Chapter 6
Sulfhydryl-Reactive Phytochemicals as Dual Activators of Transcription Factors NRF2 and HSF1

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Abstract Two central regulators, nuclear factor-erythroid 2 p45-related factor 2 (NRF2) and heat shock factor 1 (HSF1), control the KEAP1/NRF2/ARE pathway and the heat shock response, two essential cellular defense mechanisms. Both systems are highly inducible under conditions of stress. Many small molecules, including certain phytochemicals, such as isothiocyanates and phenylpropanoids, and/or their metabolites, have the capacity to induce the KEAP1/NRF2/ARE pathway. Recent results suggest that a common signal that is sensed through cysteine modification(s) within Kelch-like ECH-associated protein 1 (KEAP1) and HSF1, or possibly within a negative regulator of HSF1, is responsible for triggering both pathways. Celastrol, withaferin A, gedunin, curcumin, and sulforaphane are examples of structurally diverse phytochemicals with a common chemical signature: reactivity with sulfhydryl groups. This reactivity underlies their biological activities as multitarget agents for which protective effects have been documented in numerous animal models of human disease and which include induction of large networks of transcriptional programs regulated by transcription factors NRF2 and HSF1.

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D. R. Gang (ed.), *50 Years of Phytochemistry Research,*
Recent Advances in Phytochemistry 43, DOI 10.1007/978-3-319-00581-2_6,

6.1 Introduction

The KEAP1/NRF2/ARE pathway and the heat shock response represent two essential cellular defense mechanisms that are controlled by two central regulators, nuclear factor-erythroid 2 p45-related factor 2 (NRF2) and heat shock factor 1 (HSF1), respectively. Both systems do not normally operate at their maximal capacity, but are highly inducible under conditions of stress. Induction leads to the transcriptional upregulation of networks of proteins that protect against the potentially devastating consequences of thermal, oxidative, and electrophilic stress, and chronic inflammation. Nearly 25 years ago, Paul Talalay and his colleagues discovered that many small molecules, including certain phytochemicals, such as isothiocyanates and phenylpropanoids, and/or their metabolites, have the capacity to induce the KEAP1/NRF2/ARE pathway. Although structurally very diverse, excluding the possibility of ligand–receptor interactions as the underlying mechanism, all inducers possess a common chemical signature: reactivity with sulfhydryl groups [1]. Based on this finding, it was predicted that there exists in the cell a protein sensor for inducers that is endowed with highly reactive cysteine residues [2], which was later identified by Masayuki Yamamoto and his colleagues as Kelch-like ECH-associated protein 1 (KEAP1) [3], the main negative regulator of transcription factor NRF2 [4]. More recently, reactivity with sulfhydryl groups has emerged as also being important for the activation of HSF1 by various small molecules [5–9]. Thus, it appears that a common signal that is sensed through cysteine modification(s) within KEAP1 and HSF1, or possibly within a negative regulator of HSF1, is responsible for triggering both pathways.

6.2 The KEAP1/NRF2/ARE Pathway

The KEAP1/NRF2/ARE pathway is at the forefront of the cellular defense. In numerous experimental systems, induction of this pathway has been shown to be protective against various conditions of stress. Conversely, failure to upregulate the pathway (such as under conditions of NRF2 deficiency) leads to increased sensitization and accelerated disease pathogenesis. Under basal conditions, transcription factor NRF2 is continuously targeted for ubiquitination and proteasomal degradation by the repressor protein KEAP1, which serves as a substrate adaptor for Cullin 3 (Cul3)-based E3 ubiquitin ligase (Fig. 6.1) [10–12]. In addition to KEAP1, the levels of NRF2 within the cell are also controlled by the action of glycogen synthase kinase-3β (GSK3β) and β-transducin repeat-containing protein (β-TrCP) which serves as a substrate adaptor for Cullin 1 (Cul1)-based ubiquitin ligase [13]. The precise mechanistic details of regulation of the KEAP1/NRF2/ARE pathway are not completely understood and several different models have been proposed [14]. It is clear, however, that many inducers of the pathway chemically modify specific cysteine residues within KEAP1, leading to loss of its ability to target NRF2

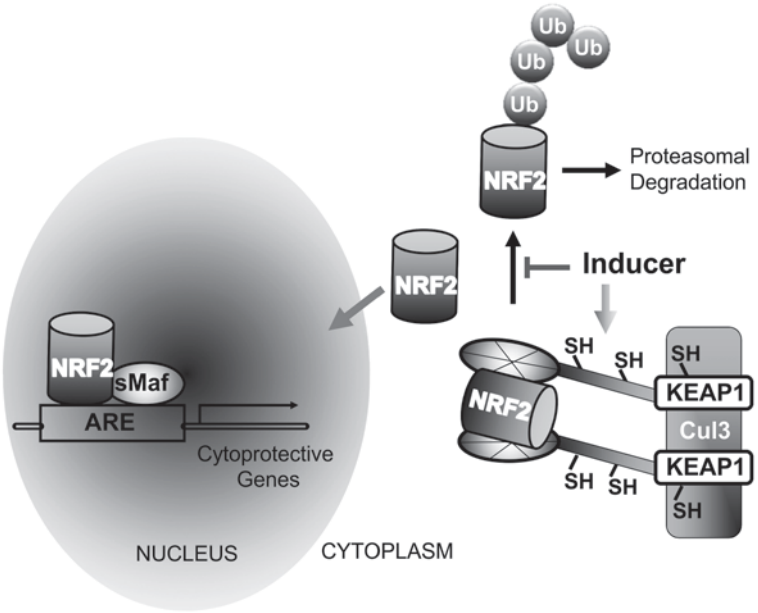

Fig. 6.1 The KEAP1/NRF2/ARE pathway. Under basal conditions, NRF2 is targeted for ubiquitination and proteasomal degradation by its repressor KEAP1, which serves as a substrate adaptor for Cullin 3 (Cul3)-based ubiquitin ligase. Inducers chemically react with cysteine residues of KEAP1, rendering it unable to target NRF2 for degradation. As a result, NRF2 accumulates and undergoes nuclear translocation, where it binds to antioxidant response elements (AREs) as a heterodimer with a small Maf protein, driving the expression of cytoprotective genes

for ubiquitination and proteasomal degradation. Subsequently, NRF2 accumulates, enters the nucleus, binds as a heterodimer with a small Maf transcription factor to antioxidant response elements (AREs, specific sequences that are present in the promoter regions of NRF2-target genes), and activates transcription [15–17].

