menadione exposure than wild-type cells. It was found that 54% and 79% of cells were dead by 6.25 and 12.5 μM menadione incubation in *nrf2*-deficient cells, while 10% and 46% cells were killed by same concentrations of menadione, respectively, in wild-type cells. However, *keap1*-deficient cells, which express high levels of NQO1, were very resistant to menadione, and only 9% cells were killed by 25 μM menadione. Regulation of NQO1 through the Nrf2-Keap1 pathway is an important determinant of cellular sensitivity to quinones and is an attractive target for chemopreventive interventions.

[20] M.-K. Kwak, N. Wakabayashi, K. Itoh, H. Motohashi, M. Yamamoto, and T. W. Kensler, *J. Biol. Chem*. **278,** 8135 (2003).
[21] J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell, *Cancer Res*. **47,** 936 (1987).

[23] Chemical Structures of Inducers of Nicotinamide Quinone Oxidoreductase 1 (NQO1)

By ALBENA T. DINKOVA-KOSTOVA, JED W. FAHEY, and PAUL TALALAY

Introduction

More than 40 years ago, Williams-Ashman and Huggins (1961) made the fortuitous observation that the enzyme discovered by Lars Ernster,[1] DT-diaphorase, NAD(P)H:quinone oxidoreductase 1, NQO1 (EC 1.6.99.2), was highly inducible in rat tissues.[2] While developing the now widely used DMBA mammary tumor model in Sprague-Dawley rats, Huggins and his colleagues showed that administration of small doses of aromatic hydrocarbons before challenge with DMBA protected against the lethal effects of this potent carcinogen.[3] This protection was accompanied by elevation of NQO1 activity in the liver and other organs.[4,5] Furthermore, the potencies of structurally related azo dyes in protecting against DMBA carcinogenesis paralleled their effectiveness in inducing NQO1. This finding led Huggins to propose the use of activity of NQO1 as a rapid screening procedure for the identification of protectors against

[1] L. Ernster and F. Navazio, *Acta Chem. Scand*. **12,** 595 (1958).
[2] H. G. Williams-Ashman and C. Huggins, *Med. Exper*. **4,** 223 (1961).
[3] C. B. Huggins, *in* ''Experimental Leukemia and Mammary Cancer: Induction, Prevention, Cure.'' The University of Chicago Press, Chicago, 1979.
[4] C. Huggins and R. Fukunishi, *J. Exp. Med*. **119,** 923 (1964).
[5] C. Huggins and J. Pataki, *Proc. Natl. Acad. Sci. USA* **53,** 791 (1965).

0076-6879/04 $35.00

carcinogenesis.[3] The ultimate importance of this long-neglected prediction cannot be overestimated.

Two parallel lines of research caused this idea to be revisited. The laboratories of Frankfurt and Wattenberg demonstrated that the phenolic antioxidants 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) that are commonly used as food preservatives protect rodents against a wide range of chemical carcinogens.[6,7] Subsequently Bueding and Talalay reported that administration of BHA to mice reduced the formation of mutagenic metabolites from benzo[*a*]pyrene and raised the activity of NQO1 in many tissues parallel to the increases in the levels of classical phase 2 detoxication enzymes—for example, glutathione *S*-transferases, epoxide hydrolase.[8–11] These observations led to the suggestion that the protective function of phenolic antioxidants against tumor development in animals could be ascribed to the induction of the phase 2 response. In the ensuing years, the evidence supporting this suggestion has become progressively more convincing.[12–16]

Subsequently, structure-activity studies revealed that a wide array of compounds caused coordinate induction of phase 2 enzymes and protected against carcinogenesis. The development of a cultured cell microtiter plate bioassay for NQO1 by Hans Prochaska provided a quick and highly quantitative system for evaluation of the potencies of inducers and for screening pure compounds, as well as complex mixtures such as plant extracts, for their inducer activity.[17–20]

[6] O. S. Frankfurt, L. P. Lipchina, T. V. Bunto, and N. M. Emanuel, *Biull. Eksp. Biol. Med.* **64,** 86 (1967).
[7] L. W. Wattenberg, *Adv. Cancer Res.* **26,** 197 (1979).
[8] R. P. Batzinger, S. Y. Ou, and E. Bueding, *Cancer Res.* **38,** 4478 (1978).
[9] A. M. Benson, R. P. Batzinger, S. Y. Ou, E. Bueding, Y.-N. Cha, and P. Talalay, *Cancer Res.* **38,** 4486 (1978).
[10] A. M. Benson, Y.-N. Cha, E. Bueding, H. S. Heine, and P. Talalay, *Cancer Res.* **39,** 2971 (1979).
[11] A. M. Benson, M. J. Hunkeler, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **77,** 5216 (1980).
[12] P. Talalay, *Adv. Enzyme Regul.* **28,** 237 (1989).
[13] P. Talalay, J. W. Fahey, W. D. Holtzclaw, T. Prestera, and Y. Zhang, *Toxicol. Lett.* **82–83,** 173 (1995).
[14] T. W. Kensler, *Environ. Health Perspect.* **105,** 965 (1997).
[15] P. Talalay, *Biofactors* **12,** 5 (2000).
[16] P. Talalay and A. T. Dinkova-Kostova, *Methods Enzymol.* **382,** 355 (2004).
[17] H. J. Prochaska and A. B. Santamaria, *Anal. Biochem.* **169,** 328 (1988).
[18] H. J. Prochaska, A. B. Santamaria, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **89,** 2394 (1992).
[19] J. W. Fahey, Y. Zhang, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **94,** 10367 (1997).
[20] J. W. Fahey, A. T. Dinkova-Kostova, K. K. Stephenson, and P. Talalay, *Methods Enzymol.* **382,** 243 (2004).

Identification of the Chemical Signals for Monofunctional Phase 2 Gene Induction

Phenolic Antioxidants and Their Alkyl Ethers as Inducers

The proposal that the protection by BHA and related phenolic antioxidants against chemical carcinogens, mutagens, and other toxic agents resulted from the induction of a family of phase 2 proteins that detoxified the reactive metabolites of these agents, focused attention on the mechanisms of the transcriptional enhancement of these genes. Elucidation of these mechanisms required understanding both of the molecular events involved in regulating these genes, and of the structural specificity of the inducers. This chapter describes the development of our understanding of the nature of the chemical signals involved in the induction of NQO1 and other phase 2 enzymes. In our first efforts to identify the essential structural features of inducers, we compared the inducer potencies of the two isomers of commercial BHA ([2]- and [3]-*tert*-butyl-4-hydroxyanisole), their demethylation product *tert*-butylhydroquinone, and an extended series of synthetic mono- and dialkyl ethers of *tert*-butylhydroquinone R_1O-[$(CH_3)_3C$-C_6H_3]-OR_2 on the levels of NQO1 and glutathione transferases in mouse liver and small intestinal mucosa.[21–23] These studies revealed that there was relatively little structural specificity among these compounds and that the free diphenol, *tert*-butylhydroquinone, was more potent than its alkyl ethers. Moreover, the *tert*-butyl group influenced potency only slightly. We concluded that the proximate inducers were probably the free phenols and that the substituted alkyl phenols required metabolic dealkylation.

Inducer Potency of Phenolic Antioxidants Depends on Oxidative Lability

However, the two phenolic hydroxyl groups of all these compounds were 1,4-*(para)*-oriented. Further experiments with a variety of 1,2-diphenols (catechols), 1,3-diphenols (resorcinols), and 1,4-diphenols (hydroquinones) disclosed the important finding that resorcinols were invariably completely inactive as inducers.[22] Moreover, analogous behavior was observed with 1,2-, 1,3-, and 1,4-phenylenediamines: the 1,3-diamines were not inducers. We concluded from these observations that capacity for oxidation to

[21] H. J. Prochaska, H. S. Bregman, M. J. De Long, and P. Talalay, *Biochem. Pharmacol.* **34,** 3909 (1985).
[22] H. J. Prochaska, M. J. De Long, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **82,** 8232 (1985).
[23] M. J. De Long, H. J. Prochaska, and P. Talalay, *Cancer Res.* **45,** 546 (1985).

quinones or quinoneimines, respectively, was an essential property of inducers. The 1,3-*meta* derivatives cannot undergo such oxidation, whereas both catechols and 1,4-hydroquinones can be easily oxidized to quinones. These experiments did not, however, disclose whether the quinone (or quinoneimine) products were in fact the ultimate inducers or whether the oxidation reactions themselves participated in the induction mechanism.

