nrf2. They have been shown to react with glutathione and *N*-acetyl cysteine,[30] and importantly, MMF reacts with Cys151 of the Keap1 protein.[31] Bardoxolone methyl **5**, a reactive cyanoenone and the methyl ester of CDDO **4**, a derivative of the natural product oleanolic acid **3** (Figure 4), is also a potent activator of Nrf2 and like the fumarates is thought to act via reaction with Cys151 of the Keap1 protein, though that notion has recently been challenged.[32, 33] In addition to these compounds, other electrophilic modulators of the Keap1-Nrf2 pathway are known including curcumin, 1,2-naphthoquinone, 4-hydroxy-2-nonenal and ebselen (Figure 4).

However, perhaps one of the most interesting of the compounds known to activate Nrf2 is sulforaphane **6**, a naturally occurring isothiocyanate isolated from cruciferous vegetables.

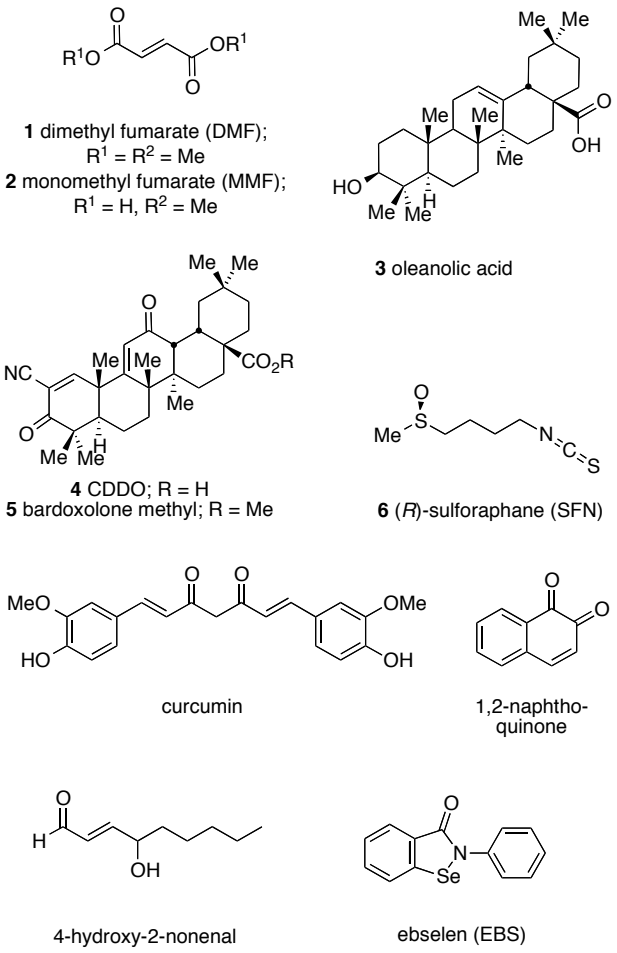


Figure 4. Known electrophilic inducers of the Keap1-Nrf2 pathway.

Many assay systems have been developed for the study of Nrf2 pathway activators. A summary of these is shown in Table 1. The current suite of available assay technologies, some of which are available as commercial kits, allows the discovery scientist to efficiently identify activators, confirm mechanism of action *in vitro*, and demonstrate pathway engagement *in vivo* in a tissue of interest. As such, the tools are available for robust drug-discovery efforts.

Table 1. Summary of assays for evaluating Nrf2 activators.

Assay Type	*Readout*	*Notes*	*Representative references where the assay was used*
Mass spectrometry	MALDI-TOF	Covalent Keap1 modifying agents	[34, 35]
Gene expression	mRNA RT-PCR amplification HO1, NQO1, GCLM, etc.	Cellular or tissue	[36]
Anti-oxidant response element (ARE) luciferase	(ARE)-dependent gene expression	Cellular assay	[36-38]
Enzymatic assay of Nrf2 dependent protein	NQO1, HO1, etc, enzymatic activity	Cellular assay	[39, 40]
Nrf2 siRNA	mRNA RT-PCR amplification HO1, NQO1, GCLM, etc	Confirm on-target activity with Nrf2 activator	[41]
Nrf2 luciferase fragment complementation	Luciferase complementation detecting Nrf2-MafK binding	Assess binding of Nrf2 to MafK	[42]
IFNγ NO production	Griess reagent detection of NO production	Used in describing the SAR of CDDO analogs	[43]
Nrf2 translocation	β-galactosidase enzyme fragment complementation	Cell based functional assay	[44]
Fluorescence polarization (FP)	Nrf2 peptide binding	Screening for Keap1-Kelch domain binders	[45]
in vivo tissue activity	Nrf2 dependent gene expression and enzymatic activity	Confirmation of *in vivo* activation of the Nrf2 pathway	[38]

