Phenolic Michael Reaction Acceptors: Combined Direct and Indirect Antioxidant Defenses Against Electrophiles and Oxidants

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Abstract: The implications of oxidative stress in the pathogenesis of many chronic human diseases has led to the widely accepted view that low molecular weight antioxidants could be beneficial and postpone or even prevent these diseases. Small molecules of either plant or synthetic origins, which contain Michael acceptor functionalities (olefins or acetylenes conjugated to electron-withdrawing groups) protect against the toxicity of oxidants and electrophiles *indirectly*, i.e., by inducing phase 2 cytoprotective enzymes. Some of these molecules, e.g., flavonoid and curcuminoid analogues that have phenolic hydroxyl groups in addition to Michael acceptor centers, are also potent *direct* antioxidants, and may therefore be appropriately designated: *bifunctional* antioxidants. By use of spectroscopic methods we identified phenolic chalcone and bis(benzylidene)acetone analogues containing one or two Michael acceptor groups, respectively, as very efficient scavengers of two different types of radicals: (a) the nitrogen-centered 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation, and (b) the oxygen-centered galvinoxyl (phenoxyl) radical. The most potent scavengers are those also bearing hydroxyl substituents on the aromatic ring(s) at the *ortho*-position(s). The initial reaction velocities are very rapid and concentration-dependent. In the human keratinocyte cell line HaCaT, the same compounds coordinately increase the intracellular levels of glutathione, glutathione reductase, and thioredoxin reductase. Thus, such *bifunctional* antioxidants could exert synergistic protective effects against oxidants and electrophiles which represent the principal biological hazards by: (i) scavenging hazardous oxidants directly and immediately; and (ii) inducing the phase 2 response to prevent and resolve the consequences of hazardous processes that are already in progress, i.e., acting indirectly, but with much more diverse and long-lasting effects.

Key Words: ABTS radical, direct antioxidant, indirect antioxidant, phase 2 inducer, phenolic Michael acceptor, phenoxyl radical, galvinoxyl radical, glutathione.

INTRODUCTION

Oxidants and electrophiles are responsible for the major toxicities that continually impact aerobic organisms and bear major responsibility for the pathogenesis of cancer, other chronic degenerative diseases, and aging [1]. Elaborate intrinsic protective mechanisms have evolved to ameliorate these damaging effects.

Electrophiles are detoxified by a group of highly diversified and inducible phase 2 enzymes which include glutathione *S*-transferases, UDP-glucuronosyltransferases, NAD(P)H: quinone oxidoreductase 1 (NQO1), and epoxide hydrolase. There is now much evidence that phase 2 enzymes do not normally operate at their full capacity, that they can be transcriptionally induced in most tissues, and that such induction is a highly effective and sufficient strategy for achieving protection against neoplasia and many types of toxicities [reviews 2-5]. Phase 2 genes are coordinately induced by common molecular mechanisms regulated by a plethora of chemical agents that belong to at least 10 chemical classes. The only common property of these inducers is their chemical reactivity with sulfhydryl groups whereby they target and modify specific and highly reactive cysteine thiols of Keap1, the intracellular sensor for inducers [6].

Cellular protection against oxidative stress is a more elaborate process than protection against electrophiles. It can be considered as involving two types of mechanisms. (a) *Direct* antioxidants. These low molecular weight compounds (e.g., ascorbate, glutathione, tocopherols, lipoic acid, vitamins K, ubiquinones, carotenes) can undergo redox reactions and scavenge reactive oxidation products (e.g., peroxides) as well as reactive and radical oxygen and nitrogen species. Direct antioxidants are all redox active, are consumed or modified in the process of their antioxidant action and require to be replenished or regenerated. (b) *Indirect* antioxidants comprise a wide range of chemically diverse inducers of the cytoprotective phase 2 response. These agents may or may not be redox active, but exert many of their effects through upregulation of phase 2 enzymes that act catalytically, have long half-lives like many other proteins, and display a wide variety of antioxidative activities, in addition to their capacities to detoxify electrophiles.

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However, the distinction between direct and indirect antioxidants, is complicated by a close reciprocal relation between these two types of agents. Some examples follow. (i) Whereas glutathione (GSH) is the principal protective direct antioxidant that is present in high (millimolar) concentrations in tissues, the rate of its synthesis is controlled by γ-glutamylcysteine synthetase, a typical phase 2 enzyme that is upregulated by phase 2 inducers. These are by definition indirect antioxidants. The complexity of this reciprocal relation is further compounded by the obligatory participation of GSH in the activities of several phase 2 enzymes (glutathione *S*-transferases, glutathione reductase, glutathione *S*-conjugate efflux pumps). (ii) At least one phase 2 enzyme, heme oxygenase 1, generates carbon monoxide and biliverdin/bilirubin which are small direct antioxidant molecules. (iii) Some direct antioxidants are inducers of the phase 2 response, e.g., the vicinal dithiol lipoic acid and reduced Michael reaction acceptors such as hydroquinones. (iv) The phase 2 enzymes NQO1 and glutathione reductase are responsible for the regeneration of the reduced and active forms of oxidized tocopherols and ubiquinone, respectively [7].

