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Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes

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Plant chlorophylls and carotenoids are highly colored, conjugated polyenes that play central roles in photosynthesis. Other porphyrins (tetrapyrroles), such as cytochromes, which are structurally related to chlorophyll, participate in redox reactions in many living systems. An unexpected new property of tetrapyrroles, including tetramethyl coproporphyrin III, tetrabenzoporphine, copper chlorin e4 ethyl ester, and of carotenoids including zeaxanthin and α-cryptoxanthin is their ability to induce mammalian phase 2 proteins that protect cells against oxidants and electrophiles. The capacity of these compounds to induce the phase 2 response depends upon their ability or that of their metabolites to react with thiol groups, a property shared with all other classes of phase 2 inducers, which show few other structural similarities. Pseudo second-order rate constants of these inducers are correlated with their potency in inducing the phase 2 enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) in murine hepatoma cells. One of the most potent inducers was isolated from chlorophyllin, a semisynthetic water-soluble chlorophyll derivative. Although chlorophyll itself is low in inducer potency, it may nevertheless account for some of the disease-protective effects attributed to diets rich in green vegetables because it occurs in much higher concentrations in those plants than the widely studied 'phytochemicals'.

Introduction

This paper describes a broad new class of phase 2 gene inducers. These conjugated cyclic and acyclic polyenes include tetrapyrroles (e.g. chlorins, corrins and porphyrins), pyrroles, as well as extended polyolefins, and polyisoprenoids (e.g. carotenoids). Induction of the phase 2 response is being progressively recognized as an effective and sufficient strategy for protecting cells against oxidants and electrophiles, which are implicated in the etiology of cancer and chronic degenerative diseases (1–3) and which contribute to aging (4). Extensive studies on the chemistry of inducers have disclosed that all inducers are chemically reactive but are without common architectural features (5), and that all react with sulfhydryl groups (6) and target Keap1 as the cellular sensor that is integrally involved in the mechanism of induction (7–10). The known inducers belong to at least nine chemical classes (5): (i) diphenols, phenylenediamines and quinones, (ii) Michael reaction acceptors, (iii) isothiocyanates/dithiocarbamates, (iv) 1,2-dithiole-3-thiones/oxathiolene oxides, (v) hydroperoxides, (vi) trivalent arsenicals, (vii) heavy metals, (viii) vicinal dimercaptans and (ix) carotenoids.

In our pursuit of strategies for chemoprotection against cancer, we have investigated a wide range of chemical species focusing special attention on the glucosinolates and isothiocyanates from Brassica vegetables (11–13), and chlorophyllin (14), which is derived from extracts of virtually any green plant. To identify and isolate new inducers, we have utilized the Prochaska microtiter plate NQO1 assay (which measures the induction of quinone reductase, one of the quintessential phase 2 proteins). The use of this assay has also guided studies of structure–activity relationships, the synthesis of analogs, elucidation of mechanisms and assessment of potency (15,16). Although the *Brassica* vegetables have long been known to contain potent inducers of mammalian phase 2 enzymes (5), chlorophyllin has not been so identified, and its successful use in a clinical intervention was based on the assumption of an alternative mechanism of action (17,18).

Tetrapyrroles are among the most ubiquitous natural compounds (19). For example, the chlorophylls are the primary photosynthetic pigments in higher plants, algae and bacteria. Other tetrapyrroles (porphyrins, chlorins and corrins) include cobalamin (vitamin B12) and heme, which is the prosthetic group of hemoglobin, and also a component of a multitude of other oxidation–reduction and oxygen transport-related cellular proteins, such as cytochromes, apoferritin, catalase, ferrichrome and peroxidases. Synthetic or semisynthetic porphyrin derivatives are widely used in photodynamic therapy against cancer, psoriasis, and age-related macular degeneration (20). Stabilization and chemical transformation of lipophilic chlorophylls into a freely water-soluble sodium or potassium salt mixture termed 'chlorophyllin' (21) involves replacing the central coordinated Mg^{2+} with Cu^{2+}, and removal of the phytyl tail (22–24). When chlorophyllin is prepared from crude plant extracts, it contains two primary chemical species, copper chlorin e6 and copper chlorin e4.

Egner *et al*. (25) recently identified copper chlorin e4 ethyl ester, as a quantitatively minor but pharmacologically important third component of commercial chlorophyllin preparations that protected individuals exposed to dietary aflatoxin. We report here that this compound is a potent inducer of phase 2 enzymes. Thus, commercial formulations of chlorophyllins, which are widely available for control of body, fecal and urinary odor in geriatric and osteomy patients, as wound

Abbreviations: CD, concentration required to double NQO1 activity in murine hepatoma cells; NQO1, NAD(P)H:quinone oxidoreductase 1 or quinone reductase; U, unit.

healing accelerators and as food colorants (26), may have more robust and broad-based chemoprotective activity than suggested previously (14,17,27), and they may have promise as prophylactic pharmaceuticals. Chlorophyllin has been shown previously to inhibit significantly the mutagenic activity of a variety of xenobiotics by mechanisms that are likely to include: (i) direct antioxidant activity and (ii) formation of complexes with mutagens/carcinogens via strong stacking interactions, thereby facilitating the excretion of these carcinogens (27). Short-term oral administration of chlorophyllin has also been reported to increase the levels of hepatic glutathione *S*-transferase in lactating mice and suckling pups (18), to inhibit a variety of cytochromes P450 (28) and to protect against heterocyclic amine-induced genotoxicity by induction of phase 2 enzymes (29). Clinical trials with chlorophyllin have reduced aflatoxin-DNA adducts in individuals at high risk for liver cancer (14,17).