Monofunctional and Bifunctional Inducers

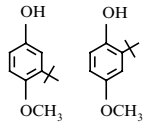
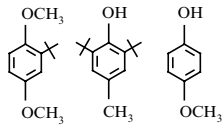
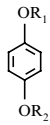
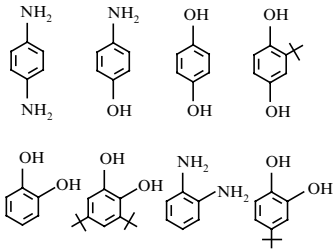
Related experiments on the mechanism of induction revealed that inducers of phase 2 enzymes are of two types: (1) bifunctional inducers (principally large planar aromatics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD], polycyclic aromatics, azo dyes, β-naphthoflavone) elevate both phase 2 and phase 1 (certain cytochromes P450) genes; and (2) monofunctional inducers (that differ widely in chemical structure) elevate phase 2 enzymes selectively.[24] In a murine hepatoma cell line (Hepa1c1c7) and its mutants defective in their ability to express certain phase 1 enzymes, and in analogous genetically defined mice, it was shown that monofunctional inducers did not depend on *Ah* (Aryl hydrocarbon) receptor function or aryl hydrocarbon hydroxylase expression, whereas bifunctional inducers did. The current review of inducer chemistry is confined to monofunctional inducers.

Michael Reaction Acceptors as Inducers

The next major advance in understanding of the chemical characteristics of inducers arose from an examination of the components of the structure of coumarin.[25] These experiments led to the realization that indeed many inducers contain a previously unrecognized olefinic or acetylenic linkage conjugated to electron-withdrawing groups, a prime example being quinones that contain olefinic functions conjugated to carbonyl groups (Tables I through V). These functional groups, designated as Michael reaction acceptors, are highly susceptible to attack by nucleophiles (on the β-carbon atom), and form the basis for the Michael reaction that is widely used in organic synthesis. The reactivity of Michael acceptors with nucleophiles depends on the strength of the electron-withdrawing function: $NO_2 > COAr > CHO > COCH_3 > CN > CONH_2 > CONR_2$. Moreover the potency of inducers of NQO1 was correlated with the power of the electron withdrawing group and consequently with the nucleophilicity of the electrophilic carbon center.

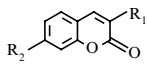
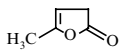
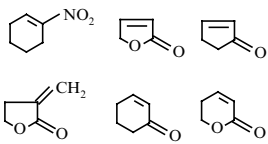
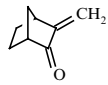
[24] H. J. Prochaska and P. Talalay, *Cancer Res.* **48,** 4776 (1988).
[25] P. Talalay, M. J. De Long, and H. J. Prochaska, *Proc. Natl. Acad. Sci. USA* **85,** 8261 (1988).

TABLE I
INDUCERS OF NQO1: PHENOLIC ANTIOXIDANTS, DIPHENOLS, AMINOPHENOLS, PHENYLENEDIAMINES

Compound	Tissue or cell line	Reference
 3-BHA 2-BHA	Liver, kidney, lung, forestomach, glandular stomach, small intestine, colon, uterus, spleen, bladder, adrenal	Benson *et al.* [11]; Talalay and Benson [26]; Cha and Heine [27]; De Long *et al.* [23]; Heine *et al.* [28]; Siegel *et al.* [29]; Shertzer and Sainsbury [30]; Munday and Munday [31]; McMahon *et al.* [32]
 BHT 4-Hydroxy-anisole	Liver, kidney, forestomach, glandular stomach, small intestine, colon, Hepa1c1c7	De Long *et al.* [23, 33]; Prochaska *et al.* [21, 22]
 $R_1 = R_2 = CH_3, C_2H_5, C_3H_7$	Liver, small intestine	Prochaska *et al.* [21]
	Hepa1c1c7	Prochaska *et al.* [21]; De Long *et al.* [33]

[26] P. Talalay and A. M. Benson, *Adv. Enzyme Regul.* **20,** 287 (1982).
[27] Y.-N. Cha and H. S. Heine, *Cancer Res.* **42,** 2609 (1982).
[28] H. S. Heine, M. K. Stoskopf, D. C. Thompson, and Y.-N. Cha, *Chem. Biol. Interact.* **59,** 219 (1986).
[29] D. Siegel, A. M. Malkinson, and D. Ross, *Toxicol. Appl. Pharmacol.* **96,** 68 (1988).
[30] H. G. Shertzer and M. Sainsbury, *Food Chem. Toxicol.* **29,** 391 (1991).
[31] R. Munday and C. M. Munday, *Nutr. Cancer* **34,** 42 (1999).

TABLE II
INDUCERS OF NQO1: COUMARINS AND OTHER CYCLIC LACTONES AND ENONES

Compound	Tissue or cell line	Reference
 $R_1 = R_2 = H$, Coumarin (weak) $R_1 = H$, $R_2 = OH$, 7-Hydroxycoumarin $R_1 = OH$, $R_2 = H$, 3-Hydroxycoumarin $R_1 = COCH_3$, $R_2 = H$, 3-Acetylcoumarin	Hepa1c1c7, small intestine	De Long *et al.* [33]; Talalay *et al.* [25]; Dinkova-Kostova *et al.* [34]; McMahon *et al.* [32]
 α-angelicalactone	Hepa1c1c7, small intestine	De Long *et al.* [33]; Talalay *et al.* [25]; McMahon *et al.* [32]
	Hepa1c1c7	Talalay *et al.* [25]
	Hepa1c1c7	Prestera *et al.* [35]

Nine Chemical Classes of Inducers

At first glance, the structural versatility of inducers seemed extraordinary. Today, inducers can be classified into 9 categories.

Class 1: Diphenols, Phenylenediamines, and Quinones

The dietary antioxidants that were first shown to induce NQO1 belong to this class.[9,11,26] Subsequently, we and many other investigators have demonstrated that members of this class induce NQO1 in various rodent

[32] M. McMahon, K. Itoh, M. Yamamoto, S. A. Chanas, C. J. Henderson, L. I. McLellan, C. R. Wolf, C. Cavin, and J. D. Hayes, *Cancer Res.* **61,** 3299 (2001).
[33] M. J. De Long, H. J. Prochaska, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **83,** 787 (1986).
[34] A. T. Dinkova-Kostova, C. Abeygunawardana, and P. Talalay, *J. Med. Chem.* **41,** 5287 (1998).
[35] T. Prestera, Y. Zhang, S. R. Spencer, C. A. Wilczak, and P. Talalay, *Adv. Enzyme Regul.* **33,** 281 (1993).

TABLE III
INDUCERS OF NQO1: MICHAEL REACTION ACCEPTORS: FUMARIC, MALEIC, ACRYLIC, CROTONIC, FERULIC, AND CAFFEIC ACID DERIVATIVES

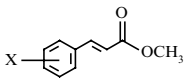
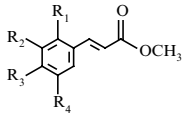
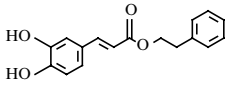
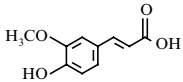
Compound	Tissue or cell line	Reference
trans $CH_3OOCCH=CHCOOCH_3$ *trans* $C_2H_5OOCCH=CHCOOC_2H_5$ *cis* $CH_3OOCCH=CHCOOCH_3$ *cis* $C_2H_5OOCCH=CHCOOC_2H_5$ $CH_3OOCC(=CH_2)CH_2COOCH_3$ $CH_3OOCC\equiv CCOOCH_3$ $CH_3CH\equiv CH-R$ R = $COOCH_3$, CHO, CHCN $CH_3CH\equiv CCOOCH_3$ $CH_2=CH-R$ R = $COOCH_3$, CHO, $COCH_3$, SO_2CH_3, CN $CH\equiv CCOOCH_3$	Forestomach, glandular stomach, intestine, colon, spleen, Hepa1c1c7	Spencer *et al.* [36]; Talalay *et al.* [25]
 X = CH_3O, NO_2, Br, Cl, F	Hepa1c1c7	Spencer *et al.* [37]; Dinkova-Kostova *et al.* [34]
 $R_1 = R_2 = R_3 = R_4 = H$ $R_1 = R_4 = H$, $R_2 = R_3 = OH$ $R_1 = OH$, $R_2 = R_3 = R_4 = H$ $R_1 = R_3 = R_4 = H$, $R_2 = OH$ $R_1 = R_2 = R_4 = H$, $R_3 = OH$ $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$ $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$	Hepa1c1c7	Talalay *et al.* [25]; Dinkova-Kostova *et al.* [34]
 Caffeic acid phenethyl ester	HepG2	Jaiswal *et al.* [38]
 Ferulic acid	Liver, colon	Kawabata *et al.* [39]