Our long-term interest in the relation of structure of phase 2 inducers to their inducer potencies identified 10 chemical classes of inducers [6]. Michael acceptors (olefins conjugated to electron-withdrawing groups) feature prominently among these agents [8,9] and their potencies are closely related to their reactivities with nucleophiles, and especially thiol groups [8,10]. Our previous studies focused on aromatic Michael acceptors, including phenylpropenoids, such as chalcones and flavonoids, bis(benzylidene) alkanones, bis(benzylidene)cycloalkanones, and curcumin derivatives [9-11]. Several generalizations emerged: (i) inducer potencies were markedly enhanced by the presence of *ortho*-hydroxyl substituents on the aromatic rings linked to the Michael acceptor groups. (ii) Michael acceptors react with certain critical and highly reactive sulfhydryl groups of Keap1, the intracellular sensor for inducers. As a consequence, Keap1 loses its ability to repress transcription factor Nrf2 which then undergoes nuclear translocation and initiates transcription of phase 2 genes [6,12]. (iii) Michael acceptor functionalities are present in many phytochemical phase 2 inducers, including flavonoids and chalcones, for which *direct* antioxidant activities have also been reported. In agreement with these findings, we showed that certain phenolic Michael reagents also powerfully inhibited the chemiluminescence arising from the reaction of lucigenin with the oxygen-centered superoxide radical, generated by the xanthine/xanthine oxidase system. Moreover the radical quenching efficiency, like the phase 2 inducer potency, was markedly increased by the presence of *ortho*-hydroxyl groups on the aromatic rings [9]. Both radical quenching and inducer potencies were also closely correlated with the velocity of reaction with model thiol compounds. This observation can now be rationalized on the basis of the reaction of these compounds with the highly reactive cysteine thiol groups of Keap1, the sensor for inducers [6,12,13].

In the present work we have gained further experimental evidence for the capacity of phenolic Michael acceptors to act both as phase 2 inducers (indirect antioxidants) and as radical quenchers (direct antioxidants). We compared the ability of these inducers to scavenge two different types of radicals: (a) the nitrogen-centered 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation (see structure in Fig. **1**); and (b) the oxygen-centered galvinoxyl (2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy) radical (see structure in Fig. **4A**). The *ortho*-hydroxylated double Michael reaction acceptor bis(2-hydroxybenzylidene)acetone **11** (Fig. **2B**) was identified as one of the most efficient scavengers of both types of radicals among a series of inducers tested. This and related compounds had been previously shown to be potent inducers of NQO1 and to elevate cellular glutathione levels in several different cell lines [10]. In the present study, using the human keratinocyte cell line HaCaT as a model system, we found that exposure to bis(2-hydroxybenzylidene)acetone **11** raised the levels of intracellular glutathione and induced glutathione reductase and thioredoxin reductase enzyme activities. Selection of keratinocytes is relevant to our studies on inhibition by phase 2 inducers of the formation of UV-light-induced skin tumors in SKH-1 hairless mice [14].

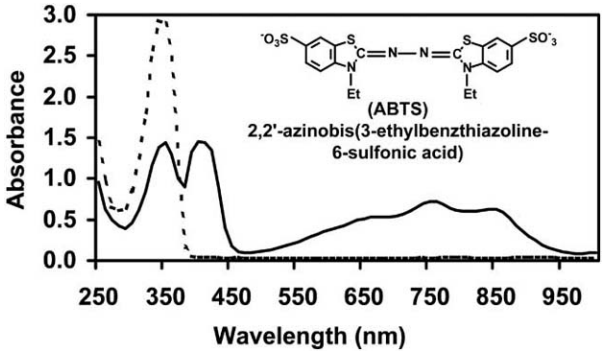

Fig. (1). Ultraviolet-visible absorption spectra of ABTS (dotted line) and $ABTS^{\bullet+}$ radical cation (solid line). $ABTS^{\bullet+}$ was obtained by the oxidation of 7.0 mM 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by 2.45 mM $K_2S_2O_8$ in deionized double distilled water, in the dark, at 25 °C, overnight. Ethanolic solutions (50 μM) were prepared and the ultraviolet-visible absorption spectra were recorded between 250-950 nm at 10-nm intervals.

These findings now suggest the intriguing possibility that phenolic Michael acceptors can play a dual protective role behaving both as indirect (through induction of phase 2 and other antioxidant enzymes), and as direct antioxidants. They may therefore be appropriately designated: *bifunctional* antioxidants. Furthermore, phenolic Michael acceptors that are also capable of scavenging free radicals could have synergistic protective effects by diminishing hazardous oxidants (direct and instantaneous action) and inducing the phase 2 response to resolve the consequences of hazardous processes that are already in progress (indirect and long-lasting action).

