



## Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation

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### ABSTRACT

For the past 40 years or so, oxidative stress has been increasingly recognized as a contributing factor in aging and in various forms of pathophysiology generally associated with aging. Our view of oxidative stress has been largely “superoxide-centric”, as we focused on the pathological sources of this oxygen-derived free radical and the types of molecular havoc it can wreak, as well as on the protection provided by the antioxidant enzymes, especially the superoxide dismutases, catalases, and glutathione peroxidases. In the last decade our view of oxidative stress has broadened considerably, and it is now often seen as an imbalance that has its origins in our genes, and the ways in which gene expression is regulated. At the center of this new focus is the transcription factor called nuclear factor (erythroid-derived 2)-like 2, or Nrf2. Nrf2 is referred to as the “master regulator” of the antioxidant response, modulating the expression of hundreds of genes, including not only the familiar antioxidant enzymes, but large numbers of genes that control seemingly disparate processes such as immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis and metastasis, and even cognitive dysfunction and addictive behavior. Thus, the dysregulation of Nrf2-regulated genes provides a logical explanation for the connections, both direct and indirect, between observable oxidative stress and perhaps 200 human diseases involving these various physiological processes, each reflecting a network involving many gene products. The evolutionary self-association of these many genes under the common control of Nrf2 suggests that the immune and inflammatory systems may present the largest demand for increased antioxidant protection, apart from constitutive oxidative stress resulting from mitochondrial oxygen consumption for metabolic purposes. Gene expression microarray data on human primary vascular endothelial cells and on the SK-N-MC human neuroblastoma-derived cell line have been obtained in response to the dietary supplement Protandim, a potent composition of highly synergistic phytochemical Nrf2 activators. Pathway analysis of results shows significant modulation by Protandim of pathways involving not only antioxidant enzymes, but of those related to colon cancer, cardiovascular disease, and Alzheimer disease.

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## 1. Introduction

### 1.1. The concept of “oxidative stress”

The term “oxidative stress” began to be used frequently in the 1970s, but its conceptual origins can be traced back to the 1950s to researchers pondering the toxic effects of ionizing radiation, free radicals, and the similar toxic effects of molecular oxygen (Gerschman et al., 1954), and the potential contribution of such processes to the phenomenon of aging (Harman, 1956). The acceptance of free radical biology was remarkably slow, probably due to the largely theoretical and hypothetical nature of its beginnings, the evanescent nature of free radicals, and the lack of experimental tools to study them. The recognition in 1968 that biological systems could produce substantial quantities of the superoxide free radical,  $O_2^{\cdot-}$ , through normal metabolic pathways (McCord and Fridovich, 1968) and that enzymes, the superoxide dismutases (SOD), had evolved with the apparent sole purpose of protecting aerobic organisms from the presumed toxicity of this free radical (McCord et al., 1969, 1971) spurred much interest. These enzymatic tools to both produce (via xanthine oxidase) and eliminate superoxide (via SOD) facilitated additional research in a number of areas of physiology and pathology.

For several decades free radical biology has been “superoxide-centric”, owing largely, perhaps, to the fact that superoxide is quantitatively the predominant free radical produced by biological systems. An example of a biologically-important free radical process that does not necessarily involve superoxide is lipid peroxidation, propagated by the characteristic “free radical chain reaction”. Oxidative stress, however, is a broader term than free radical biology, as few oxidants are actually free radicals. The superoxide radical, in fact, is a fairly good reducing agent in addition to being a mild oxidizing agent. In the dismutation reaction one superoxide radical acts as an oxidant, the other acts as a reductant. As the term “oxidative stress” came into broad usage in the 1970s it frequently described imbalances in redox couples such as reduced to oxidized glutathione (GSH/GSSG) or NADPH/NADP<sup>+</sup> ratios. Such metabolic disturbances need not involve the overproduction of reactive free radicals at all. Thus, the terms “oxidative stress” and “free radical damage” are not synonymous and may not always be interchangeable. Similarly, the terms “free radicals” and “reactive oxygen species” (ROS) are also not synonymous, as many reactive oxygen species (singlet oxygen, hydrogen peroxide, peroxynitrite) are not free radicals.

### 1.2. Oxidative stress, inflammation, reperfusion injury, fibrosis, and cancer

Even before the discovery of SOD’s enzymatic ability to scavenge the superoxide radical (McCord et al., 1969), it was recognized that the protein (also known then as *Orgotein*, *Ontosein*, and *Palosein*) had substantial anti-inflammatory activity (Cushing et al., 1973; Huber et al., 1968; Marberger et al., 1975; McGinness et al., 1977). A search for “orgotein” in PubMed returns well over 100 publications from the past 40+ years, describing many veterinary and human clinical trials. The biochemical connection between superoxide and the inflammatory process followed soon after the discovery of SOD activity. Bernard Babior reported in 1973 that phagocytosing polymorphonuclear leukocytes produced significant amounts of superoxide radical (Babior et al., 1973). It was quickly shown that the depolymerization of hyaluronic acid, as an example of molecular damage resulting from the inflammatory process, was indeed due to this ability of activated leukocytes to produce superoxide radical and to cause oxidative stress (McCord, 1974). The ability of SOD to prevent various sorts of oxidative stress-associated damage resulting from the inflammatory process supported the earlier empirical evidence that SOD appeared to be useful as an anti-inflammatory therapy, but also began to reveal that superoxide’s role in the inflammatory process is rather complex, serving constructive as well as destructive roles (McCord et al., 1980; Petrone et al., 1980; Salin and McCord, 1975). By the 1980s it became apparent that superoxide was involved in pathophysiological conditions beyond the inflammatory process, such as post-ischemic reperfusion injury (Granger et al., 1981; McCord, 1985), even though *in vivo* reperfusion injury ultimately involves inflammation as well. Overexpression of SOD2 was even found to suppress the malignant phenotype of human melanoma cells (Church et al., 1993). Furthermore, the clinical work with SOD as “orgotein” suggested that the protein may be anti-fibrotic in some applications (Ludwig, 1991; Sanchiz et al., 1996), in addition to being anti-inflammatory. Thus, for more than four decades research has suggested that superoxide-dependent oxidative stress may be involved in the pathophysiology of inflammation, fibrosis, cancer, and reperfusion injury.

### 1.3. SOD as a drug?

Much effort has been expended in the past several decades in attempts to turn SOD into a drug. Proteins and enzymes generally make very poor drugs for a variety of reasons: possible immunogenicity, high cost of production, problems associated with purification and stability, non-availability by oral administration, and poor pharmacokinetic properties. The SODs studied all suffered from these limitations—some more than others. In an effort to create an SOD with a better set of properties than any of the three human gene products, we created by genetic engineering techniques a chimeric SOD that combined the structure of the mature human mitochondrial SOD (SOD2) with the “sticky” polycationic C-terminal “tail” of the human extracellular SOD (SOD3), naming the chimeric recombinant product “SOD2/3” (Gao et al., 2003). This chimeric form of SOD did indeed possess pharmacological properties that were superior to any of the three naturally occurring forms of the human enzyme. Being smaller than SOD3 and nearly neutral in charge, it extravasated more easily into the tissue spaces. By having the ability to bind to cell surfaces, it displayed greater efficacy at lower doses than either SOD1 or

SOD2, as well as much slower renal clearance than either. This ability to bind to cell surfaces and components of the extracellular matrix also seemed to buffer the effective concentration *in vivo*. While the superiority of SOD2/3 was subsequently demonstrated in a variety of models (for a review, see [Hernandez-Saavedra et al., 2005](#)), many of the problems enumerated above remained. Was there a better solution for reducing levels of oxidative stress? More importantly, if the effective treatment of complex pathological conditions is the goal, perhaps a broader approach is called for than the administration of a single gene product such as SOD.

#### 1.4. The Keap1/Nrf2 pathway

In 1994 a transcription factor was identified as a regulator of expression of the beta-globin genes, and was named Nrf2 ([Moi et al., 1994](#)). Soon it was discovered that Nrf2 is a positive regulator of the human Antioxidant Response Element (ARE) that drives expression of antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) ([Venugopal and Jaiswal, 1996](#)). The mechanism of Nrf2 activation was described by [Itoh et al.](#), to involve a protein they named Keap1, a suppressor protein anchored in the cytoplasm that physically binds Nrf2, preventing its translocation to the nucleus and its access to ARE-containing promoters ([Itoh et al., 1999b](#)). What followed was a flurry of discoveries of additional Nrf2-regulated genes, including antioxidant-related genes such as those involved in glutathione synthesis ([Wild et al., 1999](#)), Phase II detoxification or “stress-response” genes ([Itoh et al., 1999a](#)), genes involved in limiting the inflammatory process ([Itoh et al., 2004](#)), genes involved in limiting pulmonary fibrosis ([Kikuchi et al., 2010](#)), and genes conferring protection against ischemia/reperfusion injury ([Cao et al., 2006](#)). Thus, the same spectrum of pathophysiological processes that had been found to be favorably modulated by attempts to use SOD as a drug was now also found to be favorably modulated by Nrf2 activation. Is this purely coincidental? Probably it is not. Rather, it seems likely that the process of evolution has assigned large numbers of genes, the products of which are required for survival in stressful conditions, to a common control mechanism—the Nrf2 pathway. It is reassuring to think that our cells have evolved the resources necessary to extricate themselves from many dire circumstances, in effect by making their own “medicines”. Perhaps all we need to do is assist with the signaling process—to help the cells perfect the timing and degree of Nrf2 activation. The idea becomes especially attractive in view of the fact that Nrf2 expression appears to decline with aging, leading to dysregulation of oxidative stress responses ([Tomobe et al., 2011](#); [Ungvari et al., 2011](#)). Why our antioxidant defense system appears to abandon us as we age is not clear, but one possibility is that a programmed decrease in Nrf2 expression is Nature’s way of eliminating the drain on resources imposed on the species by old, post-reproductive individuals.