In this study, we report that chlorophyllin and a number of related tetrapyrroles, highly conjugated carotenoids and sterol-binding antifungals, are inducers of phase 2 enzymes. Notably, chlorophyll itself is a sufficiently potent inducer of these detoxification enzymes that it may account for a portion of the chemoprotective effect widely ascribed to green vegetables on the basis of epidemiologic evidence (30). Furthermore, we show that each of these new classes of phase 2 enzyme inducers can react with free sulfhydryl groups, suggesting that they can bind to the Keap1 intracellular sensor for inducers, thus triggering the phase 2 gene transcription via the ARE (antioxidant response element), as recently described (3,7–9,31).

Materials and methods

Chemicals and reagents

All reagents were obtained from Sigma-Aldrich (St Louis, MO), Fisher Scientific (Fairlawn, NJ) or Frontier Scientific (Logan, UT), unless otherwise indicated. Chlorophyllin was obtained from Bush, Boake & Allen (Sudbury, Suffolk, UK) as well as Sigma-Aldrich, and copper chlorin e4 ethyl ester was isolated as described by Egner *et al*. (25). Chlorophyll *a*, in addition to being purchased from Sigma-Aldrich, was isolated from fresh spinach leaves by extraction with acetone and thin layer chromatographic separation in hexane/diethyl ether/acetone (60/30/20, by vol). All the carotenoids used were of high purity, and were donated by Dr Frederick Khachik, who isolated and purified them, and synthesized the C_{45}- and C_{50}- β-carotenes (32).

Bioassay of phase 2 enzyme induction

The 'Prochaska' bioassay was utilized to measure the induction of NQO1 in Hepa1c1c7 murine hepatoma cells (11,15,33,34). Both sulforaphane and β-naphthoflavone were used as standards in all bioassays. Aqueous solubility of almost all the compounds studied was problematic, so that compounds to be assayed were diluted into a variety of non-aqueous solvents, either tetrahydrofuran, ethanol or a triple solvent (a mixture of equal parts of dimethyl formamide, acetonitrile and dimethyl sulfoxide). Solvent concentrations of 0.5% in culture medium (0.1% for tetrahydrofuran) were never exceeded in the microtiter plates. Plates were assayed after 48 h of induction. Mutant Hepa1c1c7 cells (BP^rc1 and c1) lacking in functional cytochrome P450 activity were kindly provided by J.P.Whitlock. The former cell line is defective in the aryl hydrocarbon receptor function, and the latter is defective in the expression of aryl hydrocarbon hydroxylase. One unit of inducer activity doubles the NQO1 specific activity in 48 h in a microtiter plate well containing 150 μl of medium. CD is the **c**oncentration required to double the NQO1 specific activity.

HepG2 human hepatoma cell lines, transfected with the EpRE/TK-GFP (green fluorescent protein) plasmid, were provided by Zhu and Fahl, University of Wisconsin (35). Compounds were dosed as described above, and activity was assessed as described by Ye and Zhang (36), with the exception that the level of cellular protein was assessed using the BCA reagent (11,15).

Assessment of thiol reactivity

Compounds to be tested were incubated with 143 mM 2-mercaptoethanol at 25°C (6) in either 95% tetrahydrofuran:5% water or 50% methanol:50% water. Decreases in absorbance were monitored at the wavelengths indicated in Table V. Pseudo second-order rate constants were calculated from the initial linear region of the kinetic curve by using the molar extinction coefficient of each compound in the respective solvent.

Results

Carotenoids

There is a dramatic (14-fold) difference in the inducer potencies of α-carotene (CD = 100 μM) and β-carotene (CD = 7.2 μM) (37; Table I). Both carotenoids have 11 double bonds, but whereas in β-carotene all double bonds are conjugated, in α-carotene one of these double bonds (in one ionone ring) is not conjugated. An analogous effect of this difference in conjugation patterns is observed in comparing inducer potencies of the doubly hydroxylated zeaxanthin (CD = 2.2 μM) and lutein (CD = 17 μM). The importance of an optimal number of conjugated double bonds on inducer potency is further emphasized by the finding that β-carotene analogs in which the chain conjugation has been extended to 13 double bonds (CD = 16 μM) or 15 double bonds (CD = 30 μM) are less potent inducers than the parent β-carotene. Hydroxylation of both ionone rings increased inducer potency markedly, as shown by comparing lutein (CD = 17 μM) with α-carotene (CD = 100 μM), and zeaxanthin (CD = 2.2 μM) with β-carotene (CD = 7.2 μM). It is more difficult to interpret the very high inducer potency of the singly hydroxylated α-cryptoxanthin (CD = 1.8 μM). The β-carotene metabolite retinoic acid did not induce NQO1, and β-ionone, a precursor used in the synthesis of retinol, was a poor inducer (data not shown).