RESULTS AND DISCUSSION

Scavenging of the Preformed Nitrogen-Centered $ABTS^{\bullet+}$ Radical Cation

Our previous studies demonstrated that chalcones (Structures in Fig. **2A**) which are ubiquitous in plants can scavenge the superoxide radical, as judged by their ability to decrease the lucigenin-dependent chemiluminescence in the xanthine/

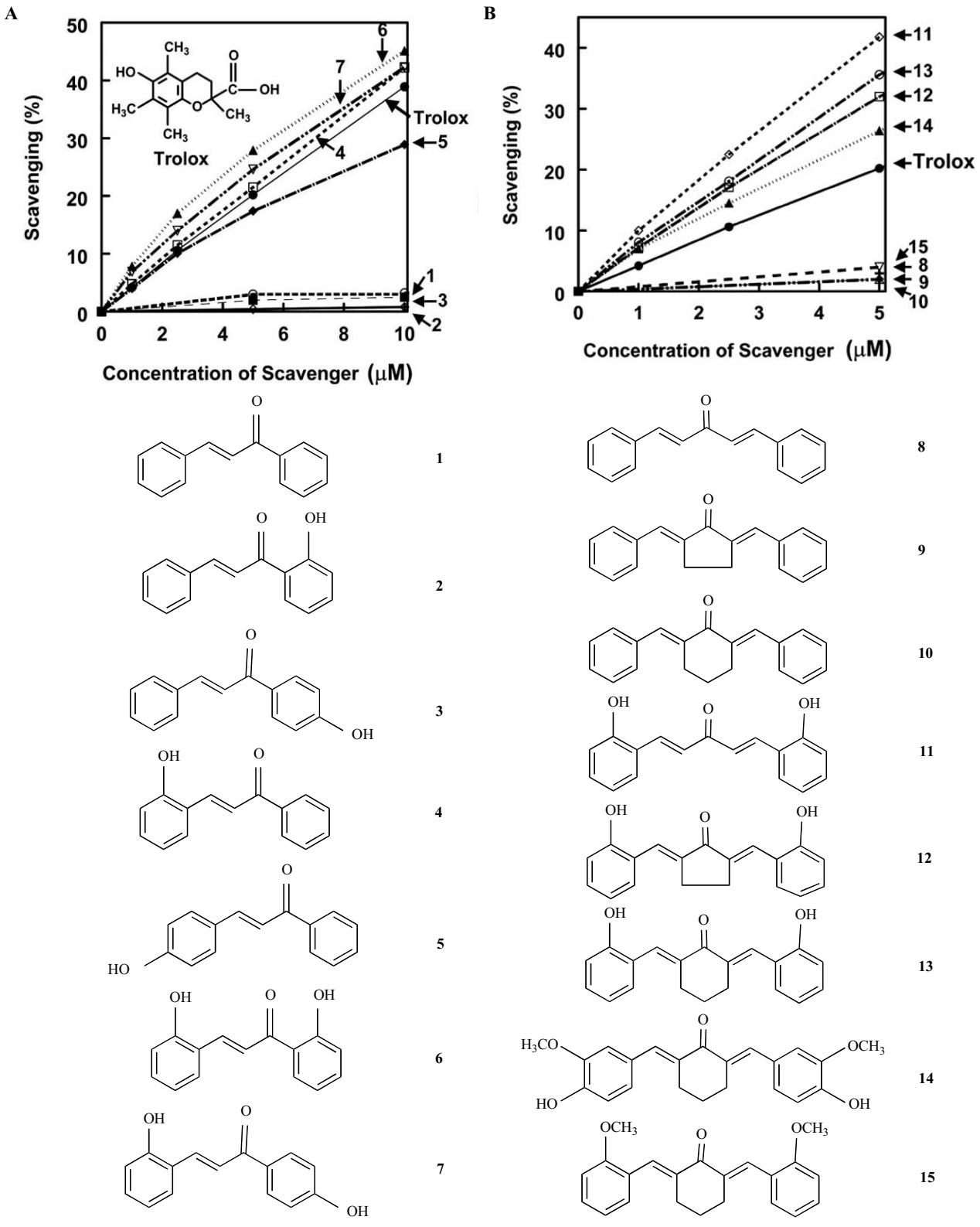

Fig. (2). Concentration dependence of scavenging of the ABTS$^{\bullet+}$ radical cation by chalcones (A) and double Michael reaction acceptors (B). The decrease in absorbance of the ABTS$^{\bullet+}$ at 734 nm (ε = 16,000 M^{-1} cm^{-1}) was monitored for 15 min at 1-min intervals. Each data point represents the average of four replicates with coefficients of variation between 5 and 10% of the mean value.