NRF2-dependent genes encode a large network of cytoprotective proteins, including those that are involved in the metabolism and transport of a wide array of endo- and xenobiotics, proteins that have antioxidant functions, as well as those that participate in the synthesis, utilization, and regeneration of glutathione and NADPH. The number of genes that are under the transcriptional control of NRF2 is fascinatingly large: a recent study integrating chromatin-immunoprecipitation with parallel sequencing (ChIP-Seq) and global transcription profiling identified 645 basal and 654 inducible direct targets of NRF2, with 244 genes at the intersection [18]. Moreover, the functional diversity of the NRF2-dependent cytoprotective proteins is extraordinary and provides the cell with multiple layers of protection. Examples of NRF2-dependent proteins include: (1) antioxidant enzymes (e.g., heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and thioredoxin reductase); (2) conjugating enzymes (e.g., glutathione *S*-transferases (GSTs) and uridine 5′-diphospho (UDP)-glucuronosyltransferases); (3) proteins

that facilitate the export of xenobiotics and/or their metabolites (e.g., solute carriers and adenosine triphosphate (ATP)-binding cassette transporters); (4) anti-inflammatory enzymes (e.g., leukotriene B_4 dehydrogenase); (5) enzymes that participate in the synthesis and regeneration of glutathione (e.g., χ-CT, the core subunit of the cystine/glutamate membrane transporter, γ-glutamate cysteine ligase catalytic (GCLC) and modulatory (GCLM) subunits, glutathione reductase); (6) enzymes that are responsible for the synthesis of reducing equivalents (e.g., glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme); (7) proteins that protect against metal overload (e.g., ferritin and metallothioneins); and (8) proteins that participate in the repair and removal of damaged proteins (e.g., subunits of the 26S proteasome).

6.3 The Heat Shock Response

The heat shock response is another critical inducible defense mechanism which is essential in protecting the cell under conditions of acute and chronic proteotoxic stress affecting the integrity of the proteome. The heat shock response is controlled by a family of heat shock (transcription) factors, among which HSF1 plays the major role [19, 20]. Under homeostatic conditions, HSF1 is an inactive monomeric phosphoprotein bound to Hsp90 (Fig. 6.2). Following stimulation, HSF1 dissociates from the Hsp90 complex, trimerizes, and binds to heat shock elements (HSEs) of its target genes, thereby driving their expression [19–23]. In addition, a number of posttranslational modifications, such as phosphorylation, sumoylation, and acetylation, are involved in regulating the transcriptional activity of HSF1, and there is also negative feedback regulation by heat shock proteins, such as Hsp70 and Hsp40. Several different ways of activation of HSF1 have been proposed and the experimental evidence for each one of them was recently reviewed [23]. Displacement of HSF1 from its negative regulator Hsp90 is one major mechanism: indeed, pharmacological inhibition of Hsp90 or its antibody-mediated depletion is sufficient to induce trimerization and DNA binding of HSF1 [24, 25].

Similarly to the KEAP1/NRF2/ARE pathway, the number of genes that are regulated by the heat shock response is strikingly large: various studies employing differential display, transcriptional profiling, or proteomic approaches have shown that, depending on the organism, approximately 50–200 genes are induced [26]. According to their functions, the proteins encoded by these genes have been grouped into seven distinct classes: (1) molecular chaperones that prevent unspecific aggregation of nonnative or partially misfolded proteins (e.g., Hsp70, Hsp40); (2) proteolytic proteins that participate in the removal of irreversibly damaged proteins (e.g., BAG3 (BCL2-associated protein), APG5 L (protein involved in autophagy), the cysteine protease, caspase 1 (CASP1), neural precursor cell-expressed developmentally downregulated 4 like (NEDD4 L), and ubiquitin-protein ligase)); (3) RNA- and DNA-modifying enzymes, which are necessary to repair DNA damage (e.g., the bacterial DNA glycosylase MutM); (4) metabolic enzymes that are needed

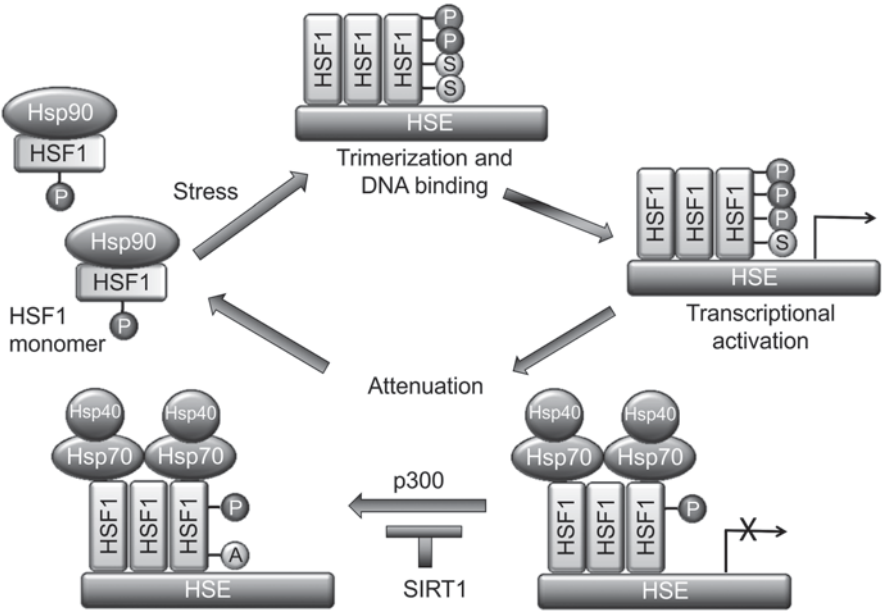

Fig. 6.2 The heat shock response. Under basal conditions, HSF1 is an inactive monomeric phosphoprotein bound to Hsp90. Following stimulation, HSF1 dissociates from Hsp90, trimerizes, and binds to heat shock elements (HSEs) of its target genes, thereby driving their expression. In addition, multiple posttranslational modifications regulate the transcriptional activity of HSF1, such as phosphorylation (P), sumoylation (S), and acetylation (A). HSF1 is also negatively feedback-regulated by heat shock proteins. (Adapted from [19])

to reorganize and maintain the energy supply of the cell (e.g., ACAT2 (acetyl-CoA acetyltransferase), ALAS1 (aminolevulinate synthase), ChGn (chondroitin α-1,4-*N*-acetylgalactosaminyltransferase)); (5) transcription factors, kinases, or phosphatases that further activate other stress response pathways (e.g., RHOH (Ras homolog), PTPG1 (tyrosine phosphatase), RGS2 (regulator of G-protein signaling), and IER5 (regulator of immediate early response)); (6) proteins involved in sustaining cellular structures such as the cytoskeleton and membranes (e.g., tight junction-associated protein (TJP4) and signal-induced proliferation-associated 1-like protein 3 (SIPA1L3)); and (7) proteins involved in transport and detoxification (e.g., the amino acid transporter SLC38A2).

It should be emphasized that the KEAP1/NRF2/ARE pathway and the heat shock response are two distinct defense mechanisms. Thus, induction of the KEAP1/NRF2/ARE pathway occurs in the absence of HSF1; likewise, induction of the heat shock response is independent of NRF2 [9]. Nevertheless, there is some functional overlap between the two pathways which is perhaps best exemplified by HO-1, also known as Hsp32. Indeed, the gene encoding HO-1 is one of the most highly inducible genes (in terms of both kinetics and magnitude of induction) in response to both heat shock as well as inducers of the KEAP1/NRF2/ARE pathway.