It was recently shown that β-carotene and lycopene increase ARE-mediated transcription of NQO1 and γ-glutamylcysteine synthetase and the levels of glutathione in two different human cell lines (38). Nrf2 undergoes nuclear translocation upon treatment with lycopene. Thus, it appears that carotenoids induce the phase 2 response through the same signal transduction pathway as do other classes of structurally unrelated inducers.

Polyene antifungals

A subsequent search for natural products with similarly unsaturated alkyl chains led us to examine the sterol-binding polyene antifungal agents. These macrocyclic conjugated polyenes, such as filipin (CD = 1.7 μM), amphotericin B (CD = 8.5 μM) and nystatin (CD = 95 μM) also induce NQO1, but this limited information does not provide insight into the relation between structure and inducer potencies in this class of agents (Table I). Although some of these compounds (e.g. filipin) are potent inducers, they are likely to have limited value as long-term protective agents because of their significant renal and other dose-dependent toxicities (39).

Tetrapyrroles—porphyrins and analogs

We thus broadened our examination to include less toxic conjugated polyenes e.g. the highly conjugated tetrapyrroles, such as chlorophyll, chlorophyllin, heme and vitamin B12, many of which occur naturally and have very favorable safety and toxicity profiles. Neither the porphyrins participating in the mammalian biosynthesis of heme, nor the metabolites of heme

Table I. Potencies of polyenes on the induction of NQO1

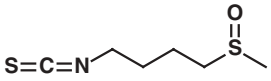
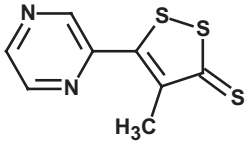
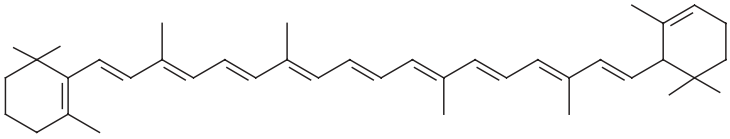
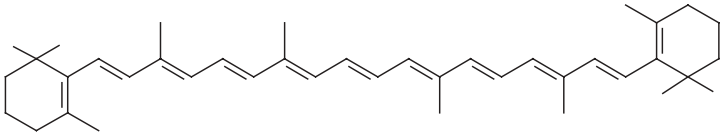
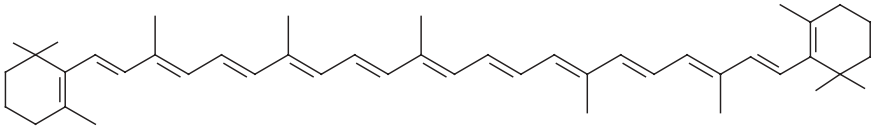
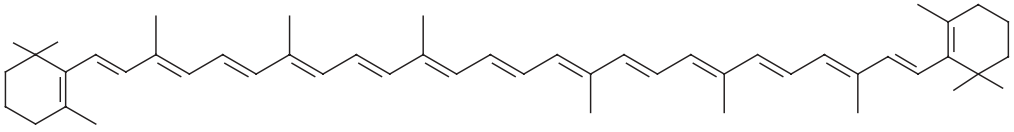
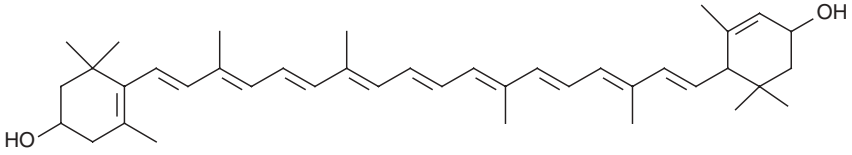
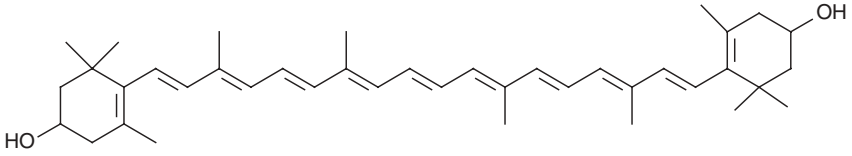
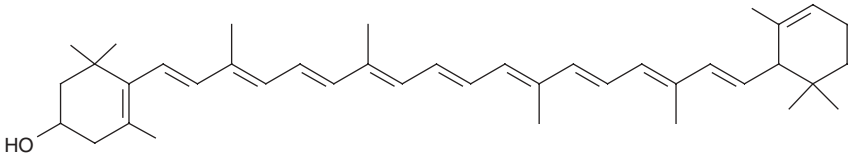
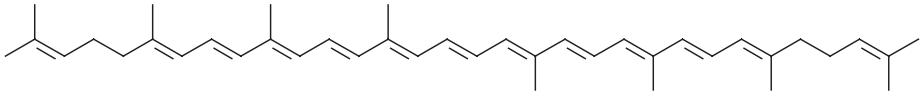
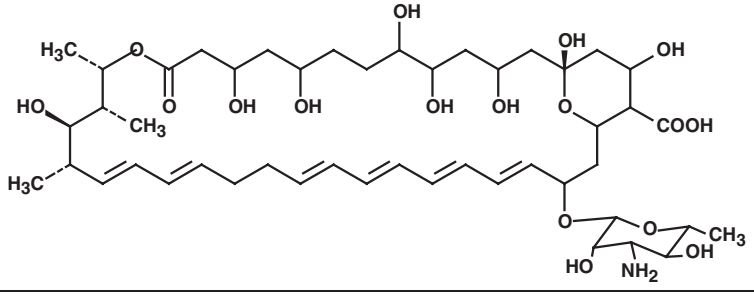
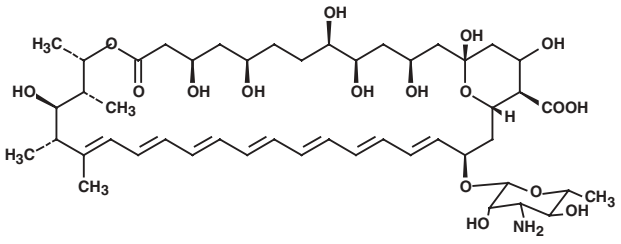
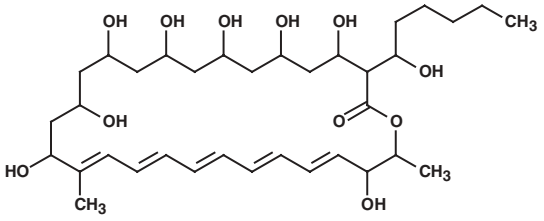
Compound	CD (μM)	Structure
Sulforaphane	0.2	
Oltipraz	22	
Carotenoids		
α-Carotene	100	
β-Carotene	7.2	
C_{45}- β-Carotene analog	16	
C_{50}- β-Carotene analog	30	
Lutein	17	
Zeaxanthin	2.2	
α-Cryptoxanthin	1.8	
Lycopene	25	
Sterol-binding antifungals		
Nystatin	95	