xanthine oxidase system [9]. In the present study, we first evaluated the ability of chalcones to scavenge the nitrogen-centered 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation. $ABTS^{\bullet+}$ was generated by addition of potassium persulfate ($K_2S_2O_8$) to a solution of 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid). Its formation or quenching can be monitored by the absorbance at 734 nm (ε = 16,000 M^{-1} cm^{-1}), which is unique to the radical species and absent in the spectrum of its precursor (Fig. **1**). Thus, the $ABTS^{\bullet+}$ radical cation decolorization assay [15] was adapted for microtiter plates. The blue/green radical initially formed in a reaction with potassium persulfate, was added to microtiter wells containing serial dilutions of each chalcone. Immediately after mixing, antioxidant activity was evaluated spectrophotometrically (734 nm) from the fraction of the total amount of radical that was decolorized. The absorbance remaining after 2 min was used to calculate the percent scavenging at each concentration of the antioxidant and was compared to that of a standard antioxidant, the synthetic vitamin E analogue Trolox (structure in Fig. **2A**). Of note, even though the degree of scavenging (i.e., the decrease in absorbance) was dependent on the concentration of the scavenger, in contrast to Trolox the progress curves for the chalcones were not linear, indicating possible occurrence of side reactions affecting the subsequent collisions (Fig. **2A**). Moreover, the stoichiometries of these reactions are very complicated such that one molecule of an active chalcone appears capable of scavenging more than one molecule of $ABTS^{\bullet+}$. This finding is in full agreement with previous reports on the reactions of $ABTS^{\bullet+}$ with other scavengers, e.g., flavonoids, glutathione, and ascorbic acid analogues [15,16]. This high efficiency of radical scavenging, i.e., the apparent ability of each phenolic molecule to quench more than one molecule of free radical, may indicate that the scavenger is being quickly recycled/regenerated.

As expected, the unsubstituted chalcone **1** was inactive (Fig. **2A**) since it has been previously demonstrated by others that phenolic hydroxyl groups are required for the antioxidant activity of flavonoids. Surprisingly, the 2'- or 4'-monohydroxylated A ring phenols **2** and **3**, respectively, were also inactive. The possibility was therefore considered whether the phenolic hydroxyl groups of **2** and **3** are hydrogen bonded and therefore unavailable for reaction with $ABTS^{\bullet+}$. Indeed, their ^{1}H NMR spectra revealed that the chemical shift corresponding to the phenolic proton of 2'-hydroxychalcone **2** is at 12.5 ppm (Table **1**) supporting its involvement in intramolecular hydrogen bonding [17]. On the other hand, the chemical shift of the phenolic proton of 4'-hydroxychalcone **3** is at 3.2 ppm, an indication of its possible participation in intermolecular hydrogen bonding [18]. In contrast, the chemical shifts of the respective phenolic protons of chalcones **4** and **5**, at positions 2 and 4 (on the B-ring) were at 10.7 and 10.1 ppm, typical for free hydroxyl groups. Importantly, the corresponding phenolic hydroxyl groups conferred antioxidant (radical quenching) activity on **4** and **5,** comparable to that of Trolox (Fig. **2A**). Surprisingly, the simultaneous presence of two hydroxyl groups at positions 2 and 2,' i.e., in 2,2'-dihydroxychalcone **6**, resulted in a more potent quencher than the monohydroxylated 2-hydroxychalcone **4**. Moreover, its potency was at least equal to that of the trihydroxylated chalcone **7**, for which the expected chemical shifts of its free phenolic protons were observed (Table **1**).

Table 1. ^{1}H NMR Chemical Shifts (δ) in ppm for Hydroxyl Protons of Chalcones

Chalcone	δ (ppm)
2'-Hydroxychalcone 2	12.5
4'-Hydroxychalcone 3	3.2
2-Hydroxychalcone 4	10.7
4-Hydroxychalcone 5	10.1
2,2'-Dihydroxychalcone 6	12.6, 10.4
2,2',4'-Trihydroxychalcone 7	10.6, 10.3, 9.5

^{1}H NMR spectra were obtained in DMSO-d_6 at 300 MHz.

Attention was next directed to the structurally related bis(benzylidene) derivatives and their ability to scavenge the $ABTS^{\bullet+}$ radical was evaluated in an analogous manner. We have previously reported that these compounds are much more potent inducers of phase 2 enzymes than the chalcones [9,10]. As shown in Fig. **2B**, this assay revealed that the hydroxylated bis(benzylidene)alkanones are also much more potent antioxidants than the chalcones. Moreover, they were found to exceed in potency even the standard antioxidant Trolox. As in the chalcone series, the presence of phenolic hydroxyl groups was found to be essential for antioxidant activity, since the unsubstituted bis(benzylidene)alkanones **8**-**10**, as well as 2,6-bis(2-methoxybenzylidene)cyclohexanone **15**, in which the hydroxyl groups at the *ortho*-positions are substituted with methoxyl, were inactive. The most potent scavengers are those bearing hydroxyl substituents on the aromatic rings at the *ortho*-positions, i.e., **11**-**13**. It should be pointed out that these are precisely the most potent phase 2 protein inducers in this series [9,10]. Furthermore, inducer potency correlates with radical-scavenging activity and the rank order of potencies in both assays are almost identical. These results are in agreement with our previous findings that: (i) the tendency of these molecules to release electrons as determined by the energy of the highest occupied molecular orbital (E_{HOMO}), correlates linearly with their inducer potency [19]; and (ii) Keap1, the cellular sensor for inducers, is endowed with essential highly reactive cysteine residues that are chemically modified by inducers by oxidation, alkylation, or thiol-disulfide interchange and appears to be at the heart of sensing electrophile and oxidative stress *via* a redox mechanism [6].