TABLE IV
INDUCERS OF NQO1: CURCUMIN AND OTHER DOUBLE MICHAEL REACTION ACCEPTORS

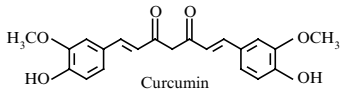
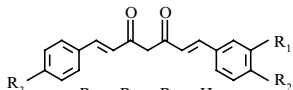
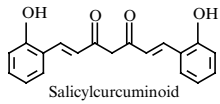
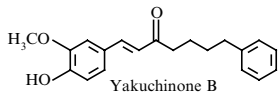
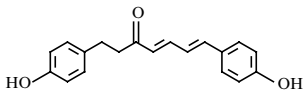
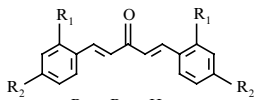
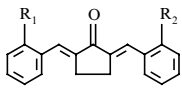
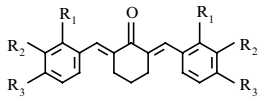
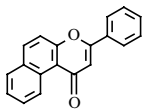
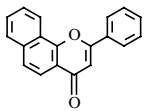
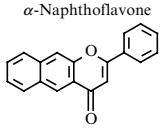
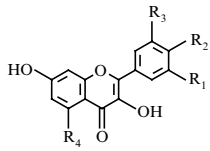
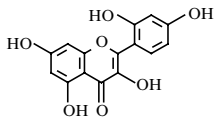
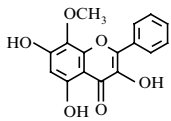
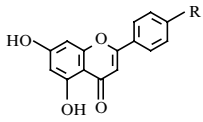
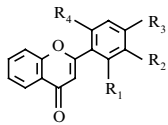
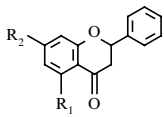
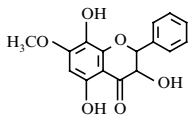
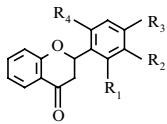
Compound	Tissue or cell line	Reference
 Curcumin	Liver, kidney, Hepa1c1c7	Iqbal *et al.* [40]; Okada *et al.* [41]; Singletary *et al.* [42]; Dinkova-Kostova and Talalay [43]; Gerhäuser *et al.* [44]
 $R_1 = R_2 = R_3 = H$ $R_1 = H, R_2 = R_3 = OH$ $R_1 = OCH_3, R_2 = R_3 = OH$	Hepa1c1c7	Dinkova-Kostova and Talalay [43]
 Salicylcurcuminoid	Hepa1c1c7	Dinkova-Kostova and Talalay [43]
 Yakuchinone B	Hepa1c1c7	Dinkova-Kostova and Talalay, unpublished
	Hepa1c1c7	Jang *et al.* [45]
 $R_1 = R_2 = H$ $R_1 = H, R_2 = OH$ $R_1 = OH, R_2 = H$	Hepa1c1c7	Dinkova-Kostova *et al.* [46]
 $R_1 = R_2 = H$ $R_1 = R_2 = OH$	Hepa1c1c7	Dinkova-Kostova *et al.* [46]
 $R_1 = R_2 = R_3 = H$ $R_1 = H, R_2 = OCH_3, R_3 = OH$ $R_1 = OH, R_2 = R_3 = H$ $R_1 = OCH_3, R_2 = R_3 = H$	Hepa1c1c7	Dinkova-Kostova *et al.* [46]

TABLE V
INDUCERS OF NQO1: FLAVONOIDS, WITHANOLIDES, AND NORWITHANOLIDES

Compound	Tissue or cell line	Reference
Flavones 	Hepa1c1c7, HepG2, MCF-7, HT-29, LNCaP, HeLa, heart	De Long *et al.* [33]; Talalay *et al.* [25]; Jiang *et al.* [47]; Floreani *et al.* [48]
β-Naphthoflavone 	Hepa1c1c7	Dinkova-Kostova [49]
α-Naphthoflavone 	Hepa1c1c7	Dinkova-Kostova [49]
γ-Naphthoflavone  $R_1 = R_2 = R_3 = R_4 = H$ $R_1 = H, R_2 = R_3 = R_4 = OH$, Quercetin $R_1 = R_3 = H, R_2 = R_4 = OH$, Kaempferol $R_1 = R_2 = R_3 = H, R_4 = OH$, Galangin $R_1 = R_2 = R_3 = R_4 = OH$, Myricetin	Hepa1c1c7, MCF-7	De Long *et al.* [33]; Dinkova-Kostova [49]; Williamson *et al.* [50]; Uda *et al.* [51]; Valerio *et al.* [52]; Fahey and Stephenson [53]
 Morin	Liver, tongue, large intestine, Hepa1c1c7	Fahey and Stephenson [53]; Tanaka *et al.* [54]; Kawabata *et al.* [55]
	Hepa1c1c7	Su *et al.* [56]

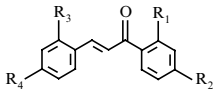
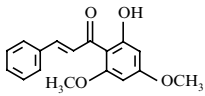
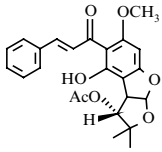
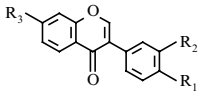
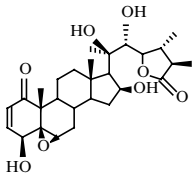
(continued)

TABLE V *(continued)*

Compound	Tissue or cell line	Reference
 R = H, Chrysin R = OH, Apigenin	Hepa1c1c7	Uda *et al.* [51]; Fahey and Stephenson [53]
 $R_1 = R_2 = R_4 = H, R_3 = Br$ $R_1 = R_2 = R_4 = H, R_3 = Cl$ $R_1 = R_2 = R_4 = H, R_3 = CF_3$ $R_1 = R_3 = R_4 = H, R_2 = Br$ $R_1 = R_3 = R_4 = H, R_2 = F$ $R_1 = R_3 = R_4 = H, R_2 = Cl$ $R_1 = F, R_2 = R_3 = R_4 = H$	Hepa1c1c7	Song *et al.* [57]
 $R_1 = OH, R_2 = OCH_3$, Pinostrobin $R_1 = OCH_3, R_2 = OH$ $R_1 = R_2 = OH$, Pinocembrin $R_1 = R_2 = OCH_3$	Hepa1c1c7	Fahey and Stephenson [53]; Su *et al.* [56]; Gu *et al.* [58]
	Hepa1c1c7	Su *et al.* [56]
 $R_1 = CH_3, R_2 = R_3 = R_4 = H$ $R_1 = OCH_3, R_2 = R_3 = R_4 = H$	Hepa1c1c7	Song *et al.* [57]

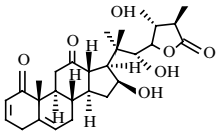
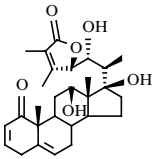
(continued)

TABLE V *(continued)*

Compound	Tissue or cell line	Reference
 Silibinin	Liver, lung, stomach, small intestine, skin	Zhao and Agarwal [59]
Chalcones		
 $R_1 = R_2 = R_3 = R_4 = H$ $R_1 = R_2 = R_3 = H, R_4 = OH$ $R_1 = R_2 = R_4 = H, R_3 = OH$ $R_1 = OH, R_2 = R_3 = R_4 = H$ $R_1 = R_2 = R_4 = OH, R_3 = H$ $R_1 = R_3 = OH, R_2 = R_4 = H$ $R_1 = R_2 = R_3 = OH, R_4 = H$	Hepa1c1c7	Dinkova-Kostova *et al.* [34]; Su *et al.* [56]
	Hepa1c1c7	Gu *et al.* [58]
 (+)-Tephropurpurin	Hepa1c1c7	Chang *et al.* [60]
Isoflavones		
 $R_1 = R_2 = H, R_3 = OH$ $R_1 = R_2 = R_3 = OCH_3$	Hepa1c1c7	Su *et al.* [56]
Withanolides		
	Hepa1c1c7	Gu *et al.* [61]