Table I. *Continued*

Compound	CD (μM)	Structure
Amphotericin B	8.5	
Filipin	1.7	

The CD values of sulforaphane and oltipraz are provided as standards for comparison. CD values of some of the carotenoids listed herein have already been reported in Khachik *et al*. (37).

(e.g. biliverdin and bilirubin) are effective phase 2 enzyme inducers (Table II). We examined a few such porphyrins containing alternate metal ions (e.g. Sn, Zn, Cr and Mn), and these also were ineffective as inducers. On the other hand, the substituted porphyrin, coproporphyrin III tetramethyl ester was an effective inducer (CD = 20 μM) with comparable potency to oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] (40) and resveratrol (41), and with very low cytotoxicity.

Some synthetic porphyrin compounds (Table III), including the basic porphyrin nucleus, porphine, are only marginally effective as inducers (CD = 250 μM). However, when the porphine ring is further conjugated with four additional 6-member aromatic rings, which permit charge delocalization and modify electrophilicity, as exemplified by tetrabenzoporphine, it becomes a much better inducer (CD = 25 μM) with very low cytotoxicity. Alteration of the methylene groups bridging the four pyrrole rings of porphine by substituting carbon for nitrogen yielded a completely inactive compound (phthalocyanine), but the introduction of a divalent copper ion to the center of this molecule (copper phthalocyanin) led to a moderately potent inducer (CD = 37 μM). Vitamin B12, a corrin with an asymmetrically modified porphyin nucleus, is also a moderately potent inducer of NQO1 (CD = 50 μM), although it is likely that efficacy *in vivo* would require levels vastly in excess of those required for nutritional purposes.