Isothiocyanates: The Sulforaphane Story

Consumption of cruciferous vegetables and in particular broccoli has long been associated with a range of effects beneficial to health, including the prevention and treatment of certain types of cancer.[46] However there is not a single compound responsible for these effects, rather they are

attributed to a range of different compounds, including indoles and glucosinolate-derived degradation products such as sulforaphane **6**,[47, 48] all of which have distinct physiological modes of action. Articles in the mainstream media commonly attribute the beneficial effects of these foods as being due to the 'anti-oxidant properties' whilst food items having higher levels of these beneficial compounds are often branded as 'super-foods'.[49]

The natural occurring compound glucoraphanin is found in many cruciferous vegetables including broccoli, brussel sprouts, cabbage, cauliflower, mustard, watercress and radish, occurring in particularly high levels in the young shoots of broccoli and cauliflower. Glucoraphanin is converted by the enzyme myrosinase into sulforaphane and raphanin upon mechanical damage to the plant tissue, such as that caused by chewing.[50] The conversion involves myrosinase mediated cleavage of glucose, followed by a Lossen-type rearrangement of the aglycone, with loss of sulfate, to generate the isothiocyanate. The production of this strong tasting, isothiocyanate compound is believed to be a defense mechanism against predation by herbivores (host-plant resistance).[51, 52] However sulforaphane is not the only isothiocyanate containing compound found in nature, since a range of similar compounds is found in related species (Figure 5). The bitter taste of uncooked brassica vegetables and members of the mustard family is partly due to the products of myrosinase activity on a range of glucosinolates that produces a range of isothiocyanate containing compounds, often referred to as the mustard oils. These reactive isothiocyanates are also produced by the enzymatic activity of myrosinase on glucosinolate natural products commonly found in virtually all plants of the *Brassicales* order and many members of the *Drypetes* genus. Notable examples include allyl isothiocyanate, produced from the glucosinolate sinigrin and found in high levels in black mustard, brown Indian mustard, horseradish and wasabi,[53] and benzyl isothiocyanate, high levels of which are found in

garden cress, papaya[54] and common brassica vegetables.[55] Benzyl isothiocyanate is a product of the hydrolysis of glucotropaeolin, but in addition to the beneficial effects typical of this class of compound, it has more toxic properties and in particular genotoxic effects.[56] Phenethyl isothiocyanate is one of the more studied compounds; it is found in high levels in watercress and is produced from gluconasturtiin in an identical fashion to the other isothiocyanate compounds discussed above. Like sulforaphane itself, phenethyl isothiocyanate has been studied for its beneficial chemopreventitive effects.[57-60] Whilst it may appear that the range of glucoraphanin natural products exist simply to serve as a reservoir for the corresponding activated isothiocyanate product, it should be noted that the glucosinolate compounds themselves exhibit a range of biological effects. Not all glucosinolates breakdown into the isothiocyanate.[61] Glucobrassicin, found in almost all of the cruciferous plants, would be expected to be converted into 3-indolylmethyl isothiocyanate by myrosinase. However this compound has never been detected or isolated, presumably because of its instability.