(continued)

TABLE V *(continued)*

Compound	Tissue or cell line	Reference
	Hepa1c1c7	Su *et al.* [62]
	Hepa1c1c7, liver, colon	Misico *et al.* [63]

[36] S. R. Spencer, C. A. Wilczak, and P. Talalay, *Cancer Res.* **50,** 7871 (1990).
[37] S. R. Spencer, L. A. Xue, E. M. Klenz, and P. Talalay, *Biochem. J.* **273,** 711 (1991).
[38] A. K. Jaiswal, R. Venugopal, J. Mucha, A. M. Carothers, and D. Grunberger, *Cancer Res.* **57,** 440 (1997).
[39] K. Kawabata, T. Yamamoto, A. Hara, M. Shimizu, Y. Yamada, K. Matsunaga, T. Tanaka, and H. Mori, *Cancer Lett.* **157,** 15 (2000).
[40] M. Iqbal, S. D. Sharma, Y. Okazaki, M. Fujisawa, and S. Okada, *Pharmacol. Toxicol.* **92,** 33 (2003).
[41] K. Okada, C. Wangpoengtrakul, T. Tanaka, S. Toyokuni, K. Uchida, and T. Osawa, *J. Nutr.* **131,** 2090 (2001).
[42] K. Singletary, C. MacDonald, M. Iovinelli, C. Fisher, and M. Wallig, *Carcinogenesis* **19,** 1039 (1998).
[43] A. T. Dinkova-Kostova and P. Talalay, *Carcinogenesis* **20,** 911 (1999).
[44] C. Gerhäuser, K. Klimo, E. Heiss, I. Neumann, A. Gamal-Eldeen, J. Knauft, G. Y. Liu, S. Sitthimonchai, and N. Frank, *Mutat. Res.* **523–524,** 163 (2003).
[45] D. S. Jang, E. J. Park, M. E. Hawthorne, J. S. Vigo, J. G. Graham, F. Cabieses, B. D. Santarsiero, A. D. Mesecar, H. H. Fong, R. G. Mehta, J. M. Pezzuto, and A. D. Kinghorn, *J. Agric. Food Chem.* **50,** 6330 (2002).
[46] A. T. Dinkova-Kostova, M. A. Massiah, R. E. Bozak, R. J. Hicks, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **98,** 3404 (2001).
[47] Z. Q. Jiang, C. Chen, B. Yang, V. Hebbar, and A. N. Kong, *Life Sci.* **72,** 2243 (2003).
[48] M. Floreani, E. Napoli, and P. Palatini, *Biochem. Pharmacol.* **60,** 601 (2000).
[49] A. T. Dinkova-Kostova, *Mini Rev. Med. Chem.* **2,** 595 (2002).
[50] G. Williamson, G. W. Plumb, Y. Uda, K. R. Price, and M. J. Rhodes, *Carcinogenesis* **17,** 2385 (1996).
[51] Y. Uda, K. R. Price, G. Williamson, and M. J. Rhodes, *Cancer Lett.* **120,** 213 (1997).
[52] L. G. Valerio, J. K. Kepa, G. V. Pickwell, and L. C. Quattrochi, *Toxicol. Lett.* **119,** 49 (2001).
[53] J. W. Fahey and K. K. Stephenson, *J. Agric. Food Chem.* **50,** 7472 (2002).
[54] T. Tanaka, K. Kawabata, M. Kakumoto, H. Makita, J. Ushida, S. Honjo, A. Hara, H. Tsuda, and H. Mori, *Carcinogenesis* **20,** 1477 (1999).

tissues.[27–33] Initially, 1,4-diphenols and *tert*-butylhydroquinone were found to raise the levels of NQO1 and GST in mouse liver in a concentration-dependent manner.[22] A series of analogs were synthesized (Table I) and their inducer potencies were compared. It was found that the critical structural feature of these phenolic compounds was their capacity to undergo reversible oxidation-reduction reactions. Thus catechols, hydroquinones, 1,2-, and 1,4-phenylenediamines were inducers, whereas resorcinols or 1,3-phenylenediamines were not. In contrast, the presence or absence of an alkyl side chain had little impact on the inducer potency. It was subsequently established that Michael reaction acceptors are a major class of inducers (see the following). This finding strongly suggested that the electrophilic quinone (or quinoneimine) oxidation products were the ultimate inducers and not the oxidation process *per se*.

Class 2: Michael Reaction Acceptors

Coumarin was one of the first plant compounds that was shown to induce NQO1, although weakly.[33] This led to the evaluation of the inducer potencies of a number of lactones, cinnamates, acrylates, crotonates, and indoles—all bearing olefins (or acetylenes) conjugated to electron-withdrawing groups—that is, Michael reaction acceptors. The potency of these compounds as NQO1 inducers paralleled closely their reactivity in the Michael reaction.[25] The Michael reaction acceptor group is present in the molecules of many plant metabolites and is essential for NQO1 inducer activity of cinnamates, curcuminoids, and flavonoids. Some examples

[55] K. Kawabata, T. Tanaka, S. Honjo, M. Kakumoto, A. Hara, H. Makita, N. Tatematsu, J. Ushido, H. Tsuda, and H. Mori, *Int. J. Cancer* **83,** 381 (1999).
[56] B. N. Su, E. J. Park, J. S. Vigo, J. G. Graham, F. Cabieses, H. H. Fong, J. M. Pezzuto, and A. D. Kinghorn, *Phytochemistry* **63,** 335 (2003).
[57] L. L. Song, J. W. Kosmeder, 2nd, S. K. Lee, C. Gerhäuser, D. Lantvit, R. C. Moon, R. M. Moriarty, and J. M. Pezzuto, *Cancer Res*. **59,** 578 (1999).
[58] J. Q. Gu, E. J. Park, J. S. Vigo, J. G. Graham, H. H. Fong, J. M. Pezzuto, and A. D. Kinghorn, *J. Nat. Prod*. **65,** 1616 (2002).
[59] J. Zhao and R. Agarwal, *Carcinogenesis* **20,** 2101 (1999).
[60] L. C. Chang, C. Gerhäuser, L. Song, N. R. Farnsworth, J. M. Pezzuto, and A. D. Kinghorn, *J. Nat. Prod*. **60,** 869 (1997).
[61] J.-Q. Gu, W. Li, Y.-H. Kang, B.-N. Su, H. H. S. Fong, R. B. van Breemen, J. M. Pezzuto, and A. D. Kinghorn, *Chem. Pharm. Bull. (Tokyo)* **51,** 530 (2003).
[62] B.-N. Su, E. J. Park, D. Nikolic, B. D. Santarsiero, A. D. Mesecar, J. S. Vigo, J. G. Graham, F. Cabieses, R. B. van Breemen, H. H. Fong, N. R. Farnsworth, J. M. Pezzuto, and A. D. Kinghorn, *J. Org. Chem*. **68,** 2350 (2003).
[63] R. I. Misico, L. L. Song, A. S. Veleiro, A. M. Cirigliano, M. C. Tettamanzi, G. Burton, G. M. Bonetto, V. E. Nicotra, G. L. Silva, R. R. Gil, J. C. Oberti, A. D. Kinghorn, and J. M. Pezzuto, *J. Nat. Prod*. **65,** 677 (2002).

are given in Tables II through V. Similarly, the Michael reaction acceptor—that is, the α,β-unsaturated ketone moiety of a number of withanolides isolated from Solanaceae is required for NQO1 inducer activity.[61–63]

It should be noted that some flavanones (e.g., pinostrobin) exhibit inducer activity, implying that such molecules might be precursors rather than the ultimate inducers and that metabolism plays a role in generating the active species. This notion is supported by the fact that pinostrobin is inactive in cells with defective *Ah*-receptor function.[53] Indeed, most flavonoids are bifunctional inducers.[64] In a series of aromatic Michael reaction acceptors the presence of hydroxyl group(s) on the aromatic ring(s) at *ortho*-position(s) to the vinyl carbons enhances the inducer potency enormously, in some cases by more than 200-fold,[34,43,46] and proposed that this structural feature must function to accelerate the rate of addition of the inducer to reactive sulfhydryl(s) on a hypothetical intracellular sensor, which has subsequently been identified as Keap1.[34,46,65]