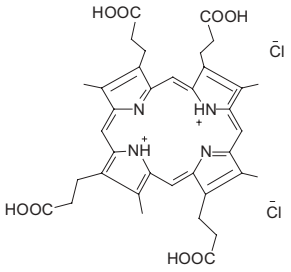
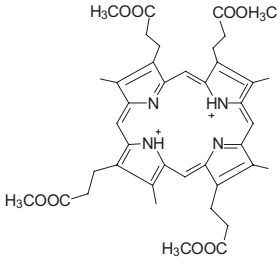
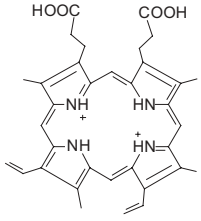
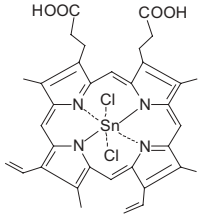
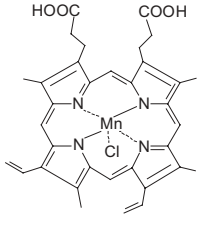
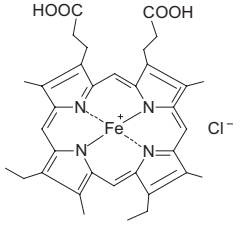
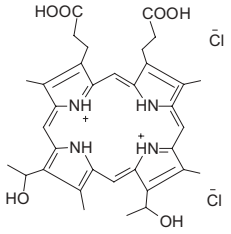
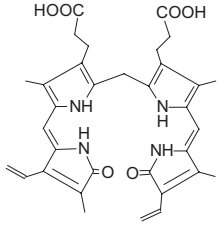
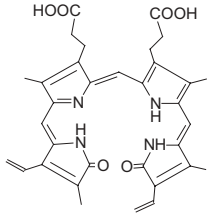
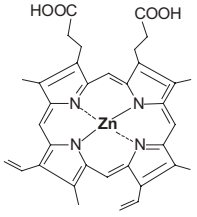
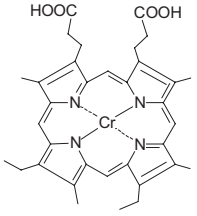
The families of substituted porphyrins occurring in highest natural abundance are the chlorophylls (containing a centrally coordinated Mg^{2+} ion) and the hemes (containing a centrally coordinated Fe^{3+} ion). Neither the precursors of heme (e.g. iron (III) mesoporphyrin IX chloride) or its oxidative metabolites (e.g. bilirubin and biliverdin), nor those of chlorophyll (e.g. pheophorbide), or porphyrins containing various metal ligands other than Mg^{2+} or Fe^{3+} (e.g. Mn-, Sn- or Zn protoporphyrin IX, or Cr mesoporphyrin IX) are active as phase 2 enzyme inducers (Table II).

It is the phase 2 enzyme induction of chlorophyll itself, its derivatives and its metabolites, however, that attracted our attention. The chlorophylls are relatively weak inducers (pure chlorophyll *a*, CD = 250 μM; a variety of chlorophyll isolates obtained from spinach chloroplasts had CD values ranging from 200 to 300 μM). The potency of pheophytin *a* (Mg^{2+}-free, phytyl-containing tetrapyrrole) was 250 μM, whereas pheophorbide *a* (lacking the phytyl 'tail') was completely inactive as an inducer. Notably, chlorophyll *a* is likely to be oxidized rapidly to chlorophyll *b* and to the pheophytins, pyropheophytins and pheophorbides, upon incubation in the microtiter plate assay. When the potency of these compounds is summed over the quantity of them that a vegetable-rich diet might reasonably be expected to contain, this minimum value could still represent a significant induction of protective phase 2 enzymes. For example, if a serving (100 g) of an average green vegetable contains 0.5% chlorophyll by weight, this would amount to about 93 000 U of NQO1 induction potential per day.[1] In comparison, this level is roughly equivalent to the NQO1 induction potential of 2.8 μmol of sulforaphane or 275 μmol of resveratrol[2] (phase 2 enzyme inducers found primarily in broccoli and red wine, respectively). By combining the benefit that we calculate could accrue from this approach, with the potential benefit that chlorophyll derivatives, such as chlorophyllin, may provide by binding to carcinogens and making them less available as mutagens, one provides an explanation for the very substantial protective effect that has already been documented for green vegetable consumption (crucifers play an additional role via their content

[1]Spinach is one of the richest sources of chlorophyll and contains 1–2% chlorophyll by weight, but if one uses a more modest figure of 0.5%, consumption of 5 servings per day of green vegetables would provide: 5 servings/d × 100 g/serving × 0.005 g chlorophyll/g vegetables × 37 300 U of NQO1 induction potential/g of chlorophyll = 93 000 U of NQO1 induction potential per day. [One unit (U) of NQO1 induction potential is defined as the quantity of compound required to double the NQO1 specific activity in a microtiter plate well containing 150 μl of medium. (CD = the concentration required to double the NQO1 activity.) Therefore, if chlorophyll has a CD of 200 μM, there are 200 nmol/ml × 0.15 ml/well = 30 nmol/well; 30 nmol/well × 0.894 μg chlorophyll/nmol = 26.8 μg chlorophyll/U or 37 300 U/g of chlorophyll].

[2]The CD of resveratrol in this bioassay system = 21 μM (41).

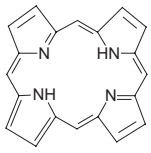
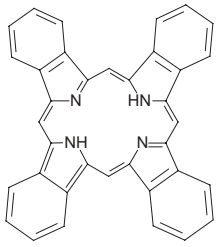
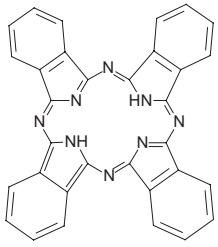
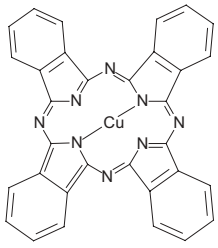
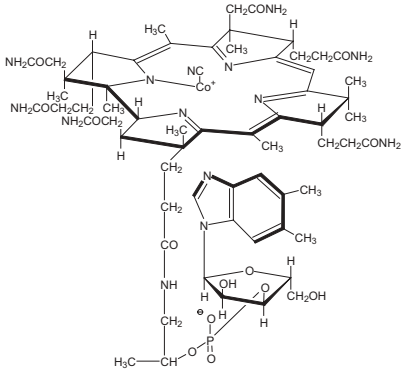
Table II. Potencies of porphyrins as inducers of NQO1