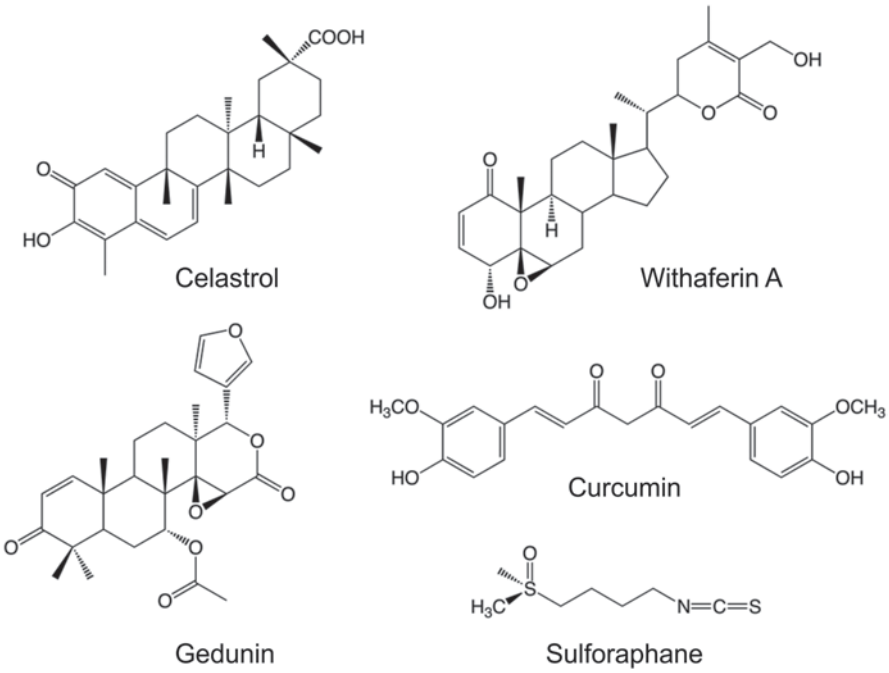

Fig. 6.3 Examples of sulfhydryl-reactive phytochemicals—dual activators of transcription factors NRF2 and HSF1

6.4 Phytochemicals that Activate Transcription Factors, NRF2 and HSF1, and the Consequences of Activation

6.4.1 Celastrol

The quinone methide triterpenoid celastrol (Fig. 6.3) was isolated from the Chinese plant *Tripterygium wilfordii*. Celastrol was reported to induce the heat shock response following a screen of bioactive small molecules in the human HeLa cell line hsp70.1pr-luc [27]. This cell line is stably transfected with a luciferase-encoding construct under the transcriptional control of the *hsp70* promoter. The same study showed that celastrol activates the *hsp70* promoter reporter in several different cell types (i.e., in the breast cancer cell lines MCF7 and BT474, the non-small-cell lung carcinoma cell line H157, and the neuroblastoma cell line SH-SY5Y) to levels comparable with those induced by heat shock (42 °C). Treatment with celastrol led to hyperphosphorylation of HSF1, enhanced binding of HSF1 to the heat shock element in the Hsp70 promoter, and transcriptional activation of endogenous heat shock genes.

Expression profiling of RNA isolated from the androgen-dependent prostate cancer cell line LNCaP that had been treated with celastrol was performed in order to

obtain a gene expression signature for celastrol activity [28]. A collection of gene expression profiles of drug-treated cell lines, termed the Connectivity Map [29], was then used to identify known drugs with similar effects on gene expression. Strikingly, the celastrol gene expression signature was found to be very similar to those of four known inhibitors of Hsp90, strongly suggesting that celastrol functions as an inhibitor of Hsp90. This conclusion was further supported by the ability of celastrol to: (1) decrease the levels of the Hsp90 client proteins AR, FLT3, EGFR, BCL-ABL1, AKT, and HER-2 in a concentration-dependent manner in several different cell lines [28, 30], (2) inhibit the ATP-binding activity of Hsp90 [28], and (3) reduce the interaction of Hsp90 with the co-chaperone p23 [28, 31].

Treatment with celastrol was found to disrupt the interaction of Hsp90 and cell division cycle protein 37 (Cdc37), a co-chaperone which is essential for the association of client proteins to Hsp90 [32]. As a result, the Hsp90 client proteins Cdk4 and AKT were destabilized and degraded via the ubiquitin proteasome, and apoptosis was initiated in the pancreatic cancer cell line Panc-1. Further mechanistic studies revealed that celastrol reacts with the C-terminus of Hsp90 and inhibits the ATPase activity of the chaperone without affecting the ATP binding pocket [33]. To identify target proteins of celastrol, stable isotope labeling with amino acids in cell culture (SILAC) approach was used by Hansen et al. [34] in cultured human lymphoblastoid cells that had been exposed to celastrol for 24 hours. It was found that 158 of the ~1,800 proteins with robust quantitation had at least a 1.5-fold change in their levels, with 112 being upregulated and 46 being downregulated. Upregulated proteins include those involved in cellular homeostatic processes, stress responses, cell death, and intracellular transport. A prominent group is that involved in protein quality control, such as the endoplasmic reticulum molecular chaperones GRP78 (HspA5), Grp94 (Hsp90B1), calnexin (CANX), calreticulin (CALR), ERp29 (ERP29), multiple protein disulfide isomerases, glucosidases, and glycosyltransferases. A second group of celastrol-induced proteins comprises those involved in the cellular defense against oxidative stress, such as peroxiredoxins, thioredoxins, and HO-1. These findings are in agreement with an earlier study by Trott et al. [5] in *Saccharomyces cerevisiae* in which transcriptional profiling showed that treatment with celastrol induced heat shock genes as well as antioxidant genes. Celastrol caused hyperphosphorylation of the yeast HSF1 and upregulation of heat shock proteins. In addition, transcription factor Yap1, which is activated in response to oxidants and electrophiles and triggers the transcription of cytoprotective genes, was also activated by celastrol treatment, via the carboxy-terminal redox center of the transcription factor. Similar to its effects in yeast, celastrol also induces antioxidant response genes (e.g., GCLM, χ-CT, and NQO1), in parallel with heat shock target genes (e.g., Hsp70) in RKO human colorectal carcinoma cells [5], Hepa1c1c7 mouse hepatoma cells, and mouse embryonic fibroblasts [9]. Induction of Hsp70 requires functional HSF1, but is independent of NRF2, whereas upregulation of NQO1 occurs in the absence of HSF1, but the presence of NRF2 is essential [9].

The ability of celastrol to upregulate the KEAP1/NRF2/ARE pathway and the heat shock response suggests that celastrol could have cytoprotective effects, a conclusion that has received experimental support in a number of different studies.