Inhibition of $ABTS^{\bullet+}$ Radical Cation Formation

With the most potent compound in this series, i.e., bis(2-hydroxybenzylidene)acetone **11** in hand, we examined its direct ability to inhibit the formation of the $ABTS^{\bullet+}$ radical cation. At an excess concentration of $K_2S_2O_8$, the rate of radical formation is linearly proportional to the concentration of its precursor (Fig. **3**, inset). Once the two reactants (ABTS and $K_2S_2O_8$) are mixed, the reaction proceeds at a constant rate, as monitored by the increase in the absorbance at 734 nm (Fig. **3**). Addition of bis(2-hydroxybenzylidene)acetone **11** leads to an apparent decrease of this rate presumably be-

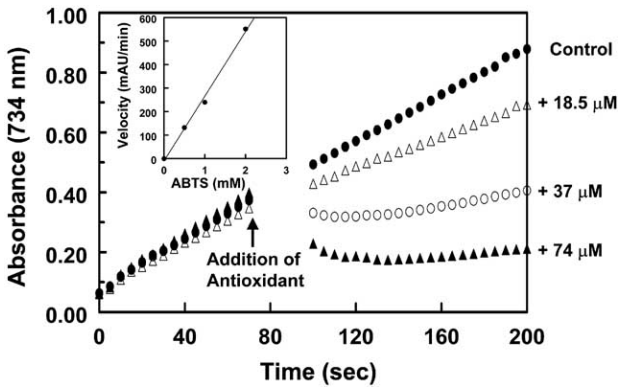

Fig. (3). Inhibition of the formation of the ABTS$^{\bullet+}$ radical cation by increasing concentrations of bis(2-hydroxybenzylidene)acetone 11. The formation of the ABTS$^{\bullet+}$ radical cation was determined by the time-dependent increase in absorbance at 734 nm after mixing equal volumes of 2 mM ABTS in ethanol and 20 mM potassium persulfate in ethanol : H_2O (1:1) in a 1-mL system. At the 70-second time point, 5 μL of each concentration of the quencher (or solvent control) were added to the reaction mixture and the absorbance was monitored for further 100 seconds. The inset shows the linear dependence of the reaction rate on the concentration of ABTS.

cause this compound is scavenging the radical as it is being formed. The observed inhibition is dependent on the concentration of the scavenger, and radical formation resumes when the scavenger is depleted. Of note, there was no evidence of a direct interaction between bis(2-hydroxybenzylidene) acetone **11** and $K_2S_2O_8$, as judged by the absence of any change in the absorption spectrum of bis(2-hydroxybenzylidene)acetone **11** in the 250-450 nm range.

Scavenging of the Oxygen-Centered Galvinoxyl Radical

To confirm that the antioxidant activity of the compounds examined is not limited to the nitrogen-centered ABTS$^{\bullet+}$ radical cation, their ability to scavenge the oxygen-centered galvinoxyl (phenoxyl) radical was next examined. The advantages of using this radical are: (a) it is resonance-stabilized; and (b) although reactions are performed in ethanol, they are only likely to occur with good H-atom donors, a characteristic feature for an antioxidant in a biological system [20]. In addition, this presented an opportunity to compare the rank order of potency of each chalcone examined in the two assays. EPR spectroscopy was employed in order to follow the reaction progress (radical scavenging) with time. Again, the single Michael reaction acceptor-containing chalcones were evaluated first. As can be seen in Fig. **4A**, there was a good agreement between radical scavenging of galvinoxyl- and ABTS$^{\bullet+}$, i.e., chalcones **1**, **2** and **3** were inactive, **4**, **5** and **7** were similar in potencies, while the dihydroxylated derivative **6** showed the highest activity in both assays.

Addition of the double Michael acceptor bis(2-hydroxybenzylidene)acetone **11** to the galvinoxyl radical resulted in a sharp decrease of the amplitude of the galvinoxyl signal (Fig. **4B**). This decrease was very rapid initially and gradually declined with time. Although it was not possible to evaluate the reaction rate under these experimental conditions, its dependence on the concentration of the scavenger was evident. Thus, bis(2-hydroxybenzylidene)acetone **11** is a very efficient scavenger of both oxygen- and nitrogen-centered radicals.

