Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8261–8265, November 1988
Medical Sciences

Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis

(Michael acceptors/quinone reductase/glutathione *S*-transferase/anticarcinogens)

PAUL TALALAY, MARY J. DE LONG, AND HANS J. PROCHASKA

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Contributed by Paul Talalay, July 19, 1988

ABSTRACT **Carcinogenesis is blocked by an extraordinary variety of agents belonging to many different classes—e.g., phenolic antioxidants, azo dyes, polycyclic aromatics, flavonoids, coumarins, cinnamates, indoles, isothiocyanates, 1,2-dithiol-3-thiones, and thiocarbamates. The only known common property of these anticarcinogens is their ability to elevate in animal cells the activities of enzymes that inactivate the reactive electrophilic forms of carcinogens. Structure–activity studies on the induction of quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione *S*-transferases have revealed that many anticarcinogenic enzyme inducers contain a distinctive and hitherto unrecognized chemical feature (or acquire this feature after metabolism) that regulates the synthesis of these protective enzymes. The inducers are Michael reaction acceptors characterized by olefinic (or acetylenic) bonds that are rendered electrophilic (positively charged) by conjugation with electron-withdrawing substituents. The potency of inducers parallels their efficiency in Michael reactions. Many inducers are also substrates for glutathione *S*-transferases, which is further evidence for their electrophilicity. These generalizations have not only provided mechanistic insight into the perplexing question of how such seemingly unrelated anticarcinogens induce chemoprotective enzymes, but also have led to the prediction of the structures of inducers with potential chemoprotective activity.**

An astonishing variety of chemical agents protects rodents against the toxic and neoplastic effects of carcinogens (1, 2). Many lines of evidence (2–5) strongly suggest that the elevation of enzymes concerned with carcinogen inactivation is one mechanism of critical importance in achieving chemoprotection. Anticarcinogenic enzyme inducers are of two types: (*i*) bifunctional inducers (polycyclic aromatic hydrocarbons, azo dyes, and flavonoids), which elevate phase II* xenobiotic metabolizing enzymes—e.g., glutathione *S*-transferases (GST), UDP-glucuronosyltransferases, and quinone reductase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]—as well as inducing phase I activities (e.g., aryl hydrocarbon hydroxylase); and (*ii*) monofunctional inducers (diphenols, thiocarbamates, 1,2-dithiol-3-thiones, isothiocyanates, cinnamates, and coumarins), which elevate phase II enzymes without inducing aryl hydrocarbon hydroxylase (3). Since phase I enzymes are the principal activators of carcinogens to their ultimate reactive forms, monofunctional inducers are more promising candidates than bifunctional inducers as useful anticarcinogens.

Although bifunctional inducers appear to elevate phase II enzymes in part by binding to the *Ah* (aryl hydrocarbon) receptor, the molecular mechanisms by which monofunctional inducers act are less clear (3). This paper extends our earlier efforts to identify structural features important for phase II enzyme induction by diphenols and phenylenediamines (5). Since the specific activity of QR in the Hepa 1c1c7 murine hepatoma cells is raised by virtually all compounds that produce coordinate elevations of phase II enzymes *in vivo* (4, 5, 7), this system was used to determine the potency of various types of enzyme inducers. Some inducers identified in cell culture were also tested as inducers of QR and GST in mouse tissues.

We report here that phase II enzyme inducers contain, or acquire by metabolism, a hitherto unrecognized and distinctive chemical and structural feature—i.e., an electrophilic olefin or related electron-deficient center. They are, therefore, Michael reaction acceptors.† This generalization has led to the identification of a number of phase II enzyme inducers that are Michael acceptors and are potential chemoprotectors. Most inducers are also substrates for GST, but whether this merely reflects their electrophilic nature or is an intrinsic aspect of the mechanism of induction is unclear.

MATERIALS AND METHODS

QR activities of Hepa 1c1c7 murine hepatoma cells were measured in cells grown in microtiter plates (8). The potency of compounds was determined from plots relating the ratio of treated to basal (vehicle only) of specific activities of QR to the concentration of inducer. Potencies are expressed as the concentrations required to double (designated CD) the basal specific activity of QR. Induction of cytosolic QR and GST activities in female CD1 mouse tissues was assessed by a standard protocol (9, 10). All compounds were of the highest quality obtainable commercially and were purified when necessary.