One protective effect of celastrol is its ability to inhibit endogenous peroxynitrite formation and to prevent endothelial barrier dysfunction [35]. Celastrol was also shown to protect against aminoglycoside-induced hair cell death and to reduce hearing loss in mice receiving systemic aminoglycoside treatment [36]. Exposure to celastrol was protective against lethal heat stress in both HeLa cells and SH-SY5Y cells, and to a similar extent as a 42° C heat shock [37]. In cells ectopically expressing a mutant polyglutamine (Q57-YFP) protein, celastrol treatment prevented the aggregation of the fusion protein and the associated cytotoxicity [37]. In rodent models for Alzheimer's disease, celastrol reduced the amyloid-α-associated pathology [38] and improved memory, learning, and psychomotor activity [39]. In mice, celastrol protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and 3-nitropropionic acid-induced neurotoxicity [40]. In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), celastrol prevented neuronal cell death, improved weight loss and motor performance, and delayed disease onset [41].

In addition to its cytoprotective effects, celastrol has also been shown to inhibit the proliferation of a number of cancer cell lines and to suppress tumor development and metastasis in various animal models of carcinogenesis. Thus, treatment of the oral leukoplakia cell line MSK-Leuk1 with celastrol inhibited the activation of AhR-dependent transcription of *CYP1A1* and *CYP1B1*, which encode proteins that are responsible for the conversion of polycyclic aromatic hydrocarbons to genotoxic metabolites [42]. Consequently, the formation of benzo(a)pyrene [B(a)P]-induced DNA adducts was reduced. The antitumor activity of celastrol has also been demonstrated using a panel of human breast cancer cell lines with selectivity toward those overexpressing the receptor tyrosine kinase ErbB2, an Hsp90 client protein [43]. Furthermore, celastrol inhibited tumor growth of ErbB2-overexpressing human breast cancer cells in a mouse xenograft model [43]. Celastrol downregulated the expression of CXCR4, a chemokine receptor that is closely linked with tumor metastasis, in breast cancer, colon cancer, squamous cell carcinoma, and pancreatic cancer cells, and inhibited invasion [44]. Reduction of hypoxia-induced angiogenesis and metastasis by celastrol has also been demonstrated and shown to be partly mediated by inhibition of Hsp90 [45]. Hsp90 inhibition was also implicated in the ability of celastrol to increase the sensitivity of the NCI-H460 lung cancer cell line to radiation [46]. In the RIP1-Tag2 transgenic mouse model of pancreatic islet carcinoma, tumor metastasis was suppressed by more than 80 % when celastrol was administered at a dose of 3 mg/kg body weight, once every 3 days, for 4 weeks [32].

6.4.2 Gedunin

Gedunin (Fig. 6.3) is a tetranortriterpenoid isolated from the Indian neem tree *Azadirachta indica* which has antimalarial, insecticidal, and anticancer activity. Using the Connectivity Map [29], it was found that, similar to celastrol-, gedunin-

induced genes were enriched in the profile of known Hsp90 inhibitors; likewise, gedunin-repressed genes were repressed by known Hsp90 inhibitors [28]. Although less potent, gedunin appears to share with celastrol some of the same mechanisms by which it inhibits Hsp90 and activates NRF2. Thus, gedunin treatment caused a decrease in ATP binding to Hsp90 and a reduction of the protein levels of the Hsp90 client proteins AR, FLT3, EGFR, and BCR-ABL1 in a concentration-dependent manner in three different cell lines: LNCaP, K562, and Ba/F3 [28]. Similarly, when MCF-7 cells were incubated with gedunin for 24 h, there occurred a dose-dependent degradation of the Hsp90 client proteins HER-2 and AKT, strongly suggesting that Hsp90 is the cellular target for this compound [30]. Indeed, gedunin has been shown to disrupt the interaction between Hsp90 and its co-chaperone, Cdc37 [30, 47].

Recently, gedunin was identified as an NRF2-dependent inducer of cytoprotective enzymes in a high-throughput screen of the Spectrum library comprising 2,000 biologically active compounds [48]. This screening assay used a Neh2-luciferase reporter system in which the Neh2 domain of NRF2, through which the transcription factor binds to its negative regulator KEAP1, was fused to firefly luciferase, thus allowing the direct monitoring of induction based on the time course of reporter activation. The same study showed that gedunin protected neurons against oxidative stress in an astrocyte-dependent manner, and via an NRF2-dependent mechanism. Thus, when primary cultured astrocytes were pretreated with gedunin for 24 h followed by the addition of neurons in the presence of the glutathione-depleting compound homocysteic acid, significant neuroprotection was observed. Protection by gedunin was accompanied by an increase in the levels of GSH and HO-1, and was abrogated by silencing of NRF2.

Gedunin was also shown to induce apoptosis and inhibit cell growth in Caco-2 (colon cancer), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small-cell lung cancer), and A375-C5 (melanoma) cells [49, 50]. In the SKOV3, OVCAR4, and OVCAR8 ovarian cancer cell lines, treatment with gedunin decreased cell proliferation by 80% [51]. When a bioinformatics analysis was performed, integrating gedunin sensitivity and gene expression data from 54 cancer cell lines, 52 genes were found to be associated with gedunin sensitivity, 49 of which had decreasing expression with increasing gedunin sensitivity [51]. Pathway analysis revealed significant alterations in signaling pathways controlled by the aryl hydrocarbon receptor, phosphatidylinositol 3-kinase (PI3K)/AKT, nitric oxide, neuregulin, and extracellular signal-regulated kinase/mitogen-activated protein kinase. In agreement with the protective effects of gedunin, three closely related compounds, deoxygedunin, deacetoxy-7-oxogedunin, and deacetylgedunin, were shown to activate HSF1 and induce Hsp70 [52]. Moreover, in an MG-132-induced protein misfolding neuronal cell culture model, the compounds protected against cell death, and RNAi knockdown of HSF1 significantly reversed the cytoprotective effect. In HeLa cells that had been transiently transfected with a polyglutamine-expanded toxic isoform (Q103) of huntingtin, a model for Huntington's disease, all three gedunin derivatives improved cell survival.

6.4.3 Withaferin A

Withaferin A (Fig. 6.3) is a withanolide found in the Indian medicinal plant *Withania somnifera*. More than 30 withanolides have been reported to induce the NRF2-dependent enzyme NQO1 [53, 54]. Withaferin A was among the active compounds which were recently identified as inducers of the HSF1-dependent heat shock response when a library of more than 80,000 natural and synthetic compounds were evaluated using a reporter cell line optimized for high-throughput screening [8]. This reporter cell line expresses enhanced green fluorescent protein (EGFP) under the transcriptional control of a minimal consensus HSE-containing promoter. Withaferin A was also shown to bind to the C-terminus of Hsp90, disrupt the Hsp90–Cdc37 complex, inhibit the activity of the chaperone, and promote the degradation of the Hsp90 client proteins AKT, Cdk4, and the glucocorticoid receptor [55]. Molecular docking analyses of withaferin A into the active Hsp90–Cdc37 complex support the hypothesis that this withanolide has the potential to inhibit the association of the chaperone with its co-chaperone by disrupting the stability of attachment of Hsp90 to Cdc37 [56, 57].