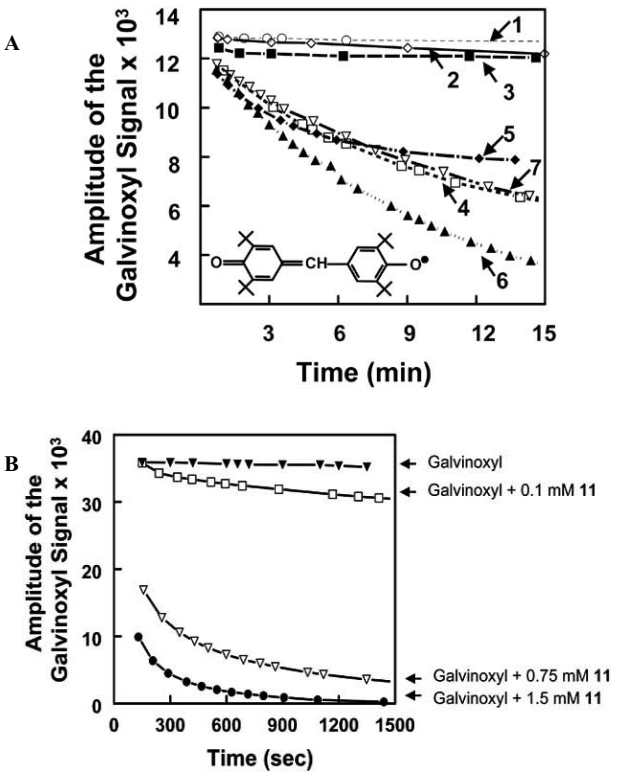

Fig. (4). Time course of the scavenging of the galvinoxyl radical cation by chalcones (A) and bis(2-hydroxybenzylidene)acetone 11 (B). Each compound was added to a solution of 0.1 mM galvinoxyl radical in ethanol and EPR spectra were immediately recorded (after ~30 seconds) at 25 ºC. The final concentration of each chalcone was 5 mM. The amount of remaining radical after mixing with the scavenger was estimated by the amplitude of its EPR signal. Each data point represents the average of three replicates with coefficient of variation less than 10% of the mean value.

Elevation of Total Glutathione Content, Glutathione Reductase and Thioredoxin Reductase Enzyme Activities

Glutathione is the primary and most abundant cellular nonprotein thiol component in both the animal and the plant kingdoms and has been implicated in virtually all major biological processes [21,22]. It constitutes a critical part of the cellular defense: it reacts readily with potentially damaging electrophiles and participates actively in the detoxification of reactive oxygen and nitrogen species and their toxic metabolites by scavenging free radicals and reducing peroxides. Most importantly, the participation of glutathione in the catalytic activities of glutathione *S*-transferases, glutathione peroxidases, glutathione reductase, thioredoxin reductase and the glutathione *S*-conjugate efflux pumps provides the living cell with multiple protection mechanisms [see 23,24 for extensive reviews]. Under normal physiological conditions, the GSH/GSSG ratio in the cell is high (>10). However, the GSH status can be compromised leading to increased suscep-

tibility to oxidative stress associated with numerous pathological conditions. It is therefore important to point out that induction of phase 2 enzymes *in vivo* has been observed more than 25 years ago to be often accompanied by elevation of intracellular glutathione [25]. Today it is known that this is a consequence, at least in part, of the induction of γ-glutamylcysteine synthetase, the enzyme catalyzing the rate-limiting step in glutathione biosynthesis [26,27].

We have previously shown that the potencies of a series of Michael reaction acceptors in elevating the specific activity of quinone reductase 1 (NQO1) correlated with those in raising GSH levels in several different cell lines and identified bis(2-hydroxybenzylidene)acetone **11** as one of the most potent inducers [10]. In the present study, we used the human keratinocyte culture HaCaT as a model system. Our choice was determined by the notions that: (a) *in vivo* the human skin is constantly exposed to pro-oxidants and pro-carcinogens and (b) increasing the antioxidant systems of skin cells could protect against the damaging effects of such noxious species. In addition, we have been interested in developing strategies for protection against UV-light-induced skin carcinogenesis through phase 2 induction [14]. We found that exposure of HaCaT cells to relatively low concentrations (1 and 5 μM) bis(2-hydroxybenzylidene)acetone **11** for 24-48 h raised the levels of glutathione, glutathione reductase and thioredoxin reductase (Fig. **5**), thus enhancing both *de novo* GSH synthesis and its regeneration.