Class 3: Isothiocyanates, Dithiocarbamates, and Related Sulfur Compounds

The characteristic $-N = C = S$ functionality of all isothiocyanates reacts very readily at its highly electrophilic central carbon atom with sulfhydryl-containing compounds to give dithiocarbamates. Isothiocyanates, thio-, dithiocarbamates, as well as related sulfur compounds, were recognized as NQO1 inducers in the early studies directed towards understanding the mechanism of induction.[25,26,66,67] At about the same time L. Wattenberg showed that, similar to BHA, dietary administration of benzyl isothiocyanate induced glutathione *S*-transferase activity in mouse esophagus, forestomach, and small intestine and this induction was associated with inhibition of benzo[*a*]pyrene-induced neoplasia of the forestomach.[68,69] Isothiocyanates are present in cruciferous vegetables as ''inert'' glucoside precursors (glucosinolates) and are compartmentally isolated from the enzyme (myrosinase) that catalyzes their conversion to the reactive isothiocyanates—for example, when the plant is injured or chewed. Sulforaphane was isolated as the principal NQO1 inducer from

[64] S. Yannai, A. J. Day, G. Williamson, and M. J. Rhodes, *Food Chem. Toxicol.* **36,** 623 (1998).
[65] A. T. Dinkova-Kostova, W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **99,** 11908 (2002).
[66] A. M. Benson, P. B. Barretto, and J. S. Stanley, *J. Natl. Cancer Inst.* **76,** 467 (1986).
[67] P. Talalay, M. J. De Long, and H. J. Prochaska, *in* ''Cancer Biology and Therapeutics.'' Plenum Publishing Corporation, 1987.
[68] V. L. Sparnins and L. W. Wattenberg, *J. Natl. Cancer Inst.* **66,** 769 (1981).
[69] V. L. Sparnins, J. Chuan, and L. W. Wattenberg, *Cancer Res.* **42,** 1205 (1982).

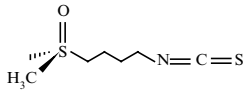
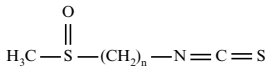
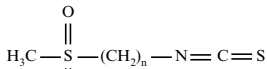
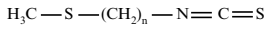
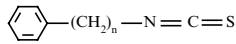
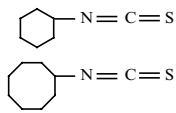
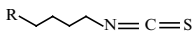
broccoli,[70] and it was subsequently found that 3-day-old broccoli sprouts (grown from selected seeds) contain 20 to 50 times higher levels of its glucosinolate, glucoraphanin than the mature broccoli plant.[19] The demonstration of the protective effect of sulforaphane against cancer in the DMBA mammary tumor model in Sprague-Dawley rats was a proof of the principle that measuring the NQO1 inducer activity is an effective strategy in the search for chemoprotective agents. A variety of isothiocyanates and synthetic analogs have been tested in this laboratory as inducers of NQO1 and their potencies have been compared (Table VI).[71] Sulforaphane remains one of the most potent NQO1 inducers known to date. Importantly, sulforaphane quickly accumulates in cells of various types, reaching intracellular concentrations in the millimolar range, which is probably one of the reasons underlying its extremely high inducer potency.[72] Intracellular accumulation is achieved through conjugation with cellular glutathione,[73] a reaction that is further accelerated by glutathione *S*-transferases,[74] and the glutathione conjugate is then exported by a transporter-mediated mechanism.[75] Furthermore, NQO1 inducer potency among a series of isothiocyanates correlates with their intracellular accumulation,[76] highlighting that transport mechanisms should also be carefully considered in addition to structure and chemical reactivity in determining inducer potency. We recently isolated 4-(rhamnopyranosyloxy)benzyl isothiocyanate as a very potent NQO1 inducer from *Moringa oleifera*. Depending on the cell type, its inducer potency was comparable to, or even exceeded that of sulforaphane.[20]

Class 4: 1,2-Dithiole-3-thiones, Oxathiolene Oxides, and Other Organosulfur Compounds

In the early 1980s, work from the laboratory of E. Bueding showed that administration of 1,2-dithiole-3-thione derivatives to mice produces biochemical effects similar to those of BHA and protected against toxicity and carcinogenicity.[77] The antischistosomal agent oltipraz and related dithiolethiones (Table VII) were then shown to induce NQO1 in many

[70] Y. Zhang, P. Talalay, C. G. Cho, and G. H. Posner, *Proc. Natl. Acad. Sci. USA* **89,** 2399 (1992).
[71] G. H. Posner, C. G. Cho, J. V. Green, Y. Zhang, and P. Talalay, *J. Med. Chem.* **37,** 170 (1994).
[72] Y. Zhang and P. Talalay, *Cancer Res.* **58,** 4632 (1998).
[73] Y. Zhang, *Carcinogenesis* **21,** 1175 (2000).
[74] Y. Zhang, *Carcinogenesis* **22,** 425 (2001).
[75] Y. Zhang and E. C. Callaway, *Biochem. J.* **364,** 301 (2002).
[76] L. Ye and Y. Zhang, *Carcinogenesis* **22,** 1987 (2001).
[77] S. S. Ansher, P. Dolan, and E. Bueding, *Hepatology* **3,** 932 (1983).

TABLE VI
INDUCERS OF NQO1: ISOTHIOCYANATES, DITHIOCARBAMATES, AND RELATED SULFUR COMPOUNDS

Compound	Tissue or cell line	Reference
 Sulforaphane	Liver, lung, forestomach, glandular stomach, small intestine, colon, Hepa1c1c7, HepG2, MCF-7, HT-29, LNCaP, HeLa	Zhang *et al.* [70]; McMahon *et al.* [32]; Jiang *et al.* [47]
 n = 3, 4, 5, 6	Liver, lung, forestomach, glandular stomach, small intestine, Hepa1c1c7	Zhang *et al.* [70]; Rose *et al.* [78]; Hou *et al.* [79]; Morimitsu *et al.* [80]
 n = 3, Cheirolin n = 4, Erysolin n = 5	Liver, forestomach, glandular stomach, small intestine, colon, Hepa1c1c7	Zhang *et al.* [70]
 n = 3, Iberverin n = 4, Erusin n = 5, Berteroin	Liver, forestomach, glandular stomach, small intestine, colon, Hepa1c1c7	Zhang *et al.* [70]
 n = 0, 1, 2, 4, 6	Liver, lung, kidney, forestomach, small intestine, colon, bladder, Hepa1c1c7	Prestera *et al.* [35]; Benson *et al.* [66]; Guo *et al.* [81]
	Hepa1c1c7	Prestera *et al.* [35]
 R = C_2H_5, N≡C, HOOC, CH_3OOC, CH_3SCO, CH_3CO, n-BuCO, $(CH_3)_2P(=O)$	Hepa1c1c7	Posner *et al.* [71]

(continued)