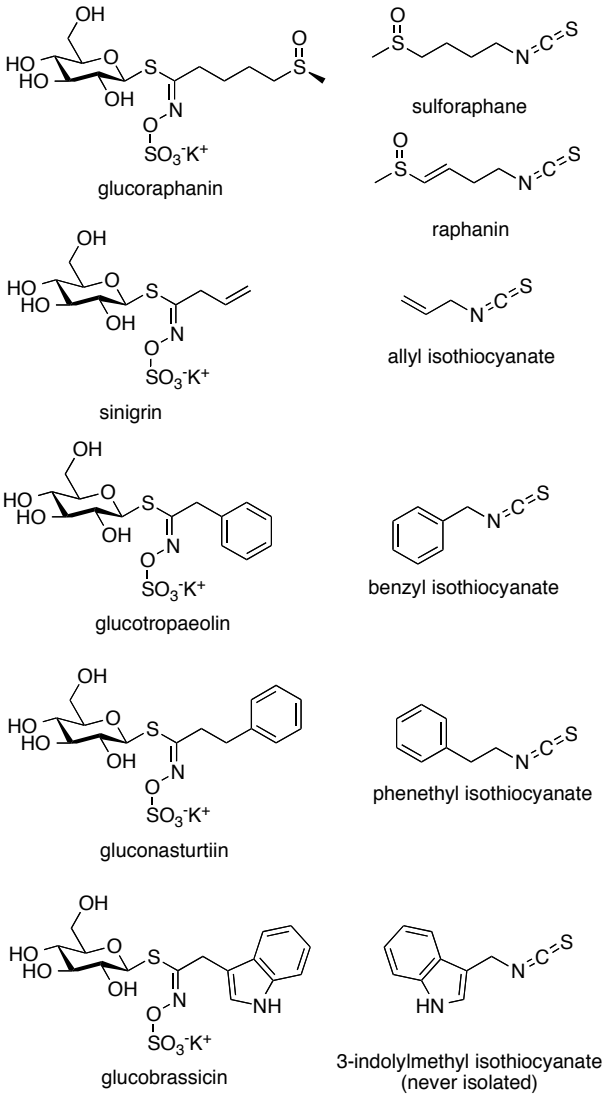


Figure 5. Structures of glucosinolates from cruciferous vegetables and the isothiocyanates formed by their myrosinase mediated degradation.

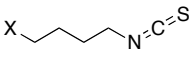
Consumption of broccoli sprouts has been shown experimentally to inhibit the growth of *Helicobactor pylori*,[62, 63] with sulforaphane being at least one of the compounds responsible for this effect.[64] Sulforaphane has also demonstrated a range of protective effects against oxidative stress,[65] and the natural broccoli extract is currently undergoing a number of investigations for use as a chemopreventative agent,[66] including several clinical trials.[67] The chemopreventative

effects of extracts containing compounds such as sulforaphane **6** are also attributed to the induction of a range of cytoprotective genes via the Nrf2 pathway,[68] as discussed previously.

Chemical synthesis work on sulforaphane **6** may be divided into two areas; early work consisted of classical SAR type investigations that explored the modifications that are possible to the sulfoxide group and the carbon backbone whilst retaining the reactive isothiocyanate, whilst more recent work has looked at the possibility of replacing the isothiocyanate group with other less reactive, and therefore more drug-like groups. An example of each approach is briefly discussed below.

In 1994 Posner *et al*. reported the synthesis and activity of a small series of linear sulforaphane analogues. This work showed that replacement of the sulfoxide with a range of other polar groups (nitrile, carboxylic acid *etc*) led to a gradual reduction in activity (as measured by the quantity of drug required to double the activity of NQO1 in murine hepatocyte cells) and substitution with an uncharged group led to a dramatic decrease in activity (Table 2). The crucial finding from this early work was that replacement of the sulfoxide group with a simple ketone resulted in a compound which retained the biological activity of sulforaphane itself.[69]

Table 2. Activity of linear analogues of sulforaphane, replacement of the sulfoxide group.[69]



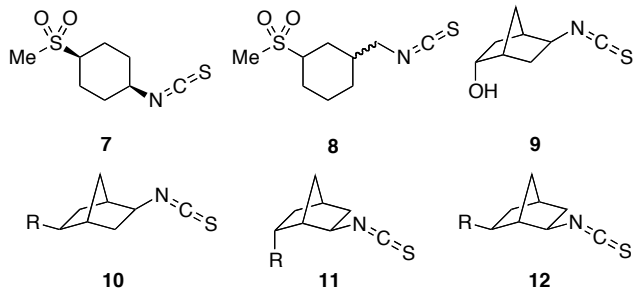
X	CD[a] (μM)	X	CD[a] (μM)
Et	15.0	MeOOC	2.8
$CH_3S(O)$ (SFN, **6**)	0.2	MeSCO	2.8
NC	2.0	MeCO	0.2

HOOC	2.2	*n*-BuCO	2.0
		$Me_2P(=O)$	0.4

[a] CD = Inducer potency for NQO1 in murine hepatoma cells.

Also examined was the effect of replacing the simple alkyl chain connecting the two functional groups with a more complex and rigid ring system. The data from these more structurally diverse backbones show the same trend as observed with the linear analogues with respect to the nature of the polar group (Table 3), and appear to suggest that the distance between the reactive isothiocyanate group and the polar group has more of an effect on activity than the shape and size of the connecting linker. This ties in with the knowledge that the existing longer chain, 6-methylsulfinylhexyl isothiocyanate (naturally occurring in wasabi) is also a potent inducer of an oxidative stress response.[70]

Table 3. Activity of cyclic analogues of sulforaphane and replacement of the sulfoxide group.[69]