The anticancer effects of withaferin A have been demonstrated in various cell culture and xenograft models. In MCF-7 cells, withaferin A downregulated estrogen receptor alpha (ERα), and caused apoptosis and cell growth inhibition [58]. Withaferin A induced apoptosis and inhibited cell proliferation in the pancreatic cancer cell lines Panc-1, MiaPaCa2, and BxPc3 [55] and in the glioma cell lines LN428, LN827, U87, and BT70, irrespective of their p53 status [8]. Furthermore, in xenograft models of pancreatic Panc-1 cells, tumor growth was reduced by withaferin A in a dose-dependent manner [55]. Tumor growth inhibition by withaferin A was also demonstrated in an orthotopic xenograft model in mice which involved intracranial implantation of BT70 glioma progenitor cells [8]. In this model, evaluation of the mRNA levels for HO-1 within the intracranial tumor mass showed a dramatic (7.7-fold) increase in the withanolide A-treated animals.

6.4.4 Curcumin

Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione, diferuloylmethane; Fig. 6.3) is a component of turmeric, a yellow spice that is obtained from the rhizomes of the East Indian plant *Curcuma longa* L. (Zingiberaceae). We have described the ability of curcumin and other related phenolic Michael acceptors to induce the NRF2-dependent enzyme NQO1 in Hepa1c1c7 cells [59, 60]. Curcumin has also been shown to increase the expression of HO-1 in rat neurons and astrocytes [61], renal epithelial cells [62], and human cardiac myoblasts, hepatocytes, monocytes, and endothelial cells [63–66]. In the human proximal tubule cell line HK-2, curcumin increased the expression of Hsp70 and protected against shiga toxin-induced apoptosis and necrosis [67]. Curcumin treatment increased the levels of HO-1 and Hsp70, and improved the functional recovery of pancreatic islets

following cryopreservation [68]. In cultured *Xenopus laevis* A6 kidney epithelial cells, curcumin led to induction of Hsp30 and Hsp70 and was protective against a subsequent cytotoxic thermal challenge [69]. In the chronic myelogenous leukemia cell line K562, exposure to curcumin disrupted the binding the Hsp90–p23 complex to its client protein p210 BCR/ABL, and thus decreased the levels of this oncogenic tyrosine kinase [70]. Hsp27, Hsp70, and Hsp40 were induced when lung adenocarcinoma (CL1–5) cells were treated with curcumin [71].

The hepatic enzyme activities of catalase and superoxide dismutase as well as the levels of Hsp70 were increased when curcumin was administered intravenously to Sprague-Dawley rats [72]. This treatment protected the liver against the damaging effects of ischemia/reperfusion, including lipid peroxidation, activation of inducible nitric oxide synthase and myeloperoxidase, and apoptosis, and improved survival. Pretreatment with curcumin enhanced induction of heat shock proteins Hsp70, Hsp27, and alpha B crystalline in liver and adrenal gland of rats that had been subjected to heat stress [73]. Numerous studies have demonstrated the protective effects of curcumin in animal models of neurodegeneration, cardiovascular disease, diabetes, and cancer; these have been recently reviewed [74–76]. In humans, curcumin is well tolerated at doses up to 12 g per day [77]. As of October 2013, curcumin has been or currently is in 85 different clinical trials targeting various disease conditions, such as psoriasis, radiation dermatitis, atopic asthma, mild cognitive impairment, Alzheimer's disease, ulcerative colitis, multiple myeloma, pancreatic cancer, colorectal cancer, and myelodysplastic syndrome (www.clinicaltrials.gov).

6.4.5 Sulforaphane

Sulforaphane (1-isothiocyanato-(4*R*)-(methylsulfinyl)butane; Fig. 6.3) is an isothiocyanate that is formed upon plant tissue injury from a glucosinolate precursor (glucoraphanin), a phytochemical present in cruciferous plants. Sulforaphane was isolated from extracts of broccoli (*Brassica oleracea*) as the principal inducer of the NRF2-dependent enzyme NQO1 using a highly quantitative bioassay in murine hepatoma Hepa1c1c7 cells [78]. Over the past 20 years, voluminous experimental evidence that is beyond the scope of this *Perspective* has convincingly shown the ability of sulforaphane to induce NRF2-dependent genes in cells and animals, and to protect against chronic degenerative conditions, such as cancer, cardiovascular disease, and neurodegenerative diseases (reviewed in [79–81]). Induction of HSF-1 dependent genes by this isothiocyanate is a more recent discovery. Global gene expression profiling of liver tissue isolated from C57BL/6J mice that had been treated with a single dose of sulforaphane (90 mg/kg, *p.o.*) revealed that, in addition to the classical NRF2-dependent genes, there was a prominent induction of heat shock proteins, including alpha B crystallin, Hsp40, and Hsp70 [82]. In HL60 and K562 cells, two human leukemic cell lines that have a chromosome abnormality known as the Philadelphia chromosome, sulforaphane treatment was reported to cause nuclear accumulation of both NRF2 and HSF1, and to increase the expression

of Hsp70; interestingly, these effects were enhanced following hGSTA1-1 overexpression [83]. Activation of HSF1 and the heat shock response was also shown in human HeLa and monkey COS-1 cells, with increased expression in Hsp27 being implicated in upregulation of the proteasomal activity [84].

In a pancreatic cancer xenograft model, co-treatment with sulforaphane enhanced the antitumor effect of the 17-allylamino 17-demethoxygeldanamycin (17-AAG), an Hsp90 inhibitor, resulting in inhibition of tumor growth by more than 70% [85]. The isothiocyanate disrupted the Hsp90–Cdc37 interaction and, synergistically with 17-AAG, downregulated several Hsp90 client proteins, including mutant p53, Raf-1, and Cdk4 [85]. Thus, inhibition of Hsp90 may underlie the ability of sulforaphane to activate HSF1 and the heat shock response. In addition to disrupting the association of Hsp90 with its co-chaperone, another potential mechanism of sulforaphane inhibiting the activity of Hsp90 is through altering the acetylation of the chaperone. Sulforaphane was discovered to downregulate the activity of histone deacetylase (HDAC) in a number of human cell lines established from colon, prostate, pancreatic, and breast cancer, as well as in leukemic cells [86–88]. Incorporation of sulforaphane in the diet was also shown to downregulate HDAC and to increase global histone acetylation, with specific increase at the *bax* and the *p21* promoter regions, in polyp tissue isolated from Apc^{min} mice and in PC-3 xenografts [89, 90]. Based on the structural similarities between the HDAC inhibitor trichostatin A and the sulforaphane metabolites sulforaphane-cysteine and sulforaphane-*N*-acetlcysteine revealed by molecular modeling, it was suggested, and then confirmed experimentally, that the metabolites were the actual inhibitors [86, 91]. Interestingly, targeted inhibition or knockdown of HDAC6 leads to acetylation of Hsp90 and disruption of its chaperone function, resulting in polyubiquitylation and depletion of Hsp90 client proteins, including BCR-ABL [92] and the androgen receptor [93]. Treatment with sulforaphane downregulates HDAC6's deacetylase activity, resulting in hyperacetylation of Hsp90 and inhibition of its association with the androgen receptor. Consequently, the proteasomal degradation of the androgen receptor is accelerated, leading to attenuation of androgen receptor-mediated signaling [93]. Thus, inhibition of Hsp90 activity by either disrupting its association with a co-chaperone or promoting its acetylation is a potential mechanism for HSF1 activation by sulforaphane.