TABLE VI *(continued)*

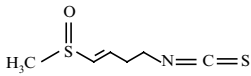
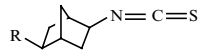
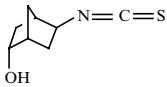
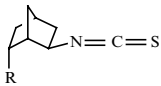
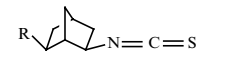
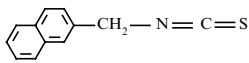
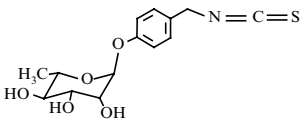
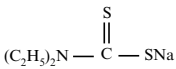
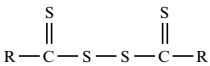
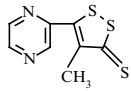
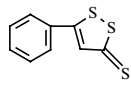
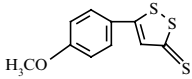
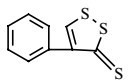
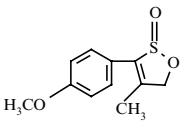
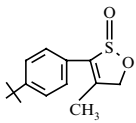
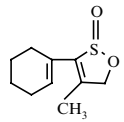
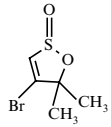
Compound	Tissue or cell line	Reference
 Sulforaphene	Hepa1c1c7	Posner *et al.* [71]
 R = O_2N, N≡C, CH_3SO_2, CH_3CO, CH_3OOC, $CH_3CH(OH)$	Hepa1c1c7	Posner *et al.* [71]
	Hepa1c1c7	Posner *et al.* [71]
 R = CH_3SO_2, CH_3CO	Hepa1c1c7	Posner *et al.* [71]
 R = CH_3SO_2, CH_3CO, CH_3OOC	Hepa1c1c7	Posner *et al.* [71]
	Liver	Leonard *et al.* [82, 83]
	Hepa1c1c7 ARPE-19 HepG2 AGS	Fahey *et al.* [20]
	Liver, lung, kidney, forestomach, small intestine, colon, bladder, Hepa1c1c7	Talalay and Benson [26]; Benson *et al.* [66]; Talalay *et al.* [67]
 R = $(C_2H_5)_2N$ Disulfiram R = C_2H_5O Bisethylxanthogen	Liver, lung, kidney, forestomach, small intestine, colon, bladder, Hepa1c1c7	Talalay and Benson [26]; Benson *et al.* [66]; Talalay *et al.* [67]

TABLE VII
INDUCERS OF NQO1: 1,2-DITHIOLE-3-THIONES, AND OXATHIOLENE OXIDES

Compound	Tissue or cell line	Reference
 1,2-dithiole-3-thione	Liver, lymphocytes	De Long *et al.* [84]; Kensler *et al.* [85]; Gordon *et al.* [86]
 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione(oltipraz)	Liver, lung, jejunum	McMahon *et al.* [32]; De Long *et al.* [84]; Ansher *et al.* [87]; Kensler *et al.* [85]
 5-phenyl-1,2-dithiole-3-thione	Liver, lung, jejunum	De Long *et al.* [84]; Ansher *et al.* [87]; Kensler *et al.* [85]
 5-(*p*-methoxyphenyl)-1,2-dithiole-3-thione	Liver, lung, jejunum	De Long *et al.* [84]; Ansher *et al.* [87]; Kensler *et al.* [85]
 4-phenyl-1,2-dithiole-3-thione	Liver, lung, jejunum	De Long *et al.* [84]; Ansher *et al.* [87]; Kensler *et al.* [85]
 3-(*p*-methoxyphenyl)-4-methyl-1,2-oxathiol-3-ene-2-oxide	Hepa1c1c7, BNLCL2	Pietsch *et al.* [88]
 3-(*p*-*t*-butylphenyl)-4-methyl-1,2-oxathiol-3-ene-2-oxide	Hepa1c1c7, BNLCL2	Pietsch *et al.* [88]

(continued)

TABLE VII *(continued)*

Compound	Tissue or cell line	Reference
 3-cyclohexenyl-4-methyl-1,2-oxathiol-3-ene-2-oxide	Hepa1c1c7, BNLCL2	Pietsch *et al.* [88]
 4-bromo-5,5-dimethyl-1,2-oxathiol-3-ene-2-oxide	Hepa1c1c7, BNLCL2	Pietsch *et al.* [88]

organs and tissues,[84,85,87] as well as in human lymphocytes in culture.[86] Oltipraz was subsequently developed as a chemopreventive agent in clinical trials in Qidong, China, an area with a very high incidence of liver cancer attributable in part to consumption of grains contaminated with aflatoxin B_1.[89,90] In such volunteers, administration of oltipraz profoundly affected the excretion of aflatoxin B_1-DNA adducts. At low doses of oltipraz the

[78] P. Rose, P. K. Faulkner, G. Williamson, and R. Mithen R, *Carcinogenesis* **21,** 1983 (2000).
[79] D. X. Hou, M. Fukuda, M. Fujii, and Y. Fuke, *Int. J. Mol. Med.* **6,** 441 (2000).
[80] Y. Morimitsu, Y. Nakagawa, K. Hayashi, H. Fujii, T. Kumagai, Y. Nakamura, T. Osawa, F. Horio, K. Itoh, K. Iida, M. Yamamoto, and K. Uchida, *J. Biol. Chem.* **277,** 3456 (2002).
[81] Z. Guo, T. J. Smith, E. Wang, K. I. Eklind, F. L. Chung, and C. S. Yang, *Carcinogenesis* **14,** 1167 (1993).
[82] T. B. Leonard, J. A. Popp, M. E. Graichen, and J. G. Dent, *Carcinogenesis* **2,** 473 (1981).
[83] T. B. Leonard, J. A. Popp, M. E. Graichen, and J. G. Dent, *Toxicol. Appl. Pharmacol.* **60,** 527 (1981).
[84] M. J. De Long, P. Dolan, A. B. Santamaria, and E. Bueding, *Carcinogenesis* **7,** 977 (1986).
[85] T. W. Kensler, P. A. Egner, P. M. Dolan, J. D. Groopman, and B. D. Roebuck, *Cancer Res.* **47,** 4271 (1987).
[86] G. B. Gordon, H. J. Prochaska, and L. Y. Yang, *Carcinogenesis* **12,** 2393 (1991).
[87] S. S. Ansher, P. Dolan, and E. Bueding, *Food Chem. Toxicol.* **24,** 405 (1986).
[88] E. C. Pietsch, A. L. Hurley, E. E. Scott, B. P. Duckworth, M. E. Welker, S. Leone-Kabler, A. J. Townsend, F. M. Torti, and S. V. Torti, *Biochem. Pharmacol.* **65,** 1261 (2003).
[89] T. W. Kensler, T. J. Curphey, Y. Maxiutenko, and R. D. Roebuck, *Drug Metabol. Drug Interact.* **17,** 3 (2000).
[90] T. W. Kensler, P. A. Egner, J. B. Wang, Y. R. Zhu, B. C. Zhang, G. S. Qian, S. Y. Kuang, S. J. Gange, L. P. Jacobson, A. Munoz, and J. D. Groopman, *Eur. J. Cancer Prev.* **11,** S58 (2002).

spectrum of urinary aflatoxin metabolites changes and is consistent with phase 2 enzyme induction.

Recently, as part of the development of new candidates for chemopreventive agents, the chemically similar oxathiolene oxides (Table VII) were synthesized and shown to be inducers of NQO1.[88] Various mono-, di-, and polysulfides from *Allium* plants (Table VIII), shown in the 1980s to elevate glutathione *S*-transferase activity in the forestomach and to protect against benzo[*a*]pyrene-induced carcinogenesis,[91] also induce NQO1, with diallyl disulfide being the most potent among them.[25,31,92–96] Structure-activity relationship studies revealed that the unsaturated allyl or propenyl functionalities are critical determinants of inducer potency.[25,91–96]

Class 5: Hydroperoxides

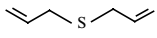
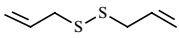
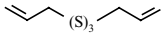
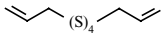
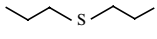
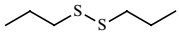
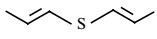
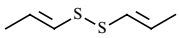
Cumene hydroperoxide, hydrogen peroxide, and *tert*-butyl hydroperoxide are, although weak, also inducers of NQO1 (Table IX).[35,37]

Class 6: Trivalent Arsenicals

Trivalent arsenicals—for example, phenylarsine oxide, are extremely potent inducers of NQO1 and this finding gave an important clue to the mechanism involved in the initial sensing of the inducer signal. In their classical studies on the development of antidotes for arsenic poisoning, Stocken and Thompson (1946)[97–99] demonstrated that vicinal dithiols are excellent reagents for trivalent arsenicals and form highly stable five-membered cyclic products, while the reaction with monothiols gives rise to ''open chain'' much more easily dissociable compounds. These properties of trivalent arsenicals form the basis for the development of arsenical affinity chromatography for proteins with sulfhydryl groups that are positioned close in space.[100] In contrast to trivalent arsenicals, pentavalent arsenicals—for example sodium arsenate, are much weaker NQO1 inducers. This finding correlates with the much higher reactivity of trivalent

[91] V. L. Sparnins, G. Barany, and L. W. Wattenberg, *Carcinogenesis* **9,** 131 (1988).
[92] S. V. Singh, S. S. Pan, S. K. Srivastava, H. Xia, X. Hu, H. A. Zaren, and J. L. Orchard, *Biochem. Biophys. Res. Commun*. **244,** 917 (1998).
[93] D. Guyonnet, M. H. Siess, A. M. Le Bon, and M. Suschetet, *Toxicol. Appl. Pharmacol.* **154,** 50 (1999).
[94] D. Guyonnet, C. Belloir, M. Suschetet, M. H. Siess, and A. M. Le Bon, *Mutat. Res*. **495,** 135 (2001).
[95] R. Munday and C. M. Munday, *Nutr. Cancer* **40** (2001).
[96] R. Munday, J. S. Munday, and C. M. Munday, *Free Radic. Biol. Med*. **34,** 1200 (2003).
[97] L. A. Stocken and R. H. S. Thompson, *Biochem. J*. **40,** 529 (1946).
[98] L. A. Stocken and R. H. S. Thompson, *Biochem. J*. **40,** 535 (1946).
[99] L. A. Stocken and R. H. S. Thompson, *Biochem. J*. **40,** 548 (1946).
[100] R. D. Hoffman and M. D. Lane, *J. Biol. Chem*. **267,** 14005 (1992).