7 **8** **9**

10 **11** **12**

Compound	R	CD (μM)	Compound	R	CD (μM)
7		0.4	**10e**	MeOOC	0.7
8*cis*		0.4	**10f**	MeCH(OH)	0.5

8*trans*		0.5	**11a**	$MeSO_2$	1.0
9		19	**11b**	MeCO	0.8
10a	$MeSO_2$	0.7	**12a**	$MeSO_2$	0.2
10b	MeCO	0.4	**12b**	MeCO	0.3
10c	NC	0.6	**12c**	MeOOC	1.6
10d	NO_2	1.1			

In their 2010 publication, Cole *et al.* described their efforts into 'electrophilic tuning' of the chemoprotective effects of sulforaphane.[71] Their work exploits the fact that the reaction between sulforaphane and cysteine residues in Keap1, which initially forms a dithiocarbamate functional group, is reversible. By replacing the highly reactive isothiocyanate group with a series of sulfoxythiocarbamate groups (Figure 6), they were able to produce a new family of compounds that still selectively target cysteine residues in proteins, but which now form stable thiocarbamate adducts, presumably irreversibly. They also demonstrate that these analogues label Keap1 on a number of key cysteine residues, as well as other cellular proteins. All of the sulfoxythiocarbamates synthesized were found to be inducers of NQO1, with the benzyl bearing compounds being particularly active (as is observed with benzyl isothiocyanate compared to linear isothiocyanates). The rate of reaction of compounds **13**, **14** and SFN with β-mercaptoethanol were compared, and the sulfoxythiocarbamates were found to have rate constants approximately 70 fold slower than for sulforaphane itself, supporting the idea that these compounds react as significantly weaker electrophiles.

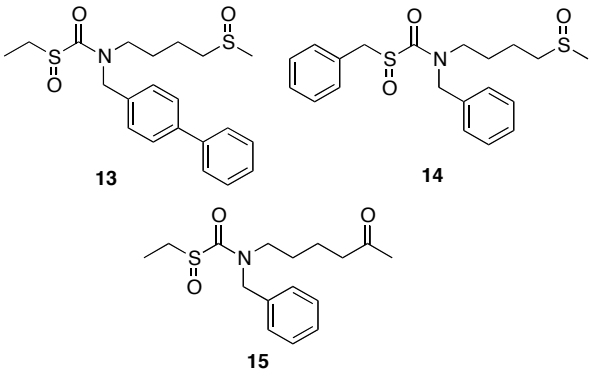
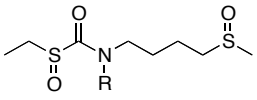


Figure 6. Sulfoxythiocarbamate activators of Keap1.

Several of the sulfoxythiocarbamate activators were assayed for their ability to induce NQO1 in a range of cell lines (Table 4). Their toxicity was also determined by measuring cell survival based on protein concentration.

Table 4. Induction potency (NQO1) and toxicity of sulfoxythiocarbamate analogues in human murine hepatoma cells.[71]



Compound	R	CD (μM)	LD_{50} (μM)
16a	Me	94	>200
16b	Pr	35	>200
16c	*c*-Pr	43	>200
16d	*c*-Hex	64	>200

16e	Ph	43	>200
16f	Bn	13	>200
16g	CH_2(1-naphthyl)	5.3	>50
16h	CH_2(4-*t*-BuC_6H_4)	3.8	32
16i	CH_2(4-PhC_6H_4)	2.8	16
16j	CH_2(2,4-F_2-C_6H_4)	7.3	>50