Compound	CD (μM)	Structure
Coproporphyrin III dihydrochloride	>300	
Coproporphyrin III tetramethyl ester	20	
Protoporphyrin IX	>300	
Sn (IV) protoporphyrin IX dichloride	>300	
Mn (III) protoporphyrin IX dichloride	>300	
Mesohemin [iron (III) mesoporphyrin IX chloride]	>300	
Hematoporphyrin IX dihydrochloride	>300	
Biliverdin IXα	95	
Bilirubin IXα	>300	
Zn (II) protoporphyrin IX	>300	
Cr (III) mesoporphyrin IX chloride	>300	

of glucosinolates/isothiocyanates). To our knowledge, chlorophyll has not hitherto been considered to play a significant role in the dietary cancer chemoprotective armamentarium, although it is perhaps one of the more obvious components of a plant-rich diet. Indeed, although chlorophyll is not a potent inducer of protective enzymes, it is ubiquitous in a plant-based diet, and could thus provide low but consistent induction of these enzymes.

Chlorophyllins (commercially prepared) are other inducers of phase 2 enzymes (CD $\approx$ 10–30 μM; see Table IV). The two major components of chlorophyllin are copper chlorin e4 and copper chlorin e6, which have CDs of 78 and $\gg$1000 μM, respectively. A third component of chlorophyllin, copper chlorin e4 ethyl ester was recently identified by some of us (25), and it is a much more potent NQO1 inducer

Table III. Potencies of porphines and a corrin (vitamin B12) as inducers of NQO1

Compound	CD (μM)	Structure
Porphine	250	
Tetrabenzoporphine	25	
Phthalocyanine	>1000	
Copper phthalocyanine	37	
Vitamin B12	50	

(CD = 5.5 μM) than its more prevalent, non-esterified analog (Table IV), perhaps reflecting cellular uptake that is expected to be much more efficient with the ethyl ester compared with the ionized disodium salt. The only other porphyrin methyl ester examined in this study was coproporphyrin III tetramethyl ester, which also may have chemoprotective utility (CD = 20 μM; Table II). For populations at high risk, the utility of copper chlorin e4 ethyl ester, a non-toxic compound that is thus ~50-fold more potent an inducer than chlorophyll, merits further investigation.

Reactivity with thiols

Sulfhydryl binding kinetics of each of these classes of newly discovered phase 2 enzyme inducers is exemplified by their reactivity with 2-mercaptoethanol and the data for representative compounds are presented in Table V. The bleaching reactions of thiols with carotenoids are well known (42), the formation of thiol adducts by porphyrins such as protoporphyrin IX has been demonstrated previously (43), and the non-enzymatic isomerization of a carotenoid derivative by sulfhydryl compounds such as mercaptoethanol has also been demonstrated (44). The only universal property of phase 2 enzyme inducers is their reactivity with sulfhydryl groups—thought to be critical in the initial 'sensing' of inducers (6,45). It was thus important to show that the diverse array of polyene inducers examined in the present study could similarly bind with a sulfhydryl reagent and to explore whether the rate constants for these reactions could be related to the ability of compounds to induce phase 2 enzymes in mammalian cells.

Because of their extended conjugation systems, all the compounds tested have favorable absorption properties, i.e. they absorb light at long wavelengths with high extinction coefficients. As exemplified in Figure 1, upon reaction with thiols, the conjugation system of β-carotene is destroyed, resulting in bleaching. Thus, reaction with sulfhydryl reagents can easily be followed by the decrease in absorbance at the corresponding wavelength for each compound. Although the stoichiometry of these reactions is unknown and the reactions are likely to involve the simultaneous reduction of multiple centers on these molecules, it was important to establish whether: (i) representatives of the polyene and tetrapyrrole classes of inducers react with sulfhydryl groups; (ii) inducer potency correlates with sulfhydryl reactivity.

Inducer potency generally correlates with sulfhydryl group reactivity (Table V). The most potent inducers, e.g. α-cryptoxanthin (CD = 1.8 μM) and zeaxanthin (CD = 2.2 μM), have the highest pseudo second-order rate constants with the sulfhydryl reagent β-mercaptoethanol ($k_2 = 8.84 \times 10^{-3}$ M^{-1} s^{-1} and $k_2 = 10.3 \times 10^{-3}$ M^{-1} s^{-1}, respectively). Vitamin B12, one of the less potent inducers (CD = 50 μM), showed the lowest reactivity with β-mercaptoethanol ($k_2 = 0.064 \times 10^{-3}$ M^{-1} s^{-1}). Similarly, chlorophyll *a*, one of the less potent inducers, did not react significantly with the sulfhydryl reagent. Chlorophyllin (CD = 30 μM), on the other hand, reacts with β-mercaptoethanol at a rate ($k_2 = 1.01 \times 10^{-3}$ M^{-1} s^{-1}) that is ~10-fold lower than the rate of reactivity of zeaxanthin ($k_2 = 10.3 \times 10^{-3}$ M^{-1} s^{-1}), which is a ~10-fold more potent inducer (CD = 2.2 μM). The only exception from this general trend is that although there is a very large difference in inducer potencies between α-carotene (CD = 100 μM), and β-carotene (CD = 7.2 μM), their reactivity with β-mercaptoethanol is essentially identical. In this isolated case, inducer potency may be largely influenced by other factors, e.g. by differences in their uptake by the cells or intracellular metabolism.