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Abbreviations: QR, quinone reductase; GSH, glutathione; GST, glutathione *S*-transferase; CD, concentration of a compound that doubles the specific activity of QR; BHA, 2(3)-*tert*-butyl-4-hydroxyanisole; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

*Enzymes of xenobiotic metabolism are of two types: (*i*) phase I enzymes (e.g., cytochromes P-450) functionalize compounds usually by oxidation or reduction; and (*ii*) phase II enzymes conjugate functionalized compounds with endogenous ligands (e.g., glutathione, glucuronic acid). Quinone reductase may be considered a phase II enzyme since it does not introduce new functional groups, is often induced coordinately with other phase II enzymes, and protects cells against toxic agents.

†In 1887 A. Michael reported that olefins conjugated with electron-withdrawing groups (Z) are susceptible to attack by nucleophiles. These so-called "Michael acceptors" have the structures $CH_2{=}CH{-}Z$, $Z'{-}CH{=}CH{-}Z$ (including quinones), or $R{-}C{\equiv}C{-}Z$ (acetylenes). The order of reactivity of $CH_2{=}CH{-}Z$ with morpholine or pyrrolidine is $Z = NO_2 > COAr > CHO > COCH_3 > CO_2CH_3 > CN > CONH_2 > CONR_2$ (6). Furthermore, alkyl substituents on the olefin decrease reactivity by electronic and steric effects.

RESULTS AND DISCUSSION

Diphenols, Phenylenediamines, and Quinones. Studies with 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) and several analogues led to the conclusion that the inductive capacity of these compounds depended on their conversion to diphenols (10). Furthermore, among such diphenols only catechols (1,2-diphenols) and hydroquinones (1,4-diphenols), but not resorcinols (1,3-diphenols), were inducers, and the presence or position of other substituents was of minor importance (5). Analogous results were obtained with phenylenediamines. These findings suggested that chemical (oxidative) reactivity rather than unique structural features was the crucial determinant of inductive activity, since 1,2- and 1,4-diphenols and corresponding phenylenediamines readily undergo oxidative conversion to quinones or quinoneimines, respectively, whereas the 1,3- analogues do not (5). We speculated that the inductive process depended on oxidative lability of the inducers. However, the present studies disclosed that the signaling of enzyme induction depended on electrophilic centers, and the inductive ability of diphenols and phenylenediamines could therefore be ascribed to their conversion to electrophilic quinones or quinoneimines, respectively.

Coumarin Analogues. Coumarin (Table 1, Compound **1**) is a widely distributed, naturally occurring flavoring agent. It protects against hydrocarbon-mediated carcinogenesis and elevates GSTs in rodent tissues (11–14). We reported that coumarin raised the QR levels in Hepa 1c1c7 cells grown on 75-cm^2 plates (CD = 100 μM; ref. 7), although it was inactive in the microtiter assay system (CD > 800 μM).

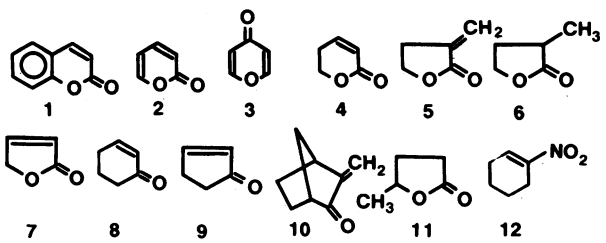
Examination of coumarin analogues to decipher the structural features required for QR induction showed that 2*H*-pyran-2-one (Compound **2**) and 4*H*-pyran-4-one (Compound **3**) were inactive, whereas 5,6-dihydro-2*H*-pyran-2-one (Compound **4**) had considerable inductive activity (CD = 45 μM). The latter finding suggested that the structural feature critical for inductive activity was an electrophilic olefin conjugated with a carbonyl group—i.e., a Michael reaction acceptor.† Hence, lack of activity of Compounds **1–3** could be related to their partial aromaticity, which lowers the electrophilicity of the α,β-unsaturated carbonyl function.