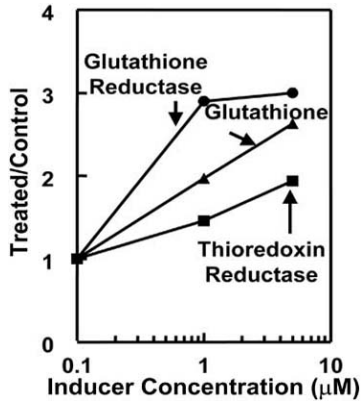

Fig. (5). Induction of glutathione (▲), glutathione reductase (•) and thioredoxin reductase (■) in HaCaT cells by bis(2-hydroxybenzylidene)acetone 11. Cells (1 x 10^6) were grown for 3 days in 10-cm dishes and then exposed to 0.1, 1.0, or 5.0 μM bis(2-hydroxybenzylidene)acetone **11** for either 24 h (for glutathione measurement) or 48 h (for enzyme activity evaluation), and finally lysed with digitonin (0.08%). The intracellular glutathione content was determined after precipitation of cellular proteins with metaphosphoric acid (50 g/L in 2 mM EDTA). Enzyme activities of cell lysate supernatants were determined spectrophotometrically in 1-mL assay systems at 25 °C by monitoring the decrease in absorbance of NADPH (340 nm) using glutathione as a substrate (glutathione reductase) or the formation of 5'-thionitrobenzoic acid (TNB) (412 nm) (thioredoxin reductase) using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) as a substrate and NADPH as a hydride donor. The specific activities are presented as ratios of treated over control cells. Each data point represents the average of four replicates with standard deviations between 5- and 10% of its ordinate value.

Elevations of the glutathione content and the activity of thioredoxin reductase by the phase 2 enzyme inducer *t*-butylhydroquinone have been observed in cortical astrocytes [28]. On this basis the authors suggested that astrocytes play a central role in protecting neurons against oxidative stress in the central nervous system. Our finding that thioredoxin reductase is induced in HaCaT cells is of particular interest, since this enzyme is known to be highly expressed in human keratinocytes and melanocytes [29,30], where it has been suggested to play a protective role against the damaging effects of free radicals on the skin surface, e.g., those generated by either UV light [31] or X-rays [30]. The ability of thioredoxin reductase to reduce dehydroascorbate and the ascorbyl free radical further supports its protective function against oxidative stress, in addition to its role in keeping thioredoxin and glutathione in their reduced states.

It is interesting to note that several research groups have reported that chalcones (e.g., 2',4'-dihydroxychalcone, 2',4', 6'-trihydroxychalcone) and bis(benzylidene) derivatives (e.g., curcumin), protect against genotoxicity and mutagenicity of a number of different mutagens (e.g, 2-nitrofluorene, 3-nitrofluoranthene, 1-nitropyrene, benzo(*a*)pyrene, isoquinoline) in both *Salmonella typhimurium* and various mammalian cell systems [32-36]. Nearly 30 years ago studies from this laboratory found that addition of the phenolic antioxidant 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) to the diet of CD-1 mice markedly decreased the levels of urinary mutagenic metabolites of benzo(*a*)pyrene [37]. This antimutagenic effect was attributed to induction of phase 2 enzymes by the protective agent leading to alterations in the metabolism of the carcinogen towards its more efficient detoxification [38].

Implications for the Mechanism of Induction of the Phase 2 Response

The requirement for a free phenolic hydroxyl group for both antioxidant and high phase 2 inducer activities and the nearly perfect correlation between radical scavenging and inducer potencies among the examined (single and double) Michael reaction acceptors raises the intriguing possibility that the ultimate chemical species that initiate the signaling cascade leading to induction of the phase 2 response by reacting with the sulfhydryl groups of the inducer sensor Keap1, might in fact be their corresponding phenoxyl radicals. Indeed, phenoxyl radicals are capable of reacting with sulfhydryl groups, including the thiols of proteins, glutathione, and thioredoxin [39,40]. We have already addressed the possibility that the most reactive cysteines of Keap1 that "sense" inducers probably have low pK_a values based on their close spatial proximity (in the context of the primary or the tertiary structure of the protein) to basic amino acid residues [12]. Their deprotonation at physiological pH will facilitate one-electron oxidation by phenoxyl radicals with the formation of thiyl radicals which may ultimately result in a disulfide bond formation within the protein. Furthermore, we have demonstrated by 2D PAGE the presence of intermolecular disulfide-linked dimers of Keap1 in extracts of cells that were treated with (2-hydroxybenzylidene)acetone **11** and their facile conversion to reduced monomers by treatment with dithiothreitol [13]. Taken together, these findings have additional implications for the

possibility that Keap1 is regenerated following reaction with phenolic Michael reaction acceptors of these types. In contrast to reaction of phenoxyl radicals with a single thiol group that may lead to irreversible thiol modification, reaction with vicinal thiols with the formation of a disulfide bridge, being fully reversible, will not lead to irreversible inactivation, and possibly subsequent destruction of the protein, but will make "regain of function" possible without the need for *de novo* synthesis.

In summary, we have found that several phenolic Michael acceptors from plants and their related synthetic analogues are potent direct antioxidants. They are also indirect antioxidants by inducing phase 2 enzymes. Especially important is the ability of such compounds to induce coordinately enzymes involved in protection against oxidative stress in cells. Taken together, these direct, as well as indirect antioxidant effects, amplify the cellular mechanisms for protection against two major classes of noxious agents, i.e., electrophiles and oxidants, both implicated in the pathogenesis of many chronic diseases.