We have used $BP^r c1$ and c1 cells to test a representative porphine and chlorin. Tetrabenzoporphine appears to be a monofunctional inducer, as is sulforaphane, in that inducer potency is roughly equivalent in wild-type and mutant

Table IV. Potencies of chlorins as inducers of NQO1

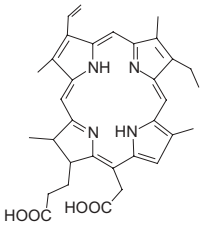
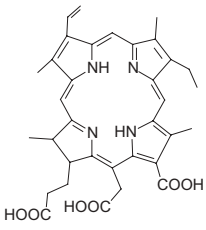
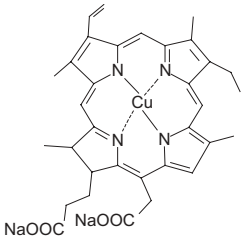
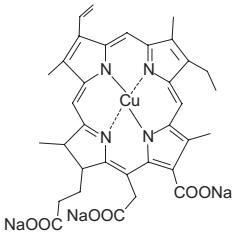
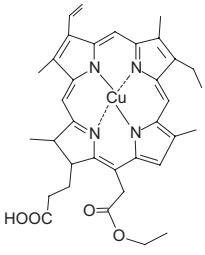
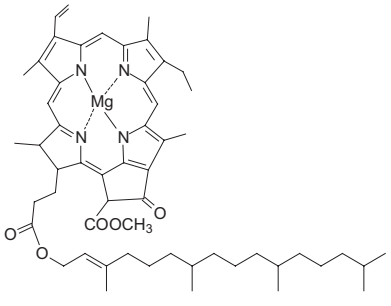
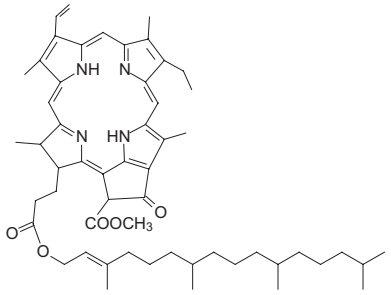
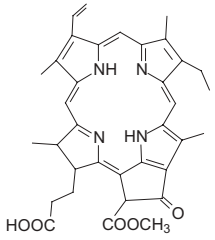
Compound	CD (μM)	Structure
Chlorin e4	>300	
Chlorin e6	>300	
Cu chlorin e4 (disodium)	78	
Cu chlorin e6 (disodium)	>300	
Cu chlorin e4 ethyl ester	5.5	
Chlorophyllin	20–40	(A variable mixture of Cu chlorin sodium salts)
Chlorophyll *a*	250–300	
Pheophytin *a*	250	
Pheophorbide	>300	

Table V. Reactivity of representatives of each class of highly conjugated phase 2 enzyme inducers with 2-mercaptoethanol

Compound	CD (μM)	λ_{max} (nm)	Solvent (%)	k_2 ($M^{-1} s^{-1}$)
Carotenoids and polyene antibiotics				
α-Carotene	100	452	95[a]	6.84×10^{-3}
β-Carotene	7.2	458	95[a]	6.77×10^{-3}
Zeaxanthin	2.2	447	95[a]	10.3×10^{-3}
α-Cryptoxanthin	1.8	450	95[a]	8.84×10^{-3}
Amphotericin B	8.5	411	95[a]	5.00×10^{-3}
Tetrapyrroles				
Vitamin B12	50	361	50[b]	0.064×10^{-3}
Chlorophyllin	30	665	50[b]	1.01×10^{-3}
Chlorophyll *a*	250	665	95[a]	Unreactive
Pheophorbide *a*	≫300	668	95[a]	0.65×10^{-3}

The solvents used are [a]tetrahydrofuran and [b]methanol.

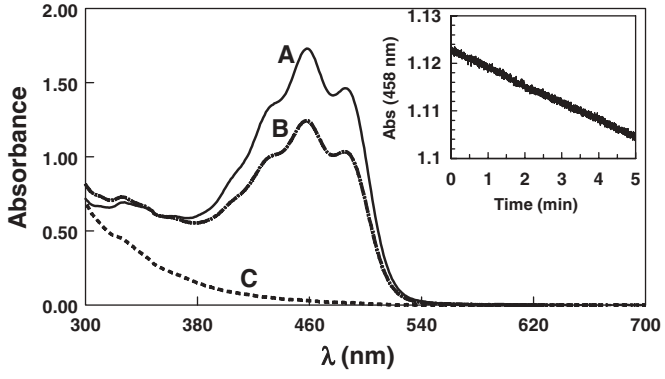


Fig. 1. Reaction of β-carotene with 2-mercaptoethanol. Absorption spectra of 9.5 μM β-carotene in 95% aqueous tetrahydrofuran (**A**), the reaction mixture of 9.5 μM β-carotene and 143 mM 2-mercaptoethanol after incubation in the same solvent at 25°C in the dark for 40 min (**B**) and 18 h (**C**). The inset shows the time course of the incubation of 6 μM β-carotene with 143 mM 2-mercaptoethanol that was used in calculating the initial rate and the pseudo second-order rate constant of the reaction.