Table 1. Concentration (CD) of coumarin and pyran analogues required to double quinone reductase in Hepa 1c1c7 cells

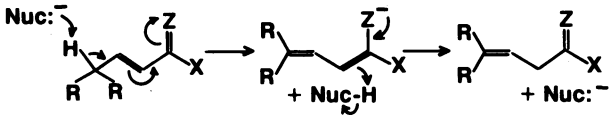
Compound		
No.	Name	CD, μM
1	Coumarin	I
2	2*H*-Pyran-2-one	I
3	4*H*-Pyran-4-one	I
4	5,6-Dihydro-2*H*-pyran-2-one	45
5	2-Methylene-4-butyrolactone	22
6	2-Methylbutyrolactone	I
7	4-Hydroxycrotonolactone	210
8	1-Cyclohexen-2-one	28
9	1-Cyclopenten-2-one	100
10	3-Methylene-2-norbornanone	6.8
11	γ-Valerolactone	I
12	1-Nitro-1-cyclohexene	1–2

I, inactive; <20% increase in specific activity at 200 μM.

The structures of **1–12** are as follows:



Comparison of the potencies of lactones with those of corresponding carbocyclics (compare Compound **4** with **8** and **7** with **9**) showed that the bridge oxygen of the lactones weakened inductive activity, either because of hydrolysis of lactones to acids or because lactones (i.e., esters) are weaker Michael acceptors than ketones.† Moreover, 2-methylene-4-butyrolactone (Compound **5**; CD = 22 μM) and 3-methylene-2-norbornanone (Compound **10**; CD = 6.8 μM) were more potent inducers than other cyclic olefins, further strengthening the evidence that these compounds induce by behaving as Michael acceptors. The higher potencies of Compounds **5** and **10** may result from the absence of acidic hydrogens on the carbon atom adjacent to the electrophilic center of the olefin (compare **5** with **4** or **7** and **10** with **8** or **9**, respectively). Such acidic protons can interfere with Michael addition by neutralizing the attacking nucleophile and by destroying the electrophilic character of the acceptor (15):



Although these protons are only weakly acidic in aqueous systems, they may decrease the reactivity of Michael acceptors in the presence of biological macromolecules that could promote deprotonation. The relationship between potency of phase II enzyme induction and Michael acceptor efficiency is also supported by the high activity of 1-nitro-1-cyclohexene (Compound **12**; CD = 1–2 μM) since nitroolefins are highly efficient Michael acceptors.† Furthermore, all cyclic compounds lacking an olefin conjugated to an electron-withdrawing group were inactive as inducers (Compounds **6** and **11**).

Acrylate, Crotonate, and Cinnamate Analogues. Identification of an α,β-unsaturated carbonyl function as essential for inductive activity among lactones and carbocyclics raised two questions: (*i*) must these olefins be part of a ring system, and (*ii*) what electron-withdrawing groups can replace the ketone function? Studies with acrylate, crotonate, and cinnamate analogues clarified both questions (Table 2).

The methyl esters of acrylic (Compound **13**) and cinnamic (Compound **28**) acids were both inducers, showing unequivocally that the activated olefin need not be cyclic. In contrast, free crotonic (Compound **22**) and cinnamic (Compound **27**) acids were inactive, as anticipated from the fact that free carboxyl groups weaken Michael acceptor efficiency, but more efficient cellular uptake of esters than free acids also may be important. The inactivity of methyl methacrylate (Compound **14**), methyl crotonate (Compound **23**), and methyl tiglate (Compound **15**) in comparison to methyl acrylate (Compound **13**; CD = 20 μM) suggested that methyl substituents on either the α- or β-carbon (or both) interfered with inductive activity, presumably because of electronic and steric effects (6), as predicted by recent molecular orbital calculations (16). Aldehydes of all three series are inducers, although crotonaldehyde (Compound **24**; CD = 9 μM) was far more potent than either acrolein (Compound **16**; CD = 130 μM) or cinnamaldehyde (Compound **29**; CD = 110 μM). The variation in inductive potencies of these aldehydes might reflect differences in rates of their metabolism.