#### 1.5. Eliminating oxidative stress by Nrf2 activation

It seems entirely plausible that cells possess all the genetic resources required to maintain proper oxidative balance, as young healthy individuals seem not to be oxidatively stressed. It seems unlikely that the condition we describe as “oxidative stress” brings forth new types of oxidizing molecules, heretofore unseen, against which our cells have evolved no specific antioxidant defenses. Rather, it seems more likely that oxidative stress merely reflects an imbalance between the quantities of oxidants our cells are producing and the quantities of antioxidant gene products (SOD, catalase, GSH peroxidases, etc.) required to restore balance. Instead of attempting to restore oxidative balance by the administration of relatively tiny amounts of one antioxidant enzyme or another (e.g. SOD), perhaps our attention should be directed at Nrf2 activation, which can modulate the expression levels of hundreds of gene products that can affect oxidative stress and the related pathophysiological states. In a number of clinical trials in osteoarthritis, the intra-articular injection of about 50,000 U of SOD has been seen to be efficacious ([McIlwain et al., 1989](#)); in a recent clinical trial of Protandim (a composition of multiple synergistic phytochemical Nrf2 activators) the average individual showed an increase of erythrocyte SOD of 34%. As the entire human body contains roughly 7 g of SOD, this 34% increase, if seen in all organs, would result in a steady-state increase of more than 6,000,000 U of SOD activity distributed throughout the body ([Nelson et al., 2006](#)). Thus, the Nrf2-induced increase produced more than 100 times the amount of SOD activity provided by a 15 mg injection of the purified enzyme. This, coupled with the fact that hundreds of other so-called “survival” genes are modulated by Nrf2 (in addition to SOD1), makes Nrf2 activation appear to be a very attractive alternative to the use of antioxidant enzymes, or of synthetic mimetics of antioxidant enzymes, or of natural or synthetic molecules touted to be “antioxidants” by virtue of their abilities to react stoichiometrically with oxidants or free radicals.

#### 1.6. How is Nrf2 activated?

The discovery of Keap1, a Nrf2-binding protein anchored to the cytoskeleton, revealed how the Keap1/Nrf2 complex functions as the cell’s “oxidative stress sensor” ([Itoh et al., 1999b](#)). Four particularly reactive cysteine residues were identified in Keap1 as the most likely candidates for being the direct sensors of oxidative stress ([Dinkova-Kostova et al., 2002](#)). The formation of adducts with electrophiles or their subsequent rearrangement to form protein disulfide linkages was suggested as the molecular basis for the cellular chemostat capable of regulating oxidative stress levels by modulating the production of ARE-regulated antioxidant enzymes. Soon, however, alternative mechanisms for Nrf2 activation were found, and they are dependent upon kinase pathways, including those of mitogen-activated protein kinases (MAPK) ([Yu et al., 1999](#)), phosphatidylinositol-3 kinase ([Kang et al., 2002](#); [Zheng et al., 2009](#)), and atypical protein kinase(s) C ([Numazawa et al., 2003](#)), among

others. Recent refinements to our understanding of Nrf2 activation suggest that the oxidant sensor function of Keap1 may be primarily to slow the ubiquitination and subsequent degradation of Nrf2 at higher levels of oxidative stress, such that more Nrf2 accumulates in the cell under these conditions. Nrf2 itself may contain an oxidant sensor that facilitates nuclear translocation, but that function remains poorly defined (Hu et al., 2010). The phosphorylation of Nrf2 at serine 40 appears to be an important event in the release of Nrf2 from Keap1 and the translocation of Nrf2 to the nucleus (Huang et al., 2002). Many early studies interpreted the action of Nrf2 activators to be mediated solely via adduct formation with, or by oxidation of, the reactive cysteine residues of Keap1, but it seems more likely that kinase signaling pathways are nearly always involved as well, with phosphorylation of Nrf2 ultimately responsible for most of its migration to the nucleus. The actions of sulforaphane and phenethyl isothiocyanate have recently been reviewed in this light (Cheung and Kong, 2010).

Literally dozens of compounds have been reported to have some ability to activate Nrf2, at least in cell culture experiments. Quantitative comparison of these compounds is nearly impossible, as there is no “standard” system in which such evaluations are made. Observed fold induction of an ARE-driven gene depends on a long list of variables, including the structure and origin of the ARE-containing promoter, the type of cell expressing the reporter gene, the concentration of the inducer, the composition of the culture medium used, the basal level of Nrf2 activation, and many other parameters. Often it is implied that an observed induction *in vitro* means that this Nrf2 activator may be useful *in vivo*, when the concentration tested *in vitro* may be impossible to achieve pharmacologically due to poor absorption, lack of bioavailability, rapid metabolism and clearance, etc.

### 1.7. Nrf2 activators as potential therapies for oxidative stress, inflammation, and chemoprevention

Many Nrf2 activators are naturally-occurring and plant-derived, but many others are synthetic compounds not found in Nature. Several Nrf2 activators have progressed to animal experiments and even to human clinical trials. Among the more interesting is bardoxolone methyl [or methyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate] (Reata Pharmaceuticals), currently in Phase 2/3 clinical trials. It was recently reported that a clinical trial of bardoxolone methyl in patients with moderate chronic kidney disease found significant and sustained improvements in estimated glomerular filtration rate with parallel improvements in other measures of kidney function, in a 52-week study (Pergola et al., 2011). BG-12 (dimethyl fumarate) (Biogen Idec) is in clinical trials for the treatment or relapsing-remitting multiple sclerosis (Kappos et al., 2008). Protandim® (LifeVantage Corp.) is a patented dietary supplement consisting of five low-dose natural Nrf2 activators that achieves its effect through a 9-fold synergy obtained when all five components are present together (Velmurugan et al., 2009). The mechanism of Nrf2 activation was concluded to be through multiple kinase pathways, including PI-3 kinase, p38 MAPK, and PKC $\delta$ . A study in humans with oral administration showed significant elevations in SOD1 and catalase, with a decrease in plasma markers of lipid peroxidation (Nelson et al., 2006). Protandim induced Nrf2 and HO-1 in a rat model of SU5416/hypoxia-induced pulmonary hypertension, reducing oxidative stress and cardiac fibrosis, preserving right ventricular microcirculation, maintaining right heart function, and reducing expression of osteopontin-1 (Bogaard et al., 2009). Osteopontin-1, a marker of fibrosis, was also decreased by oral Protandim supplementation in *mdx* mice, a model of Duchenne muscular dystrophy (Qureshi et al., 2010), a disease where fatal heart and diaphragm fibrosis are thought to be regulated by osteopontin (Vetrone et al., 2009).

A substantial literature documents the chemopreventive effect of Nrf2 activators, particularly those that are naturally occurring (such as sulforaphane and curcumin) and found in foods (Giudice and Montella, 2006; Surh et al., 2008). In a two-stage mouse skin carcinogenesis model, a Protandim-supplemented diet was found to reduce skin tumor incidence and multiplicity by 33% and 57%, respectively, compared to mice on basal diet (Liu et al., 2009). Suppression of p53 and induction of mitochondrial SOD are thought to play an important role in the tumor suppressive activity of Protandim (Robbins et al., 2010).

## 2. Materials and methods

### 2.1. Reagents

Protandim was provided by LifeVantage Corp. (Salt Lake City, UT). D,L-Sulforaphane was purchased from Axxora LLC (San Diego, CA). D-Luciferin was from Gold Biotechnology (St. Louis, MO). Bardoxolone methyl (NSC 713200, also known as “RTA 402” and “CDDO-methyl ester”) was obtained from the NCI/DTP Open Chemical Repository (<http://dtp.cancer.gov>). Unless specified, all other chemicals were from Sigma–Aldrich (St. Louis, MO).