6.5 The Importance of Reactivity with Sulfhydryl Groups for Activation of the KEAP1/NRF2/ARE Pathway and the Heat Shock Response

Reactivity with sulfhydryl groups is the only common feature of the phytochemicals discussed in this *Perspective*. Furthermore, the presence of this "chemical signature" is essential for activation of transcription factors NRF2 and HSF1. Thus, the central carbon of the isothiocyanate (—N=C=S) group of sulforaphane is highly

electrophilic and reacts avidly with sulfhydryl groups. In addition, the isothiocyanates participate in transthiocarbamoylation reactions in which they are readily transferred from one (e.g., glutathione) conjugate to another sufhydryl group-containing molecule [94, 95]. Indeed, this reactivity with sulfhydryl groups underlies the cellular uptake and metabolism of sulforaphane, and the binding to its protein targets, including KEAP1 [96]. Similarly, the electrophilicity of the α,β-unsaturated carbonyl functionality within the chemical structures of celastrol, gedunin, withaferin A, and curcumin makes them highly reactive with sulfhydryl groups, although the epoxide moiety within the structures of gedunin and withaferin A may also contribute to this reactivity. In a high-throughput screen for inducers of the HSF1-dependent heat shock response that used a library of more than 80,000 compounds, the presence of the α,β-unsaturated carbonyl moiety was found to be required for both activation of the heat-shock response as well as for inhibition of glioma tumor cell growth [8]. In a recent large-scale study of approximately 1 million small molecules, electrophilicity was a prominent feature in several of the major clusters of more than 200 activators of the heat shock response that were identified [97]. The importance of sulfhydryl reactivity of the phytochemicals discussed here for activation of the KEAP1/NRF2/ARE pathway and the heat shock response is also supported by studies which have used these agents in combination with classical nucleophiles [5, 97, 98]. Thus, both induction of NRF2-dependent genes and of HSF1 target genes by celastrol are prevented by incubation with dithiothreitol (DTT) [5]. The ability of celastrol to increase the levels of Hsp70, and to decrease the levels of the Hsp90–Cdc37 complex can be reversed by *N*-acetylcysteine or glutathione, but not vitamin C, again implying that sulfhydryl reactivity is critical for these biological effects of celastrol [98]. Similarly, the celastrol-mediated degradation of the Hsp90 client protein ErbB2 is abolished by pretreatment of celastrol with DTT [43]. By use of one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy and application of density functional theory, it was recently shown that nucleophiles (e.g., cysteine and glutathione) add to celastrol regioselectively and stereospecifically to form Michael adducts, such that nucleophilic attack is favored exclusively at the α-face with the nucleophile approach *syn* to the α-C9 methyl [99]. Taken together, these findings imply that cysteine reactivity plays a critical role in triggering both the KEAP1/NRF2/ARE pathway and the heat shock response.

It is now widely recognized that cysteine residues within KEAP1 are the sensors for NRF2 activators. KEAP1 is a multidomain homodimeric protein which has five distinct domains (Fig. 6.4): NTR, N-terminal region (amino acids 1−60), broad complex, tramtrack, bric-á-brac (BTB; amino acids 61−179) which is a dimerization domain, IVR, intervening region (amino acids 180−314) which is a particularly cysteine-rich region containing eight cysteine residues among its 134 amino acids, Kelch domain (amino acids 315−598), through which KEAP1 binds to NRF2, and CTR, C-terminal region (amino acids 599−624). Murine KEAP1 contains 25 cysteine residues among its 624 amino acids (its human homolog has 27 cysteines), nine of which (i.e., C23, C38, C151, C241, C273, C288, C297, C319, and C613) are flanked by basic amino acids. The presence of neighboring basic amino acids is

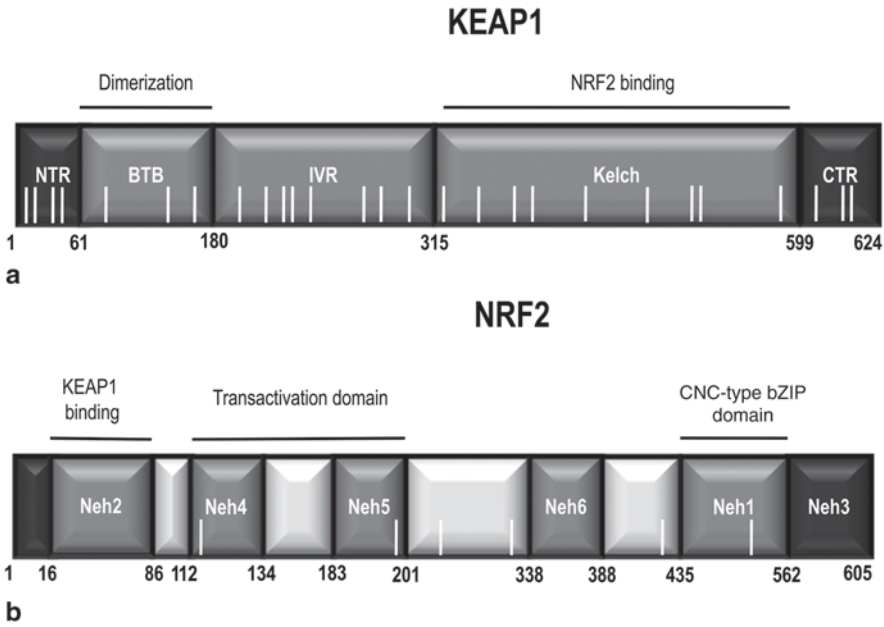

Fig. 6.4 Domain structure of KEAP1 (**a**) and NRF2 (**b**). In KEAP1, the BTB domain is the homodimerization domain and the site of interaction with Cullin 3. The Kelch domain is the NRF2-binding domain. In NRF2, the Neh2 domain is responsible for binding to KEAP1. Neh4 and Neh5 form the transactivation domain, and the Neh1 and Neh3 domains comprise the DNA-binding site of the transcription factor. The white bars indicate the distribution of the cysteine residues within the two proteins