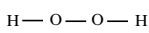
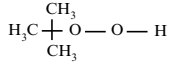
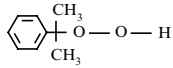
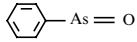
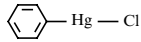
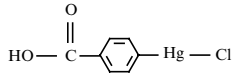
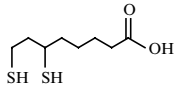
TABLE VIII
INDUCERS OF NQO1: ALKYL AND ALKENYL SULFIDES AND POLYSULFIDES

Compound	Tissue	Reference
 Diallyl sulfide	Lung, heart, bladder, forestomach, glandular stomach, duodenum, jejunum, ileum, cecum, colon, brain	Singh *et al.* [92]; Guyonnet *et al.* [93, 94]; Munday and Munday [95]; Yang *et al.* [101]
 Diallyl disulfide	Hepa1c1c7, liver, lung, heart, bladder, forestomach, glandular stomach, duodenum, jejunum, spleen, ileum, cecum, colon	Talalay *et al.* [25]; Singh *et al.* [92]; Guyonnet *et al.* [93, 94]; Munday and Munday [31]
 Diallyl trisulfide	Liver, lung, heart, kidney, bladder, forestomach, glandular stomach, duodenum, jejunum, spleen, ileum, cecum, colon	Singh *et al.* [92]; Guyonnet *et al.* [93, 94]; Munday and Munday [95]; Munday *et al.* [96]
 Diallyl tetrasulfide	Liver, lung, heart, kidney, spleen	Munday *et al.* [96]
 Dipropyl sulfide	Liver, forestomach, glandular stomach, cecum, colon	Singh *et al.* [92]; Guyonnet *et al.* [93, 94]
 Dipropyl disulfide	Liver, lung, heart, forestomach, duodenum, spleen, cecum, colon	Singh *et al.* [92]; Guyonnet *et al.* [93, 94]
 Dipropenyl sulfide	Liver, lung, heart, kidney, bladder, forestomach, glandular stomach, duodenum, jejunum, spleen, ileum, cecum, colon	Munday and Munday [95]
 Dipropenyl disulfide	Liver, lung, heart, kidney, bladder, forestomach, glandular stomach, duodenum, jejunum, spleen, ileum, cecum, colon	Munday and Munday [95]

arsenicals with sulfhydryl groups that are closely positioned in space (e.g., vicinal). Based on this, Prestera *et al.* (1993)[35] made the following prediction that has, with modern tools of molecular biology, since been shown to be correct[65]; "The potent induction of NQO1 by such compounds suggests the critical presence of two neighboring sulfhydryl groups on the protein(s) that receive and transmit the inductive signal."

[101] C. S. Yang, S. K. Chhabra, J. Y. Hong, and T. J. Smith, *J. Nutr.* **131,** 1041S (2001).

TABLE IX
INDUCERS OF NQO1: HYDROPEROXIDES, ARSENICALS, MERCURIALS, AND MERCAPTANS

Compound	Tissue or cell line	Reference
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]; Spencer *et al.* [37]
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]
	Hepa1c1c7	Prestera [35, 102]
 Lipoic acid	Astroglial cells	Flier *et al.* [103]

Class 7: Heavy Metals

Divalent metal cations induce NQO1 in an order of potency that correlates with their reactivity with sulfhydryl groups—that is, $Hg^{2+} > Cd^{2+} > Zn^{2+}$ in Hepa1c1c7 and LNCaP cells.[35,104] Hg^{2+} and Cd^{2+} increase NQO1 activity in explants of both first-trimester, as well as full-term

[102] T. Prestera, W. D. Holtzclaw, Y. Zhang, and P. Talalay, *Proc. Natl. Acad. Sci. USA* **90,** 2965 (1993).
[103] J. Flier, F. L. Van Muiswinkel, C. A. Jongenelen, and B. Drukarch, *Free Radic. Res*. **36,** 695 (2002).
[104] J. D. Brooks, M. F. Goldberg, L. A. Nelson, D. Wu, and W. G. Nelson, *Cancer Epidemiol. Biomarkers Prev*. **11,** 868 (2002).

human placentae.[105–107] In addition, the sulfhydryl reagents phenylmercuric chloride and *para*-chloromercuribenzoate are also potent inducers of NQO1.[35] Administration of triethyl lead to rats has been shown to increase the levels of NQO1 in liver and kidney.[108]

Class 8: Vicinal Dimercaptans

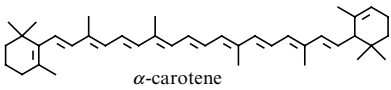
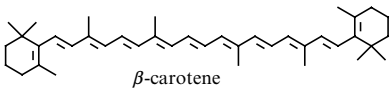
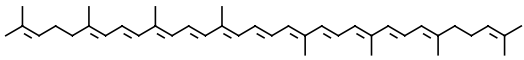
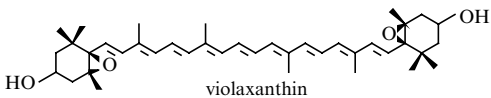
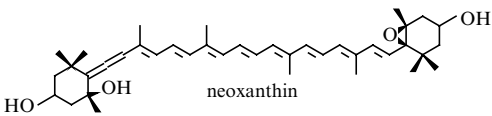
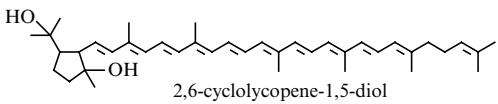
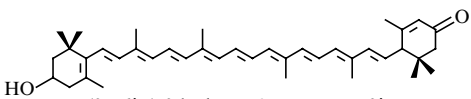
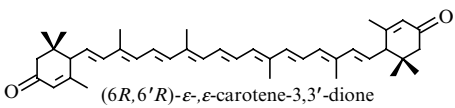
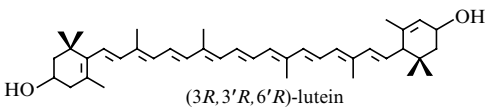
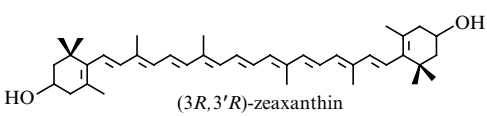
The finding that selected nucleophilic mercaptans are also NQO1 inducers was somewhat unexpected since most of the other inducers are electrophilic. The most potent members of this class have two sulfhydryl groups in close proximity—for example, 2,3-dimercapto-1-propanol (BAL) and 1,2-ethanedithiol, containing vicinal dithiols, and dihydrolipoic acid, which has 2 closely spaced thiol groups (Table IX).[35,103] Interestingly, in Hepa1c1c7 cells $HgCl_2$ and BAL act synergistically in inducing NQO1.[109] In contrast, no synergism was observed between $HgCl_2$ and monothiols, suggesting that the ultimate inducer could be a high-affinity chelate complex between Hg^{2+} and the vicinal thiol groups of BAL. Indeed, synthetic equimolar BAL-mercury chelates are much more potent inducers than $HgCl_2$. Such complexes entered and accumulated in cells more efficiently and excess BAL increased the synergism even further.