### 2.2. Bioassay for Nrf2-activation

The assay is based on the AREc32 cell line, developed and generously provided by Dr. C.R. Wolf and colleagues of the University of Dundee, Scotland (Wang et al., 2006). The AREc32 cell line is a stable transfectant derived from the MCF7 human breast cancer cell line. It contains a promoter with eight copies of the rat glutathione-S-transferase-A2 Antioxidant Response Element (ARE) and the SV40 promoter sequence upstream of a firefly luciferase reporter gene. In these cells, luciferase activity is increased up to 50-fold following treatment with 50  $\mu$ mol/L *tert*-butyl-hydroquinone. Luciferase activity is increased up to 100-fold by Protandim at 30  $\mu$ g/ml, the most potent Nrf2 activator that we have observed.

The AREc32 cells were grown in Opti-MEM (GIBCO, Carlsbad, CA) supplemented with 4% fetal bovine serum (FBS, GIBCO) and 1% Antibiotic–Antimycotic (GIBCO) at 37 °C and in a 10% CO<sub>2</sub>-supplemented air atmosphere. The cells were seeded at 1% to 5% of confluent cell density in T75 tissue culture flasks and cultivated until they approached confluence. The medium was aspirated off and the adherent cells trypsinized with 1 ml of 1× Trypsin–EDTA solution (GIBCO) for 10 min. Ten milliliters of medium was added to the flask and the cells transferred to a 50 ml centrifuge tube and centrifuged at 1000 rpm for 5 min at room temperature. Cells were washed once with 10 ml of medium, then resuspended in 10 ml of medium. Cells were counted using a hemocytometer and diluted to a concentration of 50,000 cells/ml. Four hundred microliters of this cell suspension were seeded into each well of a 24-well plate (i.e. 20,000 cells/well). Cells were then returned to the incubator for 24 h. After 18–24 h the cells were reattached and growing, and ready for treatment with putative Nrf2 activating agents. The agents were added to the wells in an appropriate concentration range, in volumes ranging from 1 to 10 µl/well. Vehicles used were aqueous or organic solvents such as ethanol or DMSO, and appropriate vehicle controls were included. The cells were then returned to the incubator for 18 h. All operations up to this point must be conducted under sterile conditions.

After 24 h the cells were checked under the microscope for any abnormalities or detachment. The medium was aspirated and the cells were washed with phosphate buffered saline, pH 7.4 (100 µl/well). Following aspiration of the wash solution, the cells were lysed by application of 0.1 M potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol and 3.5 mM sodium pyrophosphate (100 µl/well). The plate was incubated at 4 °C for 20 min. Lysate (20 µl from each well in a new 12 × 75 mm glass test tube) was assayed for luciferase activity using a Mono-light 3010 autoinject luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI), automatically injecting 50 µl of Luciferase Assay Buffer after background measurement. Luciferase Assay Buffer was prepared by mixing 9 ml of Solution A (15 mM Tricine, pH 7.8, containing 1.5 mM ATP, 7.5 mM MgSO<sub>4</sub>, and 5 mM dithiothreitol) with 1 ml of Solution B (10 mM D-luciferin). After a 4 s delay following injection, luminescence was measured for 10 s. Relative Light Units (RLU) were recorded for the contents of each well. Fold Induction of luciferase activity was calculated by dividing the RLU obtained for the test well by the average RLU obtained for control wells (which received no putative Nrf2 activator). Each assay or control was performed in duplicate. Parameters noted were the concentration of test substance providing maximal fold induction of luciferase ( $C_{\max}$ ), and the maximal fold induction observed ( $FI_{\max}$ ).

### 2.3. Gene expression experiments

Primary human umbilical vein endothelial cells (HUVEC) were obtained from Dr. Sonia Flores (University of Colorado Denver), and cultured to near confluency as two groups, Control and Protandim-treated. Protandim (as an extract of 200 mg/ml in 95% ethanol) was added to the growth medium of the treated group to produce a final concentration equivalent to 40 µg of Protandim per ml. Both groups were incubated for an additional 18 h.

#### 2.3.1. RNA preparation

For our experiments, the cell culture treatment groups were performed in triplicate, and each sample was used for an individual GeneChip array assay, resulting in three sets of gene expression data per treatment group. Total RNA was extracted from the cultured HUVEC cells (RNeasy Total RNA Isolation Kit, Qiagen, Valencia, CA), treated with DNase I, then the DNase was inactivated (DNA-free, Ambion, Austin, TX) and the sample purified further using RNeasy (Qiagen, Valencia, CA). The concentration of each sample was determined based on the absorbance at 260 nm (A<sub>260</sub>). The purity of each sample was determined based on the ratio of A<sub>260</sub> to A<sub>280</sub>, and a range of 1.9–2.1 was considered adequately pure. The integrity of Total RNA samples was verified by Agilent 2100 Bioanalyzer.

#### 2.3.2. GeneChip analysis of gene expression

Briefly, RNA samples were converted into double-stranded cDNA (ds-cDNA) using an oligodeoxythymidylic acid 24 primer with a T7 RNA polymerase promoter site added to the 3' end (Superscript cDNA Synthesis System; Life Technologies, Inc., Rockville, MD). Double-stranded cDNA was purified using a GeneChip sample cleanup module (Affymetrix, Santa Clara, CA) and then used for *in vitro* transcription with an ENZO BioArray RNA transcript labeling kit (Enzo, Farmingdale, NY), transcribing the ds-cDNA template in the presence of a mixture of biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified by affinity column (RNeasy, Qiagen, Valencia, CA) and randomly fragmented into 50–200 base cRNA fragments by incubation at 94 °C for 35 min in fragmentation buffer before hybridization to Affymetrix GeneChips Human 133 plus 2.0 arrays (45 °C, 16 h) using a GeneChip Hybridization Oven 640 (Affymetrix, Santa Clara, CA). The hybridized GeneChip microarray was stained with streptavidin–phycoerythrin using a GeneChip® Fluidics Station 450 (Affymetrix, Santa Clara, CA), and scanned at 2.5–3 µm resolution by GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

#### 2.3.3. Gene expression data analysis

Hybridization intensities were quantified and normalized across all arrays using the Robust Multichip Average (RMA) algorithm with an adjustment for guanine/cytosine content of probesets, available as an array processing tool on Partek Genomics Suite software 6.5 (St. Louis, MO) (Wu et al., 2004a). Data were filtered to remove all transcripts considered 'absent' or below "Detection Above Background (DABG)" in all samples, as determined by the Affymetrix GeneChip Operating Software (GCOS). Remaining transcripts (22,737 of 54,675) were used for all subsequent statistical and visual analysis. Partek Genomics Suite software was used to identify differentially expressed transcripts using a one-way ANOVA model with a



stringent false discovery rate of less than 2.5% (corresponding to a  $p < 0.0033$ ) to control for multiple testing. An arbitrary expression change cutoff of more than 1.5 was applied to generate a set of transcripts with differential expression between the experimental groups. These cutoff criteria resulted in discovery of 3000 gene transcripts that were significantly modulated by Protandim treatment in cultured HUVEC cells. For pathway analysis, we entered the gene probe identification numbers of the transcripts that met our cutoff criteria and corresponding fold change values into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA), which facilitated the evaluation of our Protandim-modulated gene transcripts in the context of known, published biological pathways, functions, and networks.