known to lower the pK_a value of cysteine, favoring the formation of the thiolate anion at neutral pH, and thus increasing the cysteine reactivity [100]. Modifications of specific cysteine residues of KEAP1 by sulforaphane, or its sulfoxythiocarbamate derivative STCA, have been detected using both purified recombinant protein as well as lysates of cells that are expressing ectopically KEAP1 following exposure to these compounds [101–104]. By use of mass spectroscopy and mutagenesis analysis, it was established that C151 in the BTB domain, and C273 and C288 in the IVR domain are of particular importance for the repressor function of KEAP1, although depending on the reaction conditions and the experimental system, other cysteine residues can also be modified by sulforaphane, such as C38 in the N-terminal domain, C368, C489, and C583 in the Kelch domain, and C624 in the CTR [101, 104]. Mutation of C151 rendered KEAP1 a constitutive repressor of NRF2, which was unresponsive to induction by sulforaphane [10, 105]. In contrast, substitution of C273 or C288 with either serine or alanine led to complete loss of repressor activity and KEAP1 was unable to repress NRF2 even under basal conditions [105–107]. The increased activity of NRF2 in the presence of C273S/A or C288S/A mutant KEAP1 was caused by reduced ubiquitination of NRF2, resulting in stabilization of the transcription factor [10, 11]. Experiments using transgenic mice expressing

either C273A or C288A KEAP1 mutants confirmed that these residues are required for repression of NRF2 under basal conditions [108].

Two studies—one conducted in zebrafish and another in cultured mammalian cells—have established that KEAP1 contains multiple inducer sensors. The study in zebrafish categorized a series of activators of NRF2 into two groups: those which react with C151 of KEAP1 (e.g., sulforaphane), and those which react with C273 (e.g., 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) [109]. When murine KEAP1 was ectopically expressed in mammalian cells, exposure of these cells to inducers of different types revealed that C151 and C288 each form discrete sensors, and there also exists a third sensor which is formed by H225, C226, and C613 [103]. Molecular modeling showed that C151 is in close spatial proximity with four positively charged amino acids, i.e., K131, R135, K150, and H154, an environment that most likely contributes to the increased reactivity of C151 by decreasing its pK_a and favoring the thiolate formation at physiological pH [103, 110]. Indeed, mutant KEAP1 in which K131, R135, and K150, were substituted with methionine residues had much lower sensitivity to sulforaphane [103]. Another model proposes that binding of inducers to C151 leads to a steric clash with residues in the adjacent α-helix, which may alter the interaction between KEAP1 and Cul3 [111]. It was recently suggested that gedunin may react directly with KEAP1 and impair its ability to target NRF2 for ubiquitination and proteasomal degradation by disrupting the association of KEAP1 with NRF2, although the cysteine reactivity of this phytochemical was not implicated in this model [48].

The importance of cysteine modifications for triggering the heat shock response is also apparent with regard to both HSF1 as well as its negative regulator Hsp90. Human and murine HSF1 contain five conserved cysteine residues. Based on the amino acid sequence of human HSF1, C153 and C373 might be predicted to be particularly reactive with electrophiles; they are in close proximity to basic amino acids (K148, K150, and K157 nearby C153; K372 directly adjacent to C373). An intermolecular disulfide bond formation between C36 and C103 within the human HSF1 has been reported to cause trimerization and DNA binding, whereas formation of an intramolecular disulfide bond (in which C153, C373, and C378 participate) inhibits trimerization and binding to heat shock elements in the promoter regions of heat shock genes [112]. Activation of murine HSF1 by H_2O_2 is dependent on C35 and C105, and the redox regulation of HSF1 is essential for induction of heat shock genes and for protection against thermal and oxidative stress [113]. C35 and C105 are localized within the DNA-binding domain of HSF1, and are required for disulfide bond formation in response to stress.

The reactivity of the cysteine residues of Hsp90 has also been investigated. C521 and C589/590 the rat Hsp90β, corresponding to C529 and C597/C598 in Hsp90α, have been predicted to be highly reactive [114]. Human Hsp90 is a dimeric multidomain protein that contains an N-terminal domain (amino acids 1–210) where ATP binds, a flexible linker region (amino acids 210–272) which affects the function, co-chaperone interaction, and conformation of Hsp90, a middle domain (amino acids 272–629) where many of its client proteins bind, and a C-terminal domain (amino acids 629–732) which contains a dimerization motif (Fig. 6.5) [115, 116].

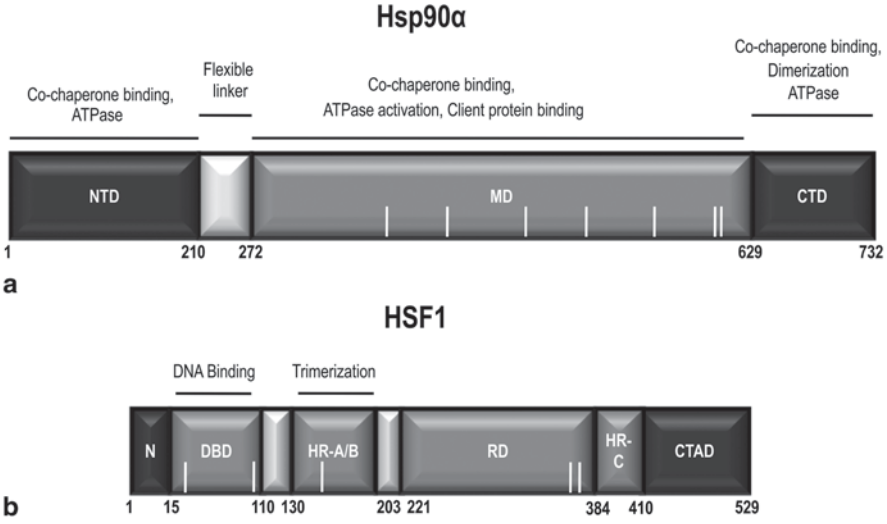

Fig. 6.5 Domain structure of human Hsp90α and HSF1. **a** In Hsp90α, the N-terminal domain (NTD) is the site of binding of ATP and some co-chaperones. The middle domain (MD) is where many of the Hsp90 client proteins and co-chaperones interact, and the C-terminal domain (CTD) contains a dimerization motif and a second ATP-binding site. **b** In HSF1, the DNA-binding domain (DBD) is at the N-terminus of the protein. Trimerization of the transcription factor is through the heptad repeat (HR-A/B) region and is negatively regulated by the HR-C domain. The transactivation domain (CTAD) is at the C-terminus of the protein. The regulatory domain (RD) has a negative regulatory function over the transactivation domain. The *white bars* indicate the distribution of the cysteine residues within the two proteins