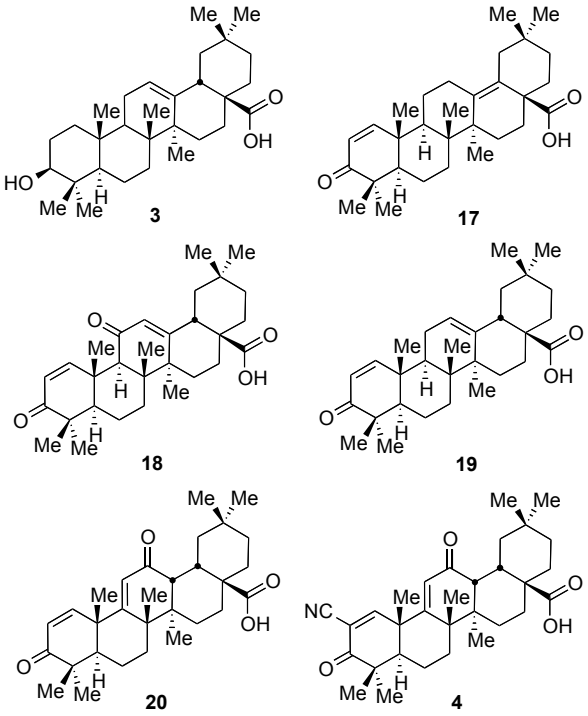
Michael acceptors

Various synthetic, semi-synthetic and natural compounds containing α,β-unsaturated ketones and esters have been shown to activate Nrf2 through reaction of one or more cysteine residues in Keap1. A distinguishing feature of these compounds is that the conjugate addition of an alkyl thiol to an α,β-unsaturated system is freely reversible with the kinetics dependent on the electronic nature of the Michael donor and acceptor, pH, temperature and, in the case of a protein, the local environment of the alkyl thiol, i.e. cysteine residue. In the case of Nrf2 activation, two compound classes containing a Michael acceptor have been evaluated in clinical trials, a structurally very simple α,β-unsaturated ester, dimethyl fumarate **1** (formulated as BG-12, Tecfidera), for multiple sclerosis (MS) and the α,β-unsaturated ketone containing triterpenoid, bardoxolone methyl ester (CDDO-Me) **5**, for chronic kidney disease.

The CDDO **4** triterpene template originated from the natural product, oleanolic acid **3**, isolated from a number of medicinally active plants. Sporn *et al.* published a series of papers on the optimization of the anti-inflammatory activity of analogues of oleanolic acid utilizing inhibition

of IFN-γ induced nitric oxide (NO) production in murine macrophages.[72, 73] The initial paper described the improved inhibition of NO production shown in analogues containing an α,β-unsaturated system in either the A or A and C rings of the triterpene template.[72] A subsequent communication described the further significant improvement of inhibitory activity seen when a cyano group is added to the 2-position of the A ring adjacent to the ketone providing compound **4**, namely CDDO (Table 5).[73] Although this change in activity can be attributed to the change in the electrophilicity of the A-ring enone, it is interesting to note that the introduction of a cyano group also increases the propensity of the Michael addition to be reversible.[23, 38]

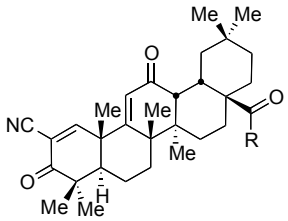
Table 5. Inhibition of production of NO induced by IFN-γ in mouse macrophages.[73]



Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
3	> 40	**19**	7.1

17	37	**20**	0.17
18	1.4	**4**	0.0004

Several additional CDDO analogues described contain further modification of the template including various analogues of the acid such as the methyl ester (CDDO-Me, bardoxolone, **5**), imidazole **21**, ethyl **22,** and trifluoroethyl **23** amides (Figure 7). The optimization of these compounds has been extensively reviewed.[74, 75] Interestingly these analogues were initially optimized using inhibition of the production of NO induced by IFN-γ in mouse macrophages. It was not until later that CDDO **4** and CDDO-imidazole **21** were shown to induce the HO-1 and Nrf2/ARE pathway.[76] The suggested mechanism of action for these compounds is the reversible conjugate addition of the reactive cysteine residues in Keap1 to the α,β-unsaturated ketone in the A-ring of CDDO. The reversibility of this reaction has been demonstrated with simple thiols where the addition adduct was too unstable to be isolated,[77, 78] and it is thought that the inherent reversibility of this reaction will provide an advantage when compared to irreversible modifiers.



4 R = OH
5 R = OMe
21 R = imidazole
22 R = $NHCH_2CH_3$
23 R = $NHCH_2CF_3$

Figure 7. Analogues of CDDO

Other triterpene scaffolds including the ursane,[72, 79] glycyrrhetic,[80] and betuline[81] natural products have also been modified to include Michael acceptors, compounds **24-26** (Figure 8). More recently work has focused on what aspects of CDDO-Me **5** are important for the interaction with Keap1. Smaller tricyclic compounds such as **27a** and **27b**, or even monocyclic compounds such as **28a-c**, containing α-cyano enones,[38] have been shown to activate the Nrf2 pathway.[82, 83] The addition of a second cyano group and acetylene substituent in **27b** (TBE31) was shown to be important for both *in vitro* and *in vivo* activity yielding a very potent inducer of the Nrf2 pathway suggesting that the reactivity, reversibility, and structural proximity of these Michael acceptors is closely related to their biological profile.[38]