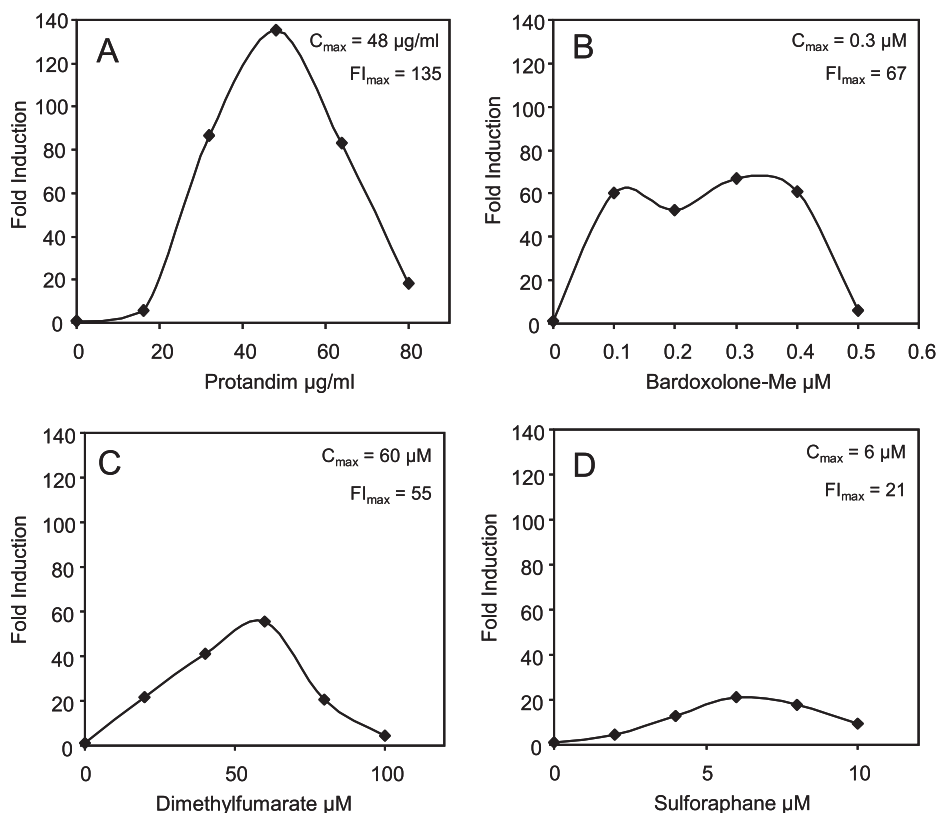
### 3. Results and discussion

#### 3.1. Quantitative comparison of Nrf2 activators using the AREc32 bioassay

Fig. 1 provides a comparison using the AREc32-based bioassay for Nrf2 activation among Protandim, sulforaphane, bardoxolone methyl, and dimethyl fumarate. Sulforaphane is often considered a “gold standard” among naturally-occurring Nrf2 activators (Agyeman et al., 2011). As seen here, the two important parameters  $C_{\max}$  and  $FI_{\max}$  are easily observed. The greatest  $FI_{\max}$  was observed with Protandim at 135-fold, followed by bardoxolone methyl at 67-fold, dimethyl fumarate at 55-fold, and sulforaphane at 21-fold. Of the three pure compounds tested, bardoxolone methyl showed the lowest  $C_{\max}$  at 0.3  $\mu\text{M}$ , with sulforaphane at 6  $\mu\text{M}$ , and dimethyl fumarate 60  $\mu\text{M}$ . Protandim, a mixture of five active ingredients, showed a  $C_{\max}$  of 48  $\mu\text{g/ml}$ . This concentration of Protandim would contain approximately 26  $\mu\text{M}$  silybinin, 13.6  $\mu\text{M}$  curcumin, 5  $\mu\text{M}$  EGCG, 0.07  $\mu\text{M}$  withaferin A, and 5  $\mu\text{M}$  bacopasides. Bardoxolone methyl appeared to produce a biphasic induction, producing near maximal FI over a range of concentrations from less than 40 nM to 0.4  $\mu\text{M}$ .

##### 3.1.1. Problems associated with quantifying Nrf2 activators

There is no universally accepted method for quantifying Nrf2 activation elicited by any given agent or for comparing potencies of agents that share this property. Sometimes the claim is based on microscopic evidence of nuclear translocation of Nrf2, detected by immunofluorescence. While important to demonstrate, this technique is qualitative, and does not



**Fig. 1.** Induction of luciferase in AREc32 cells by Protandim, bardoxolone methyl, dimethyl fumarate, and sulforaphane. Cells (20,000/well) were treated with the indicated concentrations of Nrf2 activator and incubated for 18 h. Maximum fold inductions ( $FI_{\max}$ ) and the concentrations producing those maxima ( $C_{\max}$ ) were as follows: Protandim,  $FI_{\max} = 135$  at  $C_{\max} = 48 \mu\text{g/ml}$ ; bardoxolone methyl,  $FI_{\max} = 67$  at  $C_{\max} = 0.3 \mu\text{M}$ ; dimethyl fumarate,  $FI_{\max} = 55$  at  $C_{\max} = 60 \mu\text{M}$ ; sulforaphane,  $FI_{\max} = 21$  at  $C_{\max} = 6 \mu\text{M}$ .

actually reflect gene expression. More often, Western blot analysis is used to demonstrate an increase of a particular gene product, but this is rarely used to determine  $FI_{\max}$  and  $C_{\max}$  with any degree of accuracy. Moreover, the use of these techniques is reported in many different cell types, and not all cells respond equally to any given agent due to myriad biological variables. A more quantitative technique has been transient transfection of a cell line with a reporter gene controlled by an ARE-containing promoter. While this approach works well for a given study, similar experiments performed in different laboratories generally involve use of various expression vectors, relying on different promoters and different reporter genes, transfected with different efficiencies into various cell lines. The creation of stably transfected cell lines such as the AREc32 cell line used here (Wang et al., 2006) or a similar recently described cell line derived from the human keratinocyte HaCaT cell line (Natsch and Emter, 2008) provides opportunities for defining “standard assays” that may be performed under standardized conditions in any laboratory. These standard, economical, high-throughput assays will greatly facilitate comparisons among the growing number of putative Nrf2 activators, whether phytochemicals or synthetic pharmaceuticals.

Protandim, sulforaphane, bardoxolone methyl, and dimethyl fumarate have all been tested *in vivo* in humans and are therefore of potential therapeutic interest. When compared contemporaneously in the AREc32-based assay,  $FI_{\max}$  observed was in the order Protandim > bardoxolone methyl > dimethyl fumarate > sulforaphane. A notable difference among these four agents is that Protandim consists of five active ingredients which interact with substantial synergy, whereas the other three are single compounds. The nature of the synergistic action between any two of the five active components is to increase  $FI_{\max}$  well beyond the sum of the two individual values and to substantially decrease  $C_{\max}$  for each. At the  $C_{\max}$  of Protandim, each of its five components is therefore well below that component's individual  $C_{\max}$  and  $FI_{\max}$ , such that the induction caused by the composition is up to nine times the sum of the five component contributions (Velmurugan et al., 2009).

The problems of variability in the bioassay of Nrf2 activators are not completely eliminated, even by the use of a stably transfected cell line such as AREc32. One reason is that fold induction is calculated by dividing the relative light units (RLU, representing luciferase concentration) in the presence of the inducer by the relative light units observed in the absence of the inducer, with the latter value representing the “basal” level of gene expression. Basal level is affected by growth medium composition—especially by concentration and source of the fetal bovine serum it contains. It is also affected by degree of confluency of the cells, and certainly by the type of cell. This basal level, however, may be a much smaller number than the induced number (as small as <1%) such that minor fluctuations in it have a great effect on the calculated fold induction. The variability can be largely eliminated if  $FI_{\max}$  is calculated relative to the contemporaneous standard. Using the data of Fig. 1, if sulforaphane is considered the standard, then the fold inductions relative to sulforaphane would be: Protandim, 6.4, bardoxolone methyl, 3.2, and dimethyl fumarate, 2.6. When these ratios are calculated, the basal levels of induction cancel out. Thus, the use of a stably transfected cell line such as AREc32, coupled with contemporaneous assessment of a well-characterized “standard” such as sulforaphane or *tert*-butylhydroquinone would seem to be a great improvement over currently used and often poorly controlled methods to assess Nrf2 activation.

A recent laboratory study of dimethyl fumarate found that the compound activates Nrf2 in primary astrocytes, but not in the C6 glioma-derived cell line (Wilms et al., 2010), demonstrating that different cells may respond quite differently to Nrf2 activators. These authors suggest that the increased metabolic demands of transformed cells may play a role, but data showing a strong response of the transformed MCF7-derived AREc32 cell line argues against that rationale. Rather, it seems more likely that Nrf2 activators will not be found to be “one-size-fits-all” but instead may have to be selected based on the primary mechanism of activation (i.e., via thiol alkylation versus kinase pathway activation, and even which kinase pathway predominates) or on the particular biochemical and physiological idiosyncrasies of the cell type or organ being targeted.

### 3.1.2. Why do Nrf2 activators display bell-shaped dose curves?

A feature common to all Nrf2 activators examined here is bell-shaped dose–response curves. The reason for this behavior is not understood, but may reflect self-limitation imposed by the induction, at higher levels of Nrf2 activation, of enzymes that reverse the activation process, such as deacetylases. An additional layer of complexity has been added to the Nrf2 activation story with the demonstration that acetylation–deacetylation of Nrf2 determines its nuclear translocation, its ability to promote transcription, and its egress from the nucleus to terminate its transcriptional activity (Kawai et al., 2011). Sirtuin 1 (SIRT1) was shown to decrease acetylation of Nrf2, as well as Nrf2-dependent transcription. [In our gene expression data, SIRT1 was induced 1.75-fold by Protandim ( $p = 0.015$ ).] The study also found that resveratrol, a putative activator of SIRT1 (Howitz et al., 2003), inhibited Nrf2-dependent transcription, apparently contradicting earlier reports that resveratrol activates Nrf2 (Chen et al., 2005; Ungvari et al., 2010). It may, however, do both, depending on concentration.