Class 9: Carotenoids and Related Polyenes

The finding that polyenes (Table X) are NQO1 inducers[110] was intriguing, given their known protective role against oxidants and photooxidative damage. The unsubstituted lycopene is a good NQO1 inducer, indicating that the conjugated polyene chain itself has inducer activity.[110,111] The 7-fold difference in inducer potency between α- and β-carotene is noteworthy since these compounds only differ by the position of a single double bond in the end groups (without any other structural differences)—that is, the more-potent inducer (β-carotene) has 11 conjugated double bonds vs 10 in the molecule of α-carotene. In addition to the extent of conjugation, the presence of hydroxyl as well as α, β-unsaturated ketone

[105] W. Y. Boadi, J. Urbach, E. R. Barnea, J. M. Brandes, and S. Yannai, *Pharmacol. Toxicol.* **68,** 317 (1991).
[106] W. Y. Boadi, J. Urbach, J. M. Brandes, and S. Yannai, *Environ. Res.* **57,** 96 (1992).
[107] W. Y. Boadi, J. Urbach, J. M. Brandes, and S. Yannai, *Toxicol. Lett.* **60,** 155 (1992).
[108] D. A. Daggett, E. F. Nuwaysir, S. A. Nelson, L. S. Wright, S. E. Kornguth, and F. L. Siegel, *Toxicology* **117,** 61 (1997).
[109] R. R. Putzer, Y. Zhang, T. Prestera, W. D. Holtzclaw, K. L. Wade, and P. Talalay, *Chem. Res. Toxicol.* **8,** 103 (1995).
[110] F. Khachik, J. S. Bertram, M.-T. Huang, J. W. Fahey, and P. Talalay, *in* "Antioxidant Food Supplements in Human Health," p. 203. Academic Press, 1999.
[111] V. Breinholt, S. T. Lauridsen, B. Daneshvar, and J. Jakobsen, *Cancer Lett.* **154,** 201 (2000).

TABLE X
INDUCERS OF NQO1: CAROTENOIDS AND RELATED POLYENES

Compound	Tissue or cell line	Reference
 α-carotene	Hepa1c1c7	Khachik *et al.* [110]
 β-carotene	Hepa1c1c7	Khachik *et al.* [110]
 lycopene	Hepa1c1c7, liver	Khachik *et al.* [110]; Breinholt *et al.* [111]
 violaxanthin	Hepa1c1c7	Khachik *et al.* [110]
 neoxanthin	Hepa1c1c7	Khachik *et al.* [110]
 2,6-cyclolycopene-1,5-diol	Hepa1c1c7	Khachik *et al.* [110]
 (3*R*,6′*R*)-3-hydroxy-β-,ε-carotene-3′-one	Hepa1c1c7	Khachik *et al.* [110]
 (6*R*,6′*R*)-ε-,ε-carotene-3,3′-dione	Hepa1c1c7	Khachik *et al.* [110]
 (3*R*,3′*R*,6′*R*)-lutein	Hepa1c1c7	Khachik *et al.* [110]
 (3*R*,3′*R*)-zeaxanthin	Hepa1c1c7	Khachik *et al.* [110]

functionalities increases further the inducer potency. The concentrations of the polyenes that induce NQO1 are in the micromolar range and can be achieved through dietary means. Importantly, the xanthophyll carotenoids lutein and zeaxanthin are found in high concentrations in the macula lutea region of the primate retina and are thought to protect this critical region of the eye against degeneration.[112]

Implications of the Chemical Structures of Inducers for their Mechanism of Action

The compounds that were first recognized to induce NQO1—that is, the oxidizable diphenols, were antioxidants. Paradoxically, other inducers—for example, the hydroperoxides, are powerful oxidants. Yet a third class, the isothiocyanates, have no significant redox properties. While nearly all inducers are electrophilic, the dimercaptans represent inducers that are nucleophilic. The only property that is shared by all inducers is their ability to react with sulfhydryl groups. Indeed, in the course of the systematic studies leading to the ''Michael reaction acceptor hypothesis'' of induction of NQO1,[25] it became striking that among inducers of different chemical classes were the same compounds that had been previously reported to react with glutathione and to serve as substrates for glutathione *S*-transferases.[113,114] In these classical studies, Chasseaud pointed out that the nature of the metabolic products of xenobiotic electrophiles—for example, α,β-unsaturated aldehydes, ketones, lactones, sulfones, nitro-olefins, as well as isothiocyanates, quinones, mercurials, acrylates, fumarates, allyl and epoxide derivatives—suggests the occurrence of an initial conjugation reaction with glutathione followed by catabolism via the mercapturic acid pathway.[114] Unrelated studies on the cystine and glutamate transport system from the laboratory of S. Bannai showed that while high concentrations of electrophilic agents (e.g., ethacrynic acid, cyclohex-2-en-1-one, diethyl maleate) caused depletion of glutathione and cytotoxicity, at lower concentrations the same compounds increase the intracellular glutathione levels substantially.[115] We now know that these changes in glutathione levels resulted from alkylation and enhanced synthesis, respectively. Indeed, most NQO1 inducers were found to be substrates for glutathione *S*-transferases.[25,37] Conversely, compounds that are commonly used as substrates for glutathione *S*-transferases (e.g., CDNB, DCNB, ethacrynic acid) were shown to be NQO1 inducers.[25] Moreover, the potency of

[112] B. R. Hammond, Jr., E. J. Johnson, R. M. Russell, N. I. Krinsky, K. J. Yeum, R. B. Edwards, and D. M. Snodderly, *Invest. Ophthalmol. Vis. Sci.* **38,** 1795 (1997).
[113] L. F. Chasseaud, *Biochem. J.* **131,** 765 (1973).
[114] L. F. Chasseaud, *in* ''Glutathione: Metabolism and Function.'' Raven Press, New York, 1974.
[115] S. Bannai, *J. Biol. Chem.* **259,** 2435 (1984).

inducers to elevate NQO1 paralleled their reactivity in the Michael reaction with nucleophilic donors such as mercaptans.[25,37,46] Further, within a series of structurally closely related compounds—for example, isothiocyanates, or aromatic single and double Michael reaction acceptors—the inducer potency is closely related to the second-order non-enzymatic rate constants of reactions with various sulfhydryl reagents.[46,80]

On the basis of all of these findings, we suggested the existence of a cellular ''sensor'' protein endowed with highly reactive sulfhydryl groups that recognizes and reacts with inducers in the initial event of the signal transduction leading to induction of phase 2 proteins.[46,65,102] Following the demonstration that the transcription factor Nrf2 is essential for the regulation of phase 2 genes[116] and that under basal conditions it is sequestered in the cytoplasm by the actin-bound cytosolic repressor Keap1,[117] we undertook detailed analysis of these two proteins and their interactions. Perhaps not surprisingly, Keap1 (624 amino acids) is a cysteine-rich protein: it has 25 cysteines that are conserved in its human and rat homologs. After cloning and overexpressing murine Keap1 in *Escherichia coli,* we purified the protein to homogeneity. Under reducing conditions, Keap1 binds to the N-terminal Neh2 domain of Nrf2. The complex can be visualized following non-denaturing polyacrylamide gel electrophoresis. Inducers—for example, sulforaphane, or Michael reaction acceptors—disrupt the complex in a concentration-dependent manner. Kinetic, radiolabeling and UV-spectroscopic studies demonstrated that four highly reactive cysteine residues (C257, C273, C288, and C297) located in the central intervening region (IVR) of the protein are probably the primary cellular sensors that recognize and react with inducers.[65] Subsequent mutagenesis analysis revealed that substitutions of these cysteine residues with alanine render Keap1 unable to sequester Nrf2 in the cytoplasm (our unpublished observations).

These recent experiments provide a satisfying explanation for the broad chemical specificity of phase 2 inducers: all inducers react chemically with specific cysteine thiols of the sensor protein Keap1 (by alkylation, oxidation, or thiolation) and thereby suppress its ability to bind and retain the transcription factor Nrf2 in the cytoplasm. Nrf2 is then able to migrate into the nucleus where it binds to the antioxidant response element and activates (in heterodimeric combinations with members of the small Maf family) the transcription of phase 2 genes.

[116] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, and Y. Nabeshima, *Biochem. Biophys. Res. Commun*. **236,** 313 (1997).

[117] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel, and M. Yamamoto, *Genes Dev*. **13,** 76 (1999).