Table VI. Inducer potency, reported as CD (concentration required to double the NQO1 activity in treated cells, compared with controls) in μM, measured in different cell lines

Compound	Cell line[a]			
	Hepa1c1c7	BP^rc1	c1	HepG2-ARE-GFP[b]
Sulforaphane	0.2	0.2	0.2	+
Tetrabenzoporphine	25	50	75	+
Cu chlorin e4 (disodium)	78	—	—	+

[a]Bifunctional inducers induce NQO1 in wild-type (Hepa1c1c7) murine hepatoma cell line, but not in either of BP^rc1 or c1 mutants lacking functional cytochrome P450 activity (15).
[b]'+', positive response with ARE-TK-GFP construct and not with same cell line (HepG2) transfected with only the GFP vector, but lacking ARE; '—', >1000 μM (no response at highest dose tested).

Hepa1c1c7 cells (Table VI). In contrast, copper chlorin e4 (disodium) appears to be a bifunctional inducer in that no inducer activity is seen in the mutant cell lines. This outcome suggests that this component of chlorophyllin requires activation by phase 1 enzymes. Thus, as a class, polyenes act as monofunctional or bifunctional inducers. Using a pair of HepG2 human hepatoma cell lines transfected with the ARE-linked GFP plasmid or GFP plasmid without ARE, we show that both tetrabenzoporphine and copper chlorin e4 (disodium) induce NQO1 via the ARE/Nrf2 pathway (Table VI).

Discussion

Hayes *et al*. (46) have characterized the murine *nqo1* 5′-upstream region to show that Nrf2 regulates this gene directly via an ARE, which is required for both constitutive and sulforaphane-inducible expression of NQO1. Ma *et al*. (47) have provided evidence in cultured murine cells that the AhR-dependent induction of NQO1 by bifunctional inducers (i.e. AhR ligands) cross-reacts with Nrf2 function in the induction of phase 2 enzymes. They provide suggestive evidence that at least three different pathways exist to mediate expression of NQO1, some or all of which may be involved in enzyme induction by the agents under investigation in the present study.

Since chlorophyllin is >10-fold more potent as a phase 2 enzyme inducer than chlorophyll, and since it has other detoxification properties because it is much more water-soluble than chlorophyll, its ultimate incorporation into either a dietary supplement or a pharmaceutical cancer chemoprevention strategy may be quite feasible. Indeed, administration of oral chlorophyllin supplements was responsible for the green color of the sera of study subjects. Chlorophyllin ethyl esters and chlorin e4 and e6 were present at low micromolar levels in these sera (25). The observations reported here support additional or alternate explanations for the protective effects of chlorophyllin on aflatoxin-induced carcinogenesis. For the major dietary carotenoid lutein, a doubling of NQO1 occurred at about 17 μM, but there was measurable induction at levels as low as 1.0 μM. Khachik *et al*. (48) have measured lutein levels in human liver, colon and lung tissues of 3.2, 0.85 and 0.40 μM, respectively.

Interestingly, the experimental evidence provided by Ben-Dor *et al*. (38) led them to conclude that the activation of the ARE/Nrf2-mediated phase 2 enzyme induction by a number of extremely hydrophobic carotenoids may be, in part, mediated by the more hydrophilic derivatives of these compounds. Clearly, it could be the oxidation products of some of the extremely hydrophobic compounds reported in this paper—both carotenoids and others that account for their NQO1-inducing activity. In fact, the evidence we present strongly suggests that a similar phenomenon takes place with the derivatives of the plant pigment chlorophyll, since both its water-soluble derivative (chlorophyllin), and a principal component of chlorophyllin (copper chlorin e4 ethyl ester), are substantially more potent inducers of NQO1 in Hepa1c1c7 cells (~10- and 40-fold more potent, respectively).

Concentrations of chlorophyll and pheophytin as high as almost 250 μM were required to double NQO1 activity in cell culture; however, there was some induction from these compounds even in the low micromolar range. Although the decades old dogma is that chlorophyll is not significantly taken up *in vivo*, very few studies have evaluated its uptake and tissue distribution. Recent work with human intestinal cells strongly suggests that uptake of chlorophyll and its pheophytin metabolites is substantial, and is comparable to that of lutein (49). Thus, it is still unknown whether these abundant plant pigments could reach sufficient levels in human tissues to induce phase 2 enzymes. The direct assessment of both systemic and intestinal cell induction in human subjects is clearly a challenge that must be addressed. Broadly viewed, the ability of the chlorophylls and carotenoids to induce phase 2 enzymes may, in part, explain the widely accepted protective effects of vegetable consumption against cancer.

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