### 3.2. Gene expression data

Among the 10 genes most highly upregulated by Protandim are a number of notables that encode antioxidant and anti-inflammatory proteins. SLC7A11, induced 76-fold, encodes a cystine/glutamate antiporter responsible for maintaining extracellular glutamate in the brain, and for importing cystine necessary for glutathione synthesis (Albrecht et al., 2010). This antiporter was recently found to be decreased by repeated cocaine exposure, and restoration of the activity prevented cocaine-primed drug seeking behavior in rats (Baker et al., 2003). AKR1B10, induced 72-fold, encodes aldo–keto reductase family 1 member B10 which efficiently detoxifies mutagenic and carcinogenic alpha, beta-unsaturated carbonyls such as 4-hydroxynonenal (Zhong et al., 2009). PTGR1 (aka LTB4DH), induced 68-fold, encodes leukotriene B4–12-hydroxydehydrogenase, which is considered to be a key enzyme responsible for biological inactivation of prostaglandins and related

**Table 1**

Gene symbols in bold indicate gene products targeted by specific drugs used in the clinical treatment of the disease (determined by Ingenuity IPA analysis as described under Section 2.3.3). Bolded gene symbols with an asterisk indicate genes for which Protandim treatment modulates gene expression in the same direction as achieved with the drug therapy. Within a disease section, for genes above the horizontal line Protandim opposes the change caused by the disease process, and for genes below the line Protandim changes the gene in the same direction as the disease process.

Gene symbol	Gene title	Disease process	Protandim	Fold-change
<i>Atherosclerosis (19 genes)</i>				
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	↑	↓	−2.34
<b>*DHFR</b>	Dihydrofolate reductase	↑	↓	−1.94
<b>*EDN1</b>	Endothelin 1	↑	↓	−2.00
<b>*ITGB3</b>	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	↑	↓	−2.69
MKI67	Antigen identified by monoclonal antibody Ki-67	↑	↓	−5.05
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	↑	↓	−2.75
MMP14	Matrix metalloproteinase 14 (membrane-inserted)	↑	↓	−2.11
MMP2	Matrix metalloproteinase 2	↑	↓	−1.75
<b>*PDE7A</b>	Phosphodiesterase 7A	↑	↓	−1.52
<b>*PDE7B</b>	Phosphodiesterase 7B	↑	↓	−1.62
PLAU	Plasminogen activator, urokinase	↑	↓	−3.13
<b>*PTGS1</b>	Prostaglandin-endoperoxide synthase 1	↑	↓	−2.80
SCARB1	Scavenger receptor class B, member 1	↑	↓	−1.80
<b>*TUBB3</b>	Tubulin, beta 3	↑	↓	−1.57
<b>*NR3C1</b>	Nuclear receptor subfamily 3, group C, member 1	↓	↑	1.54
<b>*PPARA</b>	Peroxisome proliferator-activated receptor alpha	↓	↑	2.21
EGR1	Early growth response 1	↑	↑	1.66
<b>PTGS2</b>	Prostaglandin-endoperoxide synthase 2	↑	↑	7.61
<b>SOAT1</b>	Sterol O-acyltransferase 1	↑	↑	2.17
<i>Colon carcinoma (28 genes)</i>				
ACLY	ATP citrate lyase	↑	↓	−1.95
ANTXR1	Anthrax toxin receptor 1	↑	↓	−3.60
C20orf27	Chromosome 20 open reading frame 27	↑	↓	−1.99
CCNA2	Cyclin A2	↑	↓	−3.35
CHAF1A	Chromatin assembly factor 1, subunit A (p150)	↑	↓	−1.68
DHFR	Dihydrofolate reductase	↑	↓	−1.94
EFCAB11	Chromosome 14 open reading frame 143	↑	↓	−1.67
FEN1	Flap structure-specific endonuclease 1	↑	↓	−2.27
GINS2	GINS complex subunit 2 (Psf2 homolog)	↑	↓	−3.59
MCM10	Minichromosome maintenance complex component 10	↑	↓	−4.42
MCM4	Minichromosome maintenance complex component 4	↑	↓	−4.80
RNASEH2A	Ribonuclease H2, subunit A	↑	↓	−1.78
SLIT2	Slit homolog 2 (Drosophila)	↑	↓	−1.70
SPC25	SPC25, NDC80 kinetochore complex component	↑	↓	−5.56
TFRC	Transferrin receptor (p90, CD71)	↑	↓	−1.69
TK1	Thymidine kinase 1, soluble	↑	↓	−3.70
TMEM97	Transmembrane protein 97	↑	↓	−2.79
TRIP13	Thyroid hormone receptor interactor 13	↑	↓	−3.22
TUBB	Tubulin, beta	↑	↓	−1.52
TUBB3	Tubulin, beta 3	↑	↓	−1.57
<b>*TYMS</b>	Thymidylate synthetase	↑	↓	−4.90
UBA1	Ubiquitin-like modifier activating enzyme 1	↑	↓	−1.57
UNG	Uracil-DNA glycosylase	↑	↓	−1.80
VRK1	Vaccinia related kinase 1	↑	↓	−1.83
ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	↓	↑	2.52
GLRX2	Glutaredoxin 2	↑	↑	2.13
MICB	MHC class I polypeptide-related sequence B	↑	↑	1.52
NQO1	NAD(P)H dehydrogenase, quinone 1	↑	↑	6.77
<i>Alzheimer disease (66 genes)</i>				
AGRN	Agtrin	↑	↓	−2.49
ANP32A	Acidic (leucine-rich) nuclear phosphoprotein 32 family, A	↑	↓	−2.20
BAX	BCL2-associated X protein	↑	↓	−2.87
<b>*BCHE</b>	Butyrylcholinesterase	↑	↓	−1.69
BGN	Biglycan	↑	↓	−2.45
BRCA1	Breast cancer 1, early onset	↑	↓	−2.09
CADPS2	Ca <sup>++</sup> -dependent secretion activator 2	↑	↓	−1.63
CAPN1	Calpain 1, (mu/l) large subunit	↑	↓	−1.53
CCNB1	Cyclin B1	↑	↓	−2.13
CDC2	Cell division cycle 2, G1 to S and G2 to M	↑	↓	−4.47
CDK2	Cyclin-dependent kinase 2	↑	↓	−1.73
CDKN2A	Cyclin-dependent kinase inhibitor 2A	↑	↓	−2.13
CXCR4	Chemokine (C-X-C motif) receptor 4	↑	↓	−3.18
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	↑	↓	−1.55

(continued on next page)



Table 1 (continued)

Gene symbol	Gene title	Disease process	Protandim	Fold-change
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	↑	↓	–3.65
HOMER1	Homer homolog 1 (Drosophila)	↑	↓	–1.90
<b>*HRH1</b>	Histamine receptor H1	↑	↓	–1.79
IGF2	Insulin-like growth factor 2 (somatomedin A)	↑	↓	–1.76
IGFBP2	Insulin-like growth factor binding protein 2, 36 kDa	↑	↓	–1.99
LDLR	Low density lipoprotein receptor	↑	↓	–3.05
<b>*MAOA</b>	Monoamine oxidase A	↑	↓	–3.02
NEFH	Neurofilament, heavy polypeptide	↑	↓	–1.53
NPDC1	Neural proliferation, differentiation and control, 1	↑	↓	–1.59
NRGN	Neurogranin (protein kinase C substrate, RC3)	↑	↓	–1.65
<b>*PREP</b>	Prolyl endopeptidase	↑	↓	–1.94
PROS1	Protein S (alpha)	↑	↓	–2.03
<b>*PTGS1</b>	Prostaglandin-endoperoxide synthase 1	↑	↓	–2.80
SELENBP1	Selenium binding protein 1	↑	↓	–2.30
TAGLN	Transgelin	↑	↓	–3.55
TGFB1	Transforming growth factor, beta 1	↑	↓	–1.58
<b>*TUBB3</b>	Tubulin, beta 3	↑	↓	–1.57
<b>*VKORC1</b>	Vitamin K epoxide reductase complex, subunit 1	↑	↓	–1.52
CANX	Calnexin	↓	↑	2.22
GCNT2	Glucosaminyl (N-acetyl) transferase 2, 1-branching enzyme	↓	↑	4.80
IDE	Insulin-degrading enzyme	↓	↑	1.82
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	↓	↑	2.79
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	↓	↑	1.84
NR3C1	Nuclear receptor subfamily 3, group C, member 1	↓	↑	1.54
<b>*PPARA</b>	Peroxisome proliferator-activated receptor alpha	↓	↑	2.21
SLC6A6	Solute carrier family 6, member 6	↓	↑	12.94
SYVN1	Synovial apoptosis inhibitor 1, synoviolin	↓	↑	1.59
TSHZ1	Teashirt zinc finger homeobox 1	↓	↑	1.55
TXN	Thioredoxin	↓	↑	1.78
ACLY	ATP citrate lyase	↓	↓	–1.95
ATAD2	ATPase family, AAA domain containing 2	↓	↓	–2.57
BECN1	Beclin 1, autophagy related	↓	↓	–1.65
DHCR24	24-Dehydrocholesterol reductase	↓	↓	–1.69
FGF2	Fibroblast growth factor 2 (basic)	↓	↓	–1.94
HTRA1	HtrA serine peptidase 1	↓	↓	–1.88
PRKCE	Protein kinase C, epsilon	↓	↓	–1.73
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	↓	↓	–1.94
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	↓	↓	–1.96
TUBB	Tubulin, beta	↓	↓	–1.52
UNG	Uracil-DNA glycosylase	↓	↓	–1.80
<b>ATP1A1</b>	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 1 polypeptide	↑	↑	1.57
CTSD	Cathepsin D	↑	↑	3.24
GLRX	Glutaredoxin (thioltransferase)	↑	↑	2.52
HMOX1	Heme oxygenase (decycling) 1	↑	↑	55.99
IL6R	Interleukin 6 receptor	↑	↑	6.53
NPTX1	Neuronal pentraxin I	↑	↑	2.42
NQO1	NAD(P)H dehydrogenase, quinone 1	↑	↑	6.77
PHF1	PHD finger protein 1	↑	↑	3.51
PRKCD	Protein kinase C, delta	↑	↑	1.74
<b>PTGS2</b>	Prostaglandin-endoperoxide synthase 2	↑	↑	7.61
RANBP9	RAN binding protein 9	↑	↑	1.59
SOD1	Superoxide dismutase 1, soluble	↑	↑	1.76