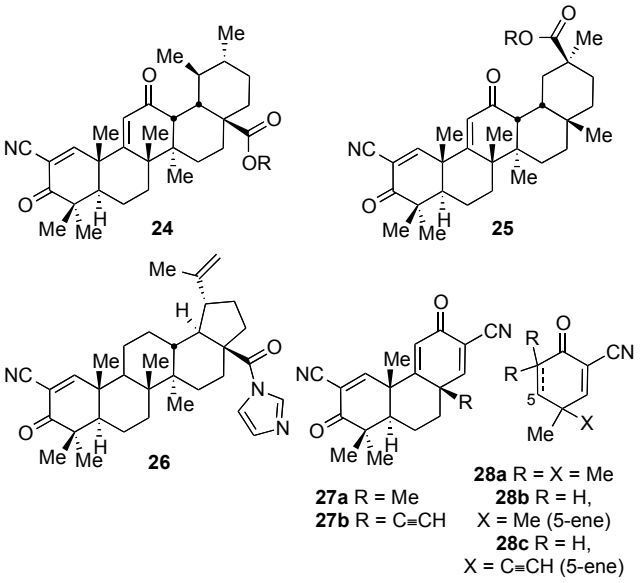


Figure 8. Cyano-enone activators of the Nrf2 pathway.

Other Michael acceptors have been reported to activate the Nrf2 pathway presumably through reaction with Cys151 or other reactive cysteine residues within Keap1. They include compounds such as isolantolactone **29**,[84] 15-deoxy-$\Delta^{12,14}$-prostagladin J_2 **30**,[85] and chalcone analogues **31** and

32.[36, 86] Although reported to activate Nrf2 like many of these Michael acceptor containing molecules they are capable of reacting with cysteine residues on other proteins, a good example of which is 15-deoxy-$\Delta^{12,14}$-prostagladin J_2 **30** that is known to also inhibit the NF-κB pathway by reacting with Cys179 in the activation domain of IKK2.[87] Care needs to be taken interpreting the reports of Nrf2 pathway activators. Many investigators assume direct interaction with the pathway by showing an increase in the production of downstream gene products of Nrf2, *e.g.* NQO1 production, while providing no direct evidence, such as knockout studies or use of siRNA or antisense gene silencing, of direct interaction with the Nrf2 pathway. An example of this are the aurone analogs reported by Go *et al.*[88] Although **33** and **34** were reported to activate Nrf2 and thus can contribute to NQO1 production, they have also been reported to activate the aryl hydrocarbon receptor that is also known to contribute to NQO1 production.

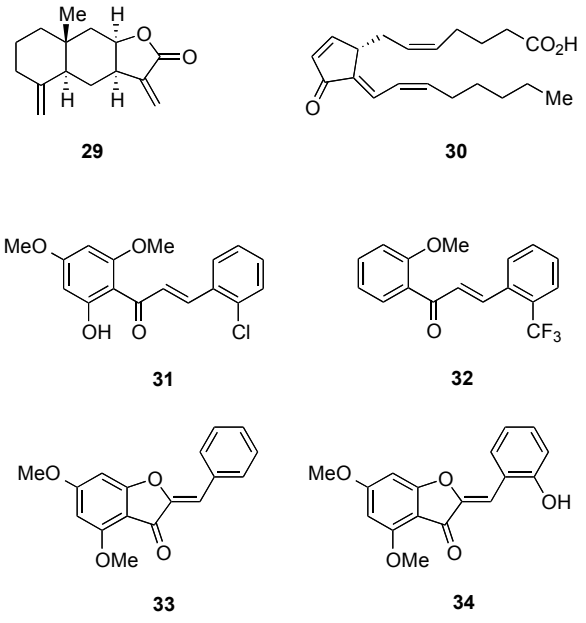


Figure 9. Other electrophilic activators of the Nrf2 pathway.

A large selection of other electrophiles have also been reported to activate the Nrf2 pathway and a number of comprehensive reviews have been recently been published summarizing their structures.[89-93] Many of these compounds contain reactive electrophiles or can easily be metabolized to reactive electrophiles and one can assume they can interact with Keap1 to activate Nrf2. The irreversible nature of many of these interactions would make these types of compounds less promising as drug discovery leads.