In addition, the C-terminal domain has a conserved MEEVD amino acid sequence implicated in binding to co-chaperones with tetratricopeptide repeat (TRP) domains [117, 118], and can also bind to ATP when the N-terminal domain contains one ATP molecule. The binding of ATP causes the N-terminal domains to dimerize and become compacted, allowing them to function as a molecular clamp. Following ATP hydrolysis, the N-terminal domains dissociate. The ATPase cycle of Hsp90 is regulated at multiple levels. It can be stimulated by the co-chaperone Aha1 [119–121] or inhibited by Hop/Sti1 [122–124] and p23/Sba1 [125–129]. Posttranslational modifications of Hsp90 such as acetylation [130], phosphorylation [132, 132], and *S*-nitrosylation [133–137], represent another level of regulation. It has been shown that in human Hsp90α, *S*-nitrosylation at C597 inhibits the ATPase activity of the chaperone [137]. Further work by Retzlaff et al. [138] reported that substituting C597 in human Hsp90α with *S*-nitrosylation-mimicking residues, such as asparagine and aspartic acid, shifts the conformational equilibrium of the protein toward the open conformation, thus decreasing its chaperone activity. This conclusion is also supported by *in silico* studies indicating that C597 is involved in regulating the conformation in Hsp90 [139]. Whereas the identity of the individual cysteine residues of Hsp90 that could be modified by the phytochemicals discussed in this

Perspective is presently unknown, it is notable that C572, adjacent to a lysine residue, has been found to be modified by the electrophilic lipid peroxidation product 4-hydroxy-2-nonenal [140], whereas C521 was identified as the site of thiocarbamoylation when recombinant Hsp90α was incubated with 6-methylsulfinylhexyl isothiocyanate [95]. In agreement, by use of proteomics and click chemistry approaches, Hsp90 was identified as being modified when HEK293 cells were exposed to the sulfoxythiocarbamate derivative of sulforaphane, STCA [9, 102]. Because all cysteine residues reside in the middle client protein-binding domain of Hsp90, it is possible that their modification may lead to disruption of the Hsp90–client protein interactions.

Another possible target for sulfhydryl-reactive phytochemicals is the Hsp90 co-chaperone Cdc37. By use of heteronuclear single quantum coherence (HSQC) NMR spectroscopy, the interaction between the $^{1}H,^{15}N$-labeled N-terminal domain of Hsp90 with unlabeled full-length Cdc37 was investigated in the absence or presence of celastrol [141]. This approach, in combination with mutagenesis analysis and chemical modification (with *N*-ethylmaleimide) of the nine cysteine residues of Cdc37, revealed that celastrol is able to react with the three cysteine residues within the N-terminal domain of Cdc37. Reaction with celastrol leads to large conformational changes both in the N-terminal and in the middle Hsp90-binding domains of Cdc37, ultimately disrupting the Cdc37–Hsp90 association. As Cdc37 is an essential component of the Hsp90 complex machinery, its displacement from the complex will undoubtedly affect the function of the chaperone. In sum, the exact protein targets of the phytochemicals discussed here, which lead to activation of the heat shock response, are incompletely understood, likely to be multiple, and represent a subject of intense investigations by many laboratories. However, it is clear that the reactivity with cysteine residues within those protein targets is critical for the underlying mechanism(s).

It is noteworthy that activation of NRF2 generally occurs at lower inducer concentrations than those that activate HSF1. The most likely reason for this observation could be the relative nucleophilicity of the cysteine residues and their accessibility within the structure of the protein targets for reaction with the electrophilic inducers. Whereas the identification of the precise reason(s) requires further investigation, based on the available experimental evidence it could be proposed that, at low concentrations (Fig. 6.6a), inducers react first with KEAP1, activating the KEAP1/NRF2/ARE pathway. At higher inducer concentrations (Fig. 6.6b), a second target, such as Hsp90, or a co-chaperone within the Hsp90 complex machinery, is also affected, leading to induction of the heat shock response.

6.6 Conclusions

Celastrol, withaferin A, gedunin, curcumin, and sulforaphane are examples of structurally diverse phytochemicals with a common chemical signature: reactivity with sulfhydryl groups. This reactivity underlies their biological activities as

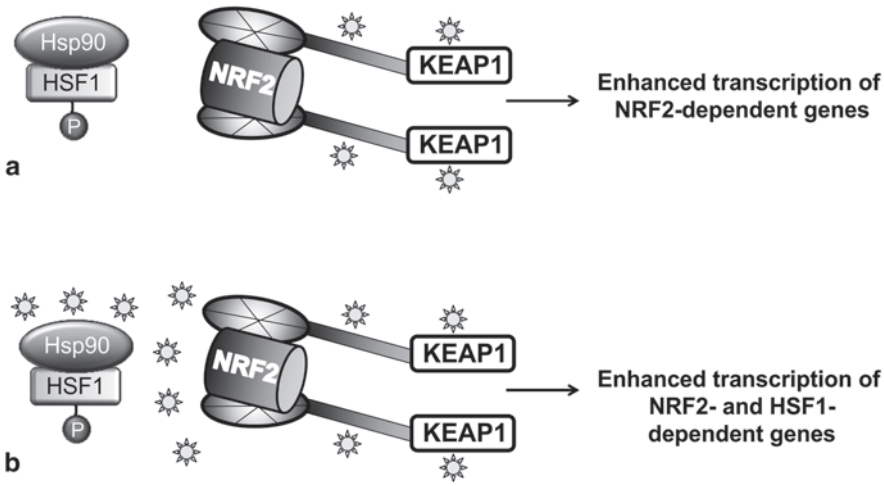

Fig. 6.6 Proposed model for concentration-dependent activation of the KEAP1/NRF2/ARE pathway and the heat shock response by sulfhydryl-reactive phytochemicals. **a** At low concentrations, inducers (depicted with solar symbols) react first with cysteine residues of KEAP1, activating transcription of NRF2-dependent genes. **b** At higher concentrations of inducers, a second target, such as Hsp90, HSF1, or a co-chaperone within the Hsp90 complex machinery, is also affected, leading to enhanced transcription of NRF2- and HSF1-dependent genes

multitarget agents for which protective effects have been documented in numerous animal models of human disease. The effects of such phytochemicals in biological systems are long lasting and comprehensive as they are due to induction of large networks of transcriptional programs regulated by transcription factors NRF2 and HSF1. The resulting upregulation of cytoprotective proteins provides the cell with multiple layers of protection against electrophiles, oxidants, and chronic inflammation, which are the major causes for the development of chronic degenerative conditions, such as cancer, cardiovascular disease, and neurodegeneration. Notably, lower concentrations of phytochemicals are required for induction of NRF2-dependent genes than those which induce HSF1-dependent responses, suggesting that activation of NRF2 precedes that of HSF1. It can be hypothesized that the KEAP1/NRF2/ARE pathway functions to defend against imminent danger. It is then followed by the heat shock response to protect against a potentially devastating damage and preserve the proteome. Collectively, the two pathways ensure adaptation and survival.

Acknowledgments The authors are extremely grateful to the Biotechnology and Biological Sciences Research Council (BBSRC, Project Grant BB/J007498/1) and Research Councils UK for financial support.

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