eicosanoids (Tai et al., 2002). It was recently found to suppress the oncogenic transformation of HepG2 cells (Wei et al., 2011). HMOX1, induced 56-fold, encodes heme oxygenase-1, an antioxidant enzyme considered a hallmark of Nrf2 activation. The induction of heme oxygenase-1 is now seen as a novel and alternative therapeutic target in the management of cardiovascular disease (Chan et al., 2011). AIFM2 (aka AMID), induced 29-fold, is implicated in caspase-independent apoptosis and was found to be downregulated in a majority of human tumors (Wu et al., 2004b). OSGIN1 (aka OKL38), induced 29-fold, is an oxidative stress response gene and a tumor suppressor gene (Yao et al., 2008). GPX3, induced 20-fold, encodes an important antioxidant enzyme, glutathione peroxidase-3, found normally in plasma and kidney but underexpressed in head and neck cancers (Chen et al., 2011). SQSTM1, induced 20-fold, encodes sequestosome-1, a participant in the autophagy pathway recently shown to be necessary to avoid premature senescence in human fibroblasts (Kang et al., 2011). HSPB8, induced 19-fold, is a heat shock protein that forms a complex with BAG3 (also induced 1.43-fold). Overexpression of the HSPB8-BAG3 complex also stimulates autophagy and facilitates the clearance of mutated aggregation-prone proteins, the accumulation of which characterizes many neurodegenerative disorders such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (Seidel et al., 2011). TNFSF9 (aka CD137L), induced 19-fold, can induce maturation of hu-

man immature monocyte-derived dendritic cells leading to an enhanced capacity of the dendritic cells to stimulate protective T cell responses, as compared to classical dendritic cells (Tang et al., 2011). It is interesting to note that even in this small sampling of the genes most highly upregulated by Protandim are genes that have been shown to be preventive or protective against cancer, cardiovascular disease, and neurodegenerative disease. These associations were further supported by pathway analysis.

### 3.2.1. Genes associated with specific disease states by pathway analysis

Ingenuity Pathway Analysis (IPA) was used to examine gene transcripts that were increased or decreased by Protandim in HUVEC cells. The analysis revealed that atherosclerosis, colon carcinoma, and Alzheimer disease are each characterized by a number of genes significantly modulated by Protandim (see Table 1). For example, 19 genes products have been associated with atherosclerosis and are up or down-regulated by Protandim. Of these 19 genes, the first 16 listed (84%) were regulated by Protandim in the opposing direction to that taken by the atherosclerosis disease process. The probable benefit of this effect of Protandim is further supported by the fact that of the 11 gene products currently being targeted by drug interventions (Table 1, in bold type), nine of them (Table 1, marked by asterisks) are modulated by Protandim in the same direction that is proposed to be beneficial and caused by the therapeutic intervention.

In colon carcinoma, IPA analysis revealed 28 genes associated with the disease that were also modulated by Protandim treatment. Of these, the first 25 listed (89%) were regulated by Protandim in the opposing direction to that taken by the colon carcinoma disease process. In addition, Protandim downregulated the one gene targeted by a chemotherapeutic drug, an antimetabolite inhibitor for that gene's product, thymidylate synthetase.

In Alzheimer disease, 66 genes were identified that are also modulated by Protandim at the gene expression level. Of these 66 genes, the first 43 of them (65%) were regulated by Protandim in the opposing direction to that taken by the Alzheimer disease process. The beneficial effect of Protandim is further supported by the fact that of the 10 gene products currently targeted by drug therapies, eight of them are modulated by Protandim in the same direction that is proposed to be beneficial and caused by the drug.

Notably, among the relatively small number of genes for which Protandim regulates in the same direction as caused by the disease processes, several are antioxidant genes that are upregulated by Protandim and reported to be upregulated in colon carcinoma and Alzheimer disease. A likely explanation for the increased expression of GLRX2 (glutaredoxin 2) and NQO1 (NAD(P)H dehydrogenase, quinone 1) in colon carcinoma and of GLRX (glutaredoxin), HMOX1 (heme oxygenase-1), NQO1, and SOD1 (superoxide dismutase 1) in Alzheimer is that it represents an adaptive attempt to partially compensate for the increased level of oxidative stress associated with these diseases. These antioxidant genes are also upregulated by Protandim, which would provide additional antioxidant protection beyond that achieved by the ROS-dependent induction of these enzymes in the diseased tissues.

### 3.2.2. Do different Nrf2 activators produce identical gene expression patterns?

While Protandim, bardoxolone methyl, BG-12, and sulforaphane all have been demonstrated to modify gene expression profiles by activation of Nrf2, they have not been compared side by side, in the same cell line, under identical conditions. It is nearly certain that none of them is *exclusively* an Nrf2 activator, so significant differences may exist among their gene expression profiles. These differences would reflect differences in activation of transcription factors other than Nrf2, and could produce additional positive effects or could be responsible for unwanted or adverse effects. A published report exists providing a comparison between gene expression profiles for Keap-1-null mice (which have constitutive and presumably *pure* Nrf2 activation) and wild-type mice treated with CDDO-Imidazole, a derivative similar to bardoxolone methyl (Yates et al., 2009). Indeed, significant differences in gene expression patterns were seen in livers of mice from these two groups, particularly with regard to genes involved in detoxification and lipid metabolism. A similar study has recently been published comparing sulforaphane modulated gene expression to Keap-1 knockdown in the non-malignant human breast epithelial cell line MCF10 (Agyeman et al., 2011). Similar patterns were observed by both microarray and proteomic analysis. Using the microarray data, only 14% of the genes modulated by sulforaphane were similarly modulated by Keap-1 knockdown, indicating that the majority of sulforaphane-regulated transcripts appear not to be regulated through the KEAP1/NRF2 pathway.

### 3.3. Prospects for human therapeutic applications of Nrf2 activators

Results of bardoxolone methyl therapy in a Phase II human clinical trial for chronic kidney disease in type II diabetics were recently reported (Pergola et al., 2011). After 52 weeks, the estimated glomerular filtration rate in the 75 mg/day treatment group had increased by  $10.5 \pm 1.8$  ml/min/1.73 m<sup>2</sup> ( $p < 0.001$ ), representing an increase of about 32% when compared to entry values. The study suggests that Nrf2 activation represents a viable new therapeutic approach for renal disease, as similar results are not achievable with currently available therapies.

Patients with relapsing-remitting multiple sclerosis treated with BG-12 for 24 weeks showed significantly fewer new gadolinium-enhancing lesions, with significantly reduced probability of their evolution to T1-hypointense lesions than patients treated with placebo (Macmanus et al., 2011). BG-12 treatment reduced the annualized relapse rate by 32% (Kappos et al., 2008). These studies suggest that Nrf2 activation may represent a promising new therapeutic approach for multiple sclerosis.

The early successes of these two experimental Nrf2-activating drugs in diseases where currently used therapies have largely failed inspire hope for the future. These Nrf2 activators may well spawn a new class of drugs to target the so-called “diseases of aging”, including cancer, cardiovascular diseases, inflammatory and autoimmune diseases, and neurodegenerative diseases.