Direct inhibition of Nrf2 binding to Keap1

Although a majority of the literature on activating Nrf2 has focused on the action of isothiocyanate electrophiles and Michael acceptors on Cys residues in Keap1 there has been growing interest in the direct disruption of the Nrf2/Keap1 binding. As mentioned previously there are high resolution X-ray structures of the Kelch domain of Keap1,[94] as well as complexes with peptides derived from ETGE,[15, 95] and DLG motifs of Nrf2 and prothymosin-α peptide.[96, 97] These structures show a highly charged protein/protein interaction with the basic residues of the Kelch domain interacting with the acidic residues of the peptides. A number of groups have developed screening assays to identify compounds that are capable of disrupting this protein/protein interaction.[45, 98, 99] Peptide analogues derived from Nrf2 have demonstrated improved affinity for the Kelch domain of Keap1.[100, 101] Recently, Searcey, *et al.* have reported a peptide analogue comprising a TAT-conjugated Nrf2 sequence that activates Nrf2 and its downstream target gene heme-oxygenase-1 (HO-1) in a dose-dependent manner in intact human THP-1 monocytes.[102] This TAT-14mer peptide analogue constitutes a useful chemical biology tool that demonstrates the potential of directly targeting this interaction to activate Nrf2. Hu *et al.* have reported using the FP assay they developed to run a pilot screen of a NCI Diversity Set.[45]

Although the assay performed well they were unable to identify any confirmed hits from this small library. More recently other groups have reported small molecule inhibitors of the Keap1 (Kelch domain)-Nrf2,[44, 103, 104] and in one case the binding site of the small molecule was confirmed by X-ray crystallography.[103]

Clinical implications of Nrf2 activation

As a cellular defense mechanism, defective regulation of Nrf2 is implicated in a number of disease processes. In the context of cancer, Nrf2 appears to have both positive and negative outcomes pre-clinically leading to the conclusion that both activation and inhibition of Nrf2 could be beneficial. Sporn has recently sought to resolve this seemingly paradoxical situation by drawing attention to the context of the experimental designs and NRF2 functions.[105]

The induction of phase 2 enzymes in WT mice (compared with Nrf2 knockouts) aimed at carcinogen detoxification has been shown to be effective in a number of animal models of cancer.[64, 106-109] In these systems, anti-oxidant, cytoprotective, and detoxifying proteins upregulated by Nrf2 play a key role in combating oxidative stress/DNA damage induced by wide variety of carcinogens with Nrf2 WT mice being most responsive to Nrf2 activation. In addition, sulforaphane **6** (SFN), a known Nrf2 activator, is protective in mouse embryonic fibroblasts (MEFs) against a wide variety of cytotoxic and carcinogenic agents.[110]

Increased activation of Nrf2 via mutations in its Keap1 partner – a large number of which are found in the IVR and DGR domains – has been implicated in a number of human cancers including lung, liver, and gallbladder.[111] As well, a quiescent KEAP1 gene resulting from increased methylation in the promoter region has been found in cells from lung and prostate cancers.[112]

In addition to mutations in Keap1, mutations in NRF2 found in a number of cancers occur exclusively in the ETGE and DLG motifs, both of which are critical for normal binding and turnover of the Nrf2 protein. Both Keap1 and Nrf2 mutations results in increased Nrf2 signaling, however, Yamamoto *et al.* report that cancer incidence is unique and exclusive with *KEAP1* mutations found in adenocarcinoma and NRF2 mutations found in squamous cell carcinoma.[113] Constitutive activation of Nrf2 does not appear sufficient for cancer initiation but rather is thought to confer advantages to some cancer cells through detoxification or increased resistance to oxidative stress making them less susceptible to drug treatments.[113-117]

While many cancers leverage the Nrf2 pathway for survival with therapeutic benefit arising from blocking Nrf2 signaling, treatment of chronic diseases with a large oxidative stress component may benefit from activation of Nrf2. Chronic obstructive pulmonary disease (COPD) is characterized by airway obstruction resulting from alveolar destruction (emphysema), mucus hypersecretion, and inflammation. Cigarette smoke and air pollution contribute to COPD development. It has been estimated that cigarette smoke contains 10^{15}-10^{17} radicals per puff.[118-120] Thus activation of Nrf2 antioxidant gene products may prove beneficial to protecting the lungs from continuous assault of pollutants and may restore corticosteroid sensitivity.[121] A couple lines of evidence support the Nrf2 hypothesis. Sulforaphane **6**, an Nrf2 activator, restores bacterial phagocytosis in alveolar macrophages from COPD patients.[122] Heme oxygenase 1 (HO-1), a gene product of Nrf2, is decreased in alveolar macrophages from severe COPD patients with pulmonary emphysema patients.[123] Importantly, Nrf2 expression is decreased in macrophages in smokers and patients with COPD.[124] While short-term cigarette smoke causes an up-regulation of HO1, longer term exposure produced decreased HO1 expression and a decrease in the

Nrf2:Bach1 ratio. In addition, COPD severity is negatively correlated with NRF2 gene expression products in lung parenchyma.[125]

Atherosclerotic lesions can result from oxidative stress. With deficient scavenging or over production of superoxide, increased superoxide concentrations convert nitric oxide to peroxynitrite resulting in hypertension and initiation of inflammatory processes. In preclinical models, broccoli extract, the natural source of the Nrf2 activator sulforaphane **6**, and sulforaphane itself are protective in the spontaneously hypertensive stroke-prone rat.[126-128] Protection was accompanied by increased glutathione (GSH) production and GSH reductase and peroxidase activity along with decreased oxidized glutathione (GSSG) and protein nitrosylation.