EXPERIMENTAL

Materials

The structures of all test compounds are shown in Fig. **2** and 3. Chalcone **1**, 4'-hydroxychalcone **3**, 4-hydroxychalcone **5**, 2,6-bis(2-methoxybenzylidene)cyclohexanone **8**, ABTS, 2, 2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione, metaphosphoric acid, and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (Milwaukee, WI); 2-hydroxychalcone **2**, 2'-hydroxychalcone **4**, 2, 2'-dihydroxychalcone **6**, and 2, 2', 4'-trihydroxychalcone **7** from Indofine (Somerville, NJ). All other bis(benzylidene) alkanone compounds were synthesized as described [9,10].

Cell Culture

HaCaT human keratinocytes were a generous gift from G. Tim Bowden, Arizona Cancer Center (Tucson, AZ). Cells were grown at 37 °C in 5 % CO_2 in Eagle's minimum essential medium (EMEM) with 8% fetal bovine serum, treated with Chelex resin (Bio-Rad) to remove Ca^{2+}.

$ABTS^{\bullet+}$ Radical Cation Decolorization Assay

$ABTS^{\bullet+}$ was prepared by the oxidation of 7.0 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) by 2.45 mM $K_2S_2O_8$ in deionized double distilled water, in the dark, at 25 °C, overnight [15]. The $ABTS^{\bullet+}$ so obtained was diluted with ethanol to a concentration of 62.5 µM. Serial dilutions of each test compound were prepared in ethanol and 50-µL aliquots transferred to columns 2 through 12 of 96-well microtiter plates, while column 1 received 50 µL ethanol. Ethanol (250 µL) was then added to rows E, F, G and H (bottom half of the plate), these serving as controls for any absorbance of the test compounds themselves, while 250 µL of the 62.5 µM ethanolic solution of $ABTS^{\bullet+}$ was added to rows A, B, C and D (top half of the plate). Immediately after this last addition, the decrease in absorbance at 734 nm (ε = 16,000 M^{-1} cm^{-1}) was monitored every minute for 15 min in an optical microtiter plate reader (Molecular Devices, Sunnyvale, CA). A standard curve (a plate with serial dilutions of the synthetic vitamin E analogue Trolox) was run in each experiment.

Inhibition of the $ABTS^{\bullet+}$ Formation

In a 1-mL quartz cuvette, to a 500-µL ethanolic solution of ABTS (1.0 mM), 500-µL of 20 mM $K_2S_2O_8$ in ethanol : H_2O (1:1) were added. The reaction rate of the $ABTS^{\bullet+}$ radical cation formation was determined by the time-dependent increase in absorbance at 734 nm, which was typically followed for 200 seconds. For the inhibition studies, at the 70-second time point, 5 µL of a series of concentrations of the quencher (or solvent control) were added to the reaction mixture and the absorbance monitored in an analogous fashion.

Scavenging of the Galvinoxyl Radical

Antioxidant potential was also evaluated by the ability to scavenge 0.1 mM galvinoxyl radical in ethanol using EPR spectroscopy. The final concentration of each chalcone was at 50-fold molar excess, i.e., 5 mM. EPR spectra were recorded at 25 ºC using either a capillary (chalcones) or a quartz flat cell (compound **11**), fitted into the cavity of a Bruker ER 300 spectrometer. The amount of remaining radical after mixing with the scavenger was estimated by the amplitude of its EPR signal.

NMR Spectroscopy

^1H NMR spectra were recorded in DMSO-d_6 at 300 MHz on a Bruker AMX-300 spectrometer. The chemical shifts are relative to tetramethylsilane.

Determination of Cellular Glutathione Levels, Glutathione Reductase and Thioredoxin Reductase Activities

Cells (1 x 10^6) were grown for 3 days in 10-cm dishes, exposed to 1- or 10 µM bis(2-hydroxybenzylidene)acetone **11** for 24 h, and finally lysed in 300 µl of 0.08 % digitonin and centrifuged to remove cell debris. Ten µl were used for protein determination using the bicinchoninic acid protein assay with bovine serum albumin as a standard [41]. For determination of the intracellular glutathione content, 50 µl cell lysate supernatant was transferred to an Eppendorf tube and received 100 µl of ice-cold metaphosphoric acid (50 g/L) in 2 mM EDTA to precipitate cellular protein. After 10 min at 4 °C, tubes were centrifuged and the supernatant fractions transferred to new tubes. Three 20 µl-aliquots of each extract were transferred to the wells of a 96-well plate. Eighty µl of 200 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA, were added to each well and total cellular glutathione levels were determined by rate measurements in a recycling assay [42,43]. Glutathione reductase activity of 20 µl cell lysate supernatant fractions was determined spectrophotometrically at 25 °C by following the decrease in absorbance of NADPH (340 nm) in a 1-ml assay system according to a classical procedure [44]. Thioredoxin reductase activity was determined by the method of Arnér [45] using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) as a substrate in the presence of NADPH and monitoring the formation of 5'-thionitrobenzoic acid (TNB) spectrophotometrically by the increase in absorbance at 412 nm.

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