#### 4. Concluding remarks

The fact that as many as 200 human diseases have been associated with increased levels of oxidative stress has always been puzzling. Oxidative stress, because it is tied to mitochondrial oxidation of foodstuff and the generation of the energy necessary to sustain life, occupies a place of central importance. Even though reactive oxygen species are capable of disrupting nearly any metabolic pathway through their attack on proteins, lipids, and nucleic acids, is it reasonable that exposure to reactive oxygen species alone could cause such a diversity of disease processes? That still may be the case, and such arguments are supported by the early experiments to use SOD as a drug. It actually appeared to work *single-handedly* in many clinical and laboratory applications. A more useful paradigm, however, may be to focus on Nrf2 as the regulator of several thousand genes, including, not coincidentally, the family of antioxidant enzymes. Thus, if the real initiator of a disease process is dysfunctional activation of Nrf2, oxidative stress would inevitably be a symptom associated with whatever else may result. That is to say, oxidative stress may indeed be *associated* with 200 diseases, and even *contributory* to all of them, but not necessarily *causative* in every case. The data in Table 1 seem to support this view. Of the 66 Protandim-regulated genes that are associated with Alzheimer disease, only five (SOD1, NQO1, HMOX1, GLRX, and TXN) appear to be in the antioxidant family. Protandim upregulated all five of them, but clearly there is more to the story than genes associated with oxidative stress. The focus on Nrf2 will not only broaden our view, it will provide practical solutions.

#### Disclosure statement

Dr. McCord is Chief Science Officer for LifeVantage Corp. (the manufacturer of Protandim, used in this study, and primary sponsor of the project). He holds equity in the Company and serves on its Board of Directors. Dr. Hybertson serves as a paid consultant to the Company, and holds equity. Dr. Gao holds equity in the Company.

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#### References

- Agyeman, A.S., Chaerkady, R., Shaw, P.G., Davidson, N.E., Visvanathan, K., Pandey, A., Kensler, T.W., 2011. Transcriptomic and proteomic profiling of KEAP1 disrupted and sulforaphane-treated human breast epithelial cells reveals common expression profiles. *Breast Cancer Res. Treat.* 2011 [Epub ahead of print].
- Albrecht, P., Lewerenz, J., Dittmer, S., Noack, R., Maher, P., Methner, A., 2010. Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system xc<sup>-</sup> as a neuroprotective drug target. *CNS Neurol. Disord.: Drug Targets* 9, 373–382.
- Babior, B.M., Kipnes, R.S., Curnutte, J.T., 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* 52, 741–744.
- Baker, D.A., McFarland, K., Lake, R.W., Shen, H., Tang, X.C., Toda, S., Kalivas, P.W., 2003. Neuroadaptations in cystine–glutamate exchange underlie cocaine relapse. *Nat. Neurosci.* 6, 743–749.
- Bogaard, H.J., Natarajan, R., Henderson, S.C., Long, C.S., Kraskauskas, D., Smithson, L., Ockaili, R., McCord, J.M., Voelkel, N.F., 2009. Chronic pulmonary artery pressure elevation is insufficient to explain right heart failure. *Circulation* 120, 1951–1960.
- Cao, Z., Zhu, H., Zhang, L., Zhao, X., Zweier, J.L., Li, Y., 2006. Antioxidants and phase 2 enzymes in cardiomyocytes: chemical inducibility and chemoprotection against oxidant and simulated ischemia–reperfusion injury. *Exp. Biol. Med. (Maywood)* 231, 1353–1364.
- Chan, K.H., Ng, M.K., Stocker, R., 2011. Haem oxygenase-1 and cardiovascular disease: mechanisms and therapeutic potential. *Clin. Sci. (Lond.)* 120, 493–504.
- Chen, B., Rao, X., House, M.G., Nephew, K.P., Cullen, K.J., Guo, Z., 2011. GPx3 promoter hypermethylation is a frequent event in human cancer and is associated with tumorigenesis and chemotherapy response. *Cancer Lett.* 309, 37–45.
- Chen, C.Y., Jang, J.H., Li, M.H., Surh, Y.J., 2005. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem. Biophys. Res. Commun.* 331, 993–1000.
- Cheung, K.L., Kong, A.N., 2010. Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. *AAPS J.* 12, 87–97.
- Church, S.L., Grant, J.W., Ridnour, L.A., Oberley, L.W., Swanson, P.E., Meltzer, P.S., Trent, J.M., 1993. Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. *Proc. Natl. Acad. Sci. USA* 90, 3113–3117.
- Cushing, L.S., Decker, W.E., Santos, F.K., Schulte, T.L., Huber, W., 1973. Orgotein therapy for inflammation in horses. *Mod. Vet. Pract.* 54, 17–21.
- Dinkova-Kostova, A.T., Holtzclaw, W.D., Cole, R.N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., Talalay, P., 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. USA* 99, 11908–11913.
- Gao, B., Flores, S.C., Leff, J.A., Bose, S.K., McCord, J.M., 2003. Synthesis and anti-inflammatory activity of a chimeric recombinant superoxide dismutase: SOD2/3. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 284, L917–L925.
- Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P., Fenn, W.O., 1954. Oxygen poisoning and X-irradiation: a mechanism in common. *Science* 119, 623–626.
- Giudice, A., Montella, M., 2006. Activation of the Nrf2-ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* 28, 169–181.
- Granger, D.N., Rutili, G., McCord, J.M., 1981. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* 81, 22–29.
- Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298–300.