Oxidative stress and inflammation are thought to be important in multiple sclerosis. Tecfidera (BG-12), an oral formulation of dimethyl fumarate (DMF) **1** have demonstrated positive efficacy in pre-clinical models,[129] and in patients with relapsing-remitting multiple sclerosis (RRMS).[130, 131] These fumarates have been shown to be activators of Nrf2 and protective of CNS cells against oxidative stress albeit at high concentrations. While mutations in either KEAP1 or NRF2 have been linked to various cancers, recent pre-clinical work has suggested that fumarate hydratase deficiency leading to succination of Keap1 by mono methyl fumarate and subsequent Nrf2 activation plays a role in renal cyst and papillary renal cell carcinoma.[132, 133] In a phase IIb study, BG-12 significantly reduced the number of new Gd+ lesions across a number of population sub-groups.[130] The phase III DEFINE trials studied 1234 RRMS patients with doses of 240 mg twice and/or three-times daily.[134] Patients in the treatment group exhibited reduced lesions and a slower rate of disability progression. During the preparation of this manuscript, Tecfidera (BG-12) was approved by the FDA for the treatment of multiple sclerosis.

Bardoxolone methyl **5**, derived from the natural product oleanolic acid **3**, is also a potent activator of Nrf2. In mouse macrophages, bardoxolone methyl inhibits iNOS production at low nanomolar (nM) concentrations.[72, 73] Recently, this compound has been reported to improve estimated glomerular filtration rates (eGFR) at doses of 75 and 150 mg in patients with type 2 diabetes and chronic kidney disease (CKD).[135, 136] In addition, bardoxolone methyl has been dosed in patients with advanced solid tumors and lymphomas.[137] While bardoxolone methyl is an activator of Nrf2, the rationale for dosing in cancer patients derives from its activity in inhibiting NF-κB. This is done through direct inhibition of upstream kinases JAK1, STAT3, and IKKβ. In the case of IKKβ, inhibition is through conjugate addition of Cys-179 on the kinase activation loop.[138] Importantly in this study, both activation of Nrf2 and inhibition of NF-κB were demonstrated.

Though the clinical results with bardoxolone methyl **5** have been very encouraging and demonstrate the promise of interacting with the Nrf2 pathway, during the preparation of this manuscript, bardoxolone methyl trials were halted due to severe adverse events including mortalities in a Phase III study. Although there has been extensive speculation in the literature,[139] to date no additional information as to the cause of the adverse events beyond an initial press release has been shared by the sponsors of the trail. An initial reaction to these data might point to the electrophilic nature of the activator. However, based on the extensive pre-clinical and clinical data collected for this compound, it is unlikely this alone can explain the recent unexpected clinical results.

Outlook

While the promise of Nrf2 is apparent, the challenge for medicinal chemists is how to regulate this transcription factor. The most direct strategy for activation is apparent from known activators

SFN, dimethyl and monomethyl fumarate, and bardoxolone methyl, among others all of which leverage the electrophile sensing faculty of Keap1 targeting key cysteine residues including Cys151 of the BTB domain along with other key cysteines in Keap1 like Cys273 and Cys288. While this whole approach is predicated on the use of electrophilic molecules that react covalently with Keap1, these activators are unique in that they present a mechanism for reversibility. Indeed, this feature is key to understanding this class of activators. Reversibility certainly does not preclude selectivity or off-target issues; however these may be mitigated relative to irreversible agents. Though bardoxolone methyl has recently failed in the clinic due to adverse events, the approval of Tecfidera (BG-12) gives added confidence in this approach. Although not the major focus of this perspective, a second possibility for activating the Nrf2 pathway arises via disruption of the binding of Nrf2 to the Kelch domain of Keap1. Evidence for the viability of this strategy comes from recent publications showing peptide and small molecule inhibitors of Nrf2 binding.[44, 100, 102-104] It remains to be seen, which of these approaches proves more successful.

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