- Hernandez-Saavedra, D., Zhou, H., McCord, J.M., 2005. Anti-inflammatory properties of a chimeric recombinant superoxide dismutase: SOD2/3. *Biomed. Pharmacother.* 59, 204–208.
- Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.L., Scherer, B., Sinclair, D.A., 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425, 191–196.
- Hu, R., Saw, C.L., Yu, R., Kong, A.N., 2010. Regulation of Nrf2 signaling for cancer chemoprevention: antioxidant coupled with anti-inflammatory. *Antioxid. Redox Signal.* 13, 1679–1698.
- Huang, H.C., Nguyen, T., Pickett, C.B., 2002. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* 277, 42769–42774.
- Huber, W., Schulte, T.L., Carson, S., Goldhamer, R.E., Vogin, E.E., 1968. Some chemical and pharmacologic properties of a novel anti-inflammatory protein. *Toxicol. Appl. Pharmacol.* 12, 308.
- Itoh, K., Ishii, T., Wakabayashi, N., Yamamoto, M., 1999a. Regulatory mechanisms of cellular response to oxidative stress. *Free Radic. Res.* 31, 319–324.
- Itoh, K., Mochizuki, M., Ishii, Y., Ishii, T., Shibata, T., Kawamoto, Y., Kelly, V., Sekizawa, K., Uchida, K., Yamamoto, M., 2004. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2). *Mol. Cell. Biol.* 24, 36–45.
- Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., Yamamoto, M., 1999b. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86.
- Kang, H.T., Lee, K.B., Kim, S.Y., Choi, H.R., Park, S.C., 2011. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS ONE* 6, e23367.
- Kang, K.W., Lee, S.J., Park, J.W., Kim, S.G., 2002. Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. *Mol. Pharmacol.* 62, 1001–1010.
- Kappos, L., Gold, R., Miller, D.H., Macmanus, D.G., Havrdova, E., Limmroth, V., Polman, C.H., Schmierer, K., Yousry, T.A., Yang, M., Eraksoy, M., Meluzinova, E., Rektor, I., Dawson, K.T., Sandrock, A.W., O'Neill, G.N., 2008. Efficacy and safety of oral fumarate in patients with relapsing–remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. *Lancet* 372, 1463–1472.
- Kawai, Y., Garduno, L., Theodore, M., Yang, J., Arinze, I.J., 2011. Acetylation–deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *J. Biol. Chem.* 286, 7629–7640.
- Kikuchi, N., Ishii, Y., Morishima, Y., Yageta, Y., Haraguchi, N., Itoh, K., Yamamoto, M., Hizawa, N., 2010. Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. *Respir. Res.* 11, e31.
- Liu, J., Gu, X., Robbins, D., Li, G., Shi, R., McCord, J.M., Zhao, Y., 2009. Protandim, a fundamentally new antioxidant approach in chemoprevention using mouse two-stage skin carcinogenesis as a model. *PLoS ONE* 4, e5284.
- Ludwig, G., 1991. Evaluation of conservative therapeutic approaches to Peyronie's disease (fibrotic induration of the penis). *Urol. Int.* 47, 236–239.
- Macmanus, D.G., Miller, D.H., Kappos, L., Gold, R., Havrdova, E., Limmroth, V., Polman, C.H., Schmierer, K., Yousry, T.A., Eraksoy, M., Meluzinova, E., Dufek, M., Yang, M., O'Neill, G.N., Dawson, K., 2011. BG-12 reduces evolution of new enhancing lesions to T1-hypointense lesions in patients with multiple sclerosis. *J. Neurol.* 258, 449–456.
- Marberger, H., Bartsch, G., Huber, W., Menander, K.B., Schulte, T.L., 1975. Orgotein: a new drug for the treatment of radiation cystitis. *Curr. Ther. Res.* 18, 466–475.
- McCord, J.M., 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* 185, 529–531.
- McCord, J.M., 1985. The role of superoxide in post-ischemic tissue injury. In: Oberley, L. (Ed.), *Superoxide Dismutase*, vol. III. Pathological States CRC Press, Boca Raton, pp. 143–150.
- McCord, J.M., Fridovich, I., 1968. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* 243, 5753–5760.
- McCord, J.M., Keele Jr., B.B., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244, 6049–6055.
- McCord, J.M., Keele Jr., B.B., Fridovich, I., 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 68, 1024–1027.
- McCord, J.M., Wong, K., Stokes, S.H., Petrone, W.F., English, D., 1980. Superoxide and inflammation: a mechanism for the anti-inflammatory activity of superoxide dismutase. *Acta Physiol. Scand. Suppl.* 492, 25–30.
- McGinness, J.E., Proctor, P.H., Demopoulos, D.S., Hokanson, A., Kirkpatrick, D.S., 1977. Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase). *Physiol. Chem. Phys.* 10, 267–277.
- McIlwain, H., Silverfield, J.C., Cheatum, D.E., Pooley, J., Taborn, J., Ignaczak, T., Multz, C.V., 1989. Intra-articular orgotein in osteoarthritis of the knee: a placebo-controlled efficacy, safety, and dosage comparison. *Am. J. Med.* 87, 295–300.
- Moi, P., Chan, K., Asunis, I., Cao, A., Kan, Y.W., 1994. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. USA* 91, 9926–9930.
- Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicol. Sci.* 102, 110–119.
- Nelson, S.K., Bose, S.K., Grunwald, G.K., Myhill, P., McCord, J.M., 2006. The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy. *Free Radic. Biol. Med.* 40, 341–347.
- Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S., Yoshida, T., 2003. Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am. J. Physiol. Cell Physiol.* 285, C334–C342.
- Pergola, P.E., Raskin, P., Toto, R.D., Meyer, C.J., Huff, J.W., Grossman, E.B., Krauth, M., Ruiz, S., Audhya, P., Christ-Schmidt, H., Wittes, J., Warnock, D.G., 2011. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N. Engl. J. Med.* 365, 327–336.
- Petrone, W.F., English, D.K., Wong, K., McCord, J.M., 1980. Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. *Proc. Natl. Acad. Sci. USA* 77, 1159–1163.
- Qureshi, M.M., McClure, W.C., Arevalo, N.L., Rabon, R.E., Mohr, B., Bose, S.K., McCord, J.M., Tseng, B.S., 2010. The dietary supplement Protandim decreases plasma osteopontin and markers of oxidative damage in muscular dystrophy mdx mice. *J. Diet. Suppl.* 7, 159–178.
- Robbins, D., Gu, X., Shi, R., Liu, J., Wang, F., Ponville, J., McCord, J.M., Zhao, Y., 2010. The chemopreventive effects of Protandim: modulation of p53 mitochondrial translocation and apoptosis during skin carcinogenesis. *PLoS ONE* 5, e11902.
- Salin, M.L., McCord, J.M., 1975. Free radicals and inflammation. Protection of phagocytosine leukocytes by superoxide dismutase. *J. Clin. Invest.* 56, 1319–1323.
- Sanchiz, F., Milla, A., Artola, N., Julia, J.C., Moya, L.M., Pedro, A., Vila, A., 1996. Prevention of radioinduced cystitis by orgotein: a randomized study. *Anticancer Res.* 16, 2025–2028.
- Seidel, K., Vinet, J., den Dunnen, W.F., Brunt, E.R., Meister, M., Boncoraglio, A., Zijlstra, M.P., Boddeke, H.W., Rub, U., Kampinga, H.H., Carra, S., 2011. The HSPB8–BAG3 chaperone complex is upregulated in astrocytes in the human brain affected by protein aggregation diseases. *Neuropathol. Appl. Neurobiol.* 2011. doi:10.1111/j.1365-2990.2011.01198.x [Epub ahead of print].
- Surh, Y.J., Kundu, J.K., Na, H.K., 2008. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med.* 74, 1526–1539.
- Tai, H.H., Ensor, C.M., Tong, M., Zhou, H., Yan, F., 2002. Prostaglandin catabolizing enzymes. *Prostaglandins Other Lipid Mediat.* 68–69, 483–493.
- Tang, Q., Jiang, D., Shao, Z., Martinez Gomez, J.M., Schwarz, H., 2011. Species difference of CD137 ligand signaling in human and murine monocytes. *PLoS ONE* 6, e16129.
- Tomobe, K., Shinozuka, T., Kuroiwa, M., Nomura, Y., 2011. Age-related changes of Nrf2 and phosphorylated GSK-3beta in a mouse model of accelerated aging (SAMP8). *Arch. Gerontol. Geriatr.* 2011 [Epub ahead of print].
- Ungvari, Z., Bagi, Z., Feher, A., Recchia, F.A., Sonntag, W.E., Pearson, K., de Cabo, R., Csizsar, A., 2010. Resveratrol confers endothelial protection via activation of the antioxidant transcription factor Nrf2. *Am. J. Physiol. Heart Circ. Physiol.* 299, H18–H24.

- Ungvari, Z.I., Bailey-Downs, L., Sosnowska, D., Gautam, T., Koncz, P., Losonczy, G., Ballabh, P., de Cabo, R., Sonntag, W.E., Csiszar, A., 2011. Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of Nrf2-mediated antioxidant response. *Am. J. Physiol. Heart Circ. Physiol.* 301, H363–H372.
- Velmurugan, K., Alam, J., McCord, J.M., Pugazhenth, S., 2009. Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim. *Free Radic. Biol. Med.* 46, 430–440.
- Venugopal, R., Jaiswal, A.K., 1996. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. USA* 93, 14960–14965.
- Vetrone, S.A., Montecino-Rodriguez, E., Kudryashova, E., Kramerova, I., Hoffman, E.P., Liu, S.D., Miceli, M.C., Spencer, M.J., 2009. Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-beta. *J. Clin. Invest.* 119, 1583–1594.
- Wang, X.J., Hayes, J.D., Wolf, C.R., 2006. Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of Nrf2 by cancer chemotherapeutic agents. *Cancer Res.* 66, 10983–10994.
- Wei, L., Liu, J., Le, X.C., Han, Y., Tong, Y., Lau, A.S., Rong, J., 2011. Pharmacological induction of leukotriene B4-12-hydroxydehydrogenase suppresses the oncogenic transformation of human hepatoma HepG2 cells. *Int. J. Oncol.* 39, 735–745.
- Wild, A.C., Moinova, H.R., Mulcahy, R.T., 1999. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J. Biol. Chem.* 274, 33627–33636.
- Wilms, H., Sievers, J., Rickert, U., Rostami-Yazdi, M., Mrowietz, U., Lucius, R., 2010. Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1beta, TNF-alpha and IL-6 in an in-vitro model of brain inflammation. *J. Neuroinflamm.* 7, 30.
- Wu, M., Xu, L.G., Su, T., Tian, Y., Zhai, Z., Shu, H.B., 2004a. AMID is a p53-inducible gene downregulated in tumors. *Oncogene* 23, 6815–6819.
- Wu, Z.J., Irizarry, R.A., Gentleman, R., Martinez-Murillo, F., Spencer, F., 2004b. A model-based background adjustment for oligonucleotide expression arrays. *J. Am. Stat. Assoc.* 99, 909–917.
- Yao, H., Li, P., Venters, B.J., Zheng, S., Thompson, P.R., Pugh, B.F., Wang, Y., 2008. Histone Arg modifications and p53 regulate the expression of OKL38, a mediator of apoptosis. *J. Biol. Chem.* 283, 20060–20068.
- Yates, M.S., Tran, Q.T., Dolan, P.M., Osburn, W.O., Shin, S., McCulloch, C.C., Silkworth, J.B., Taguchi, K., Yamamoto, M., Williams, C.R., Libby, K.T., Sporn, M.B., Sutter, T.R., Kensler, T.W., 2009. Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid treated mice. *Carcinogenesis* 30, 1024–1031.
- Yu, R., Lei, W., Mandlekar, S., Weber, M.J., Der, C.J., Wu, J., Kong, A.N., 1999. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *J. Biol. Chem.* 274, 27545–27552.
- Zheng, J., Chen, Y., Pat, B., Dell'italia, L.A., Tillson, M., Dillon, A.R., Powell, P.C., Shi, K., Shah, N., Denney, T., Husain, A., Dell'Italia, L.J., 2009. Microarray identifies extensive downregulation of noncollagen extracellular matrix and profibrotic growth factor genes in chronic isolated mitral regurgitation in the dog. *Circulation* 119, 2086–2095.
- Zhong, L., Liu, Z., Yan, R., Johnson, S., Zhao, Y., Fang, X., Cao, D., 2009. Aldo-keto reductase family 1 B10 protein detoxifies dietary and lipid-derived alpha, beta-unsaturated carbonyls at physiological levels. *Biochem. Biophys. Res. Commun.* 387, 245–250.