In the acrylate series, inductive activity is preserved if the oxygen function is part of a ketone (i.e., methyl vinyl ketone, Compound **17**; CD = 40 μM) or of a sulfone (i.e., methyl vinyl sulfone, Compound **18**; CD = 25 μM), but not of an amide (i.e., acrylamide; Compound **20**). The carbonyl function may also be replaced by a nitrile (acrylonitrile, Compound **19**; CD = 50 μM), although not in the crotonate

Table 2. Concentration (CD) of acrylate, crotonate, and cinnamate analogues required to double quinone reductase in Hepa 1c1c7 cells

Compound No.	Name	Structure	CD, μM
		Acrylates	
13	Methyl acrylate	CH_2=$CHCOOCH_3$	20
14	Methyl methacrylate	CH_2=$C(CH_3)COOCH_3$	I
15	Methyl tiglate	CH_3CH=$C(CH_3)COOCH_3$	I
16	Acrolein	CH_2=$CHCHO$	130
17	Methyl vinyl ketone	CH_2=$CHCOCH_3$	40
18	Methyl vinyl sulfone	CH_2=$CHSO_2CH_3$	25
19	Acrylonitrile	CH_2=CHC≡N	50
20	Acrylamide	CH_2=$CHCONH_2$	I
21	Methyl propiolate	CH≡$CCOOCH_3$	5
		Crotonates	
22	Crotonic acid	CH_3CH=$CHCOOH$	I
23	Methyl crotonate	CH_3CH=$CHCOOCH_3$	I
24	Crotonaldehyde	CH_3CH=$CHCHO$	9
25	Crotononitrile	CH_3CH=CHC≡N	I
26	Methyl tetrolate	CH_3C≡$CCOOCH_3$	15
		trans-Cinnamates	
27	Cinnamic acid	C_6H_5CH=$CHCOOH$	I
28	Methyl cinnamate	C_6H_5CH=$CHCOOCH_3$	125
29	Cinnamaldehyde	C_6H_5CH=$CHCHO$	110
30	Cinnamonitrile	C_6H_5CH=CHC≡N	I
31	Cinnamamide	C_6H_5CH=$CHCONH_2$	600
32	β-Nitrostyrene	C_6H_5CH=$CHNO_2$	25

I, inactive; <20% increase in specific activity at 200 μM.

(Compound **25**) and cinnamate (Compound **30**) series. Electrophilic acetylenes, such as methyl propiolate (Compound **21**) and methyl tetrolate (Compound **26**), are very efficient Michael acceptors and were also very potent inducers. As predicted from the high potency of 1-nitro-1-cyclohexene in relation to other cyclic olefins, β-nitrostyrene (Compound **32**; CD = 25 μM) was by far the most efficient inducer among the cinnamates. We conclude that the electrophilic olefin need not be cyclic and that inductive activity generally parallels the potency of the electron-withdrawing group.†

Unsaturated Dicarboxylic Acids. Fumaric (Table 3, Compound **33**) and maleic (Compound **36**) acids were inactive as inducers of QR in Hepa 1c1c7 cells, whereas their dimethyl esters (Compounds **34** and **37**, respectively) and dimethyl itaconate (Compound **40**) were moderately potent inducers with CD values of 20–35 μM. The olefinic structure is essential since the saturated dimethyl succinate (Compound **39**) was inactive. The esters of the unsaturated dicarboxylic acids were more active than were the free acids. Furthermore, the lower potency of the diethyl compared with the

Table 3. Concentration (CD) of fumarate, maleate, and itaconate derivatives required to double quinone reductase in Hepa 1c1c7 cells

Compound No.	Name	Structure	CD, μM
33	Fumaric acid	HOOCCH=CHCOOH (*trans*)	I
34	Dimethyl fumarate	CH_3OOCCH=$CHCOOCH_3$ (*trans*)	22
35	Diethyl fumarate	C_2H_5OOCH=$CHCOOC_2H_5$ (*trans*)	100
36	Maleic acid	HOOCCH=CHCOOH (*cis*)	I
37	Dimethyl maleate	CH_3OOCCH=$CHCOOCH_3$ (*cis*)	20
38	Diethyl maleate	C_2H_5OOCCH=$CHCOOC_2H_5$ (*cis*)	40
39	Dimethyl succinate	$CH_3OOCCH_2CH_2COOCH_3$	I
40	Dimethyl itaconate	$CH_3OOCC(=CH_2)CH_2COOCH_3$	35
41	Dimethyl acetylene dicarboxylate	CH_3OOCC≡$CCOOCH_3$	87

I, inactive; <20% increase in specific activity at 200 μM.

dimethyl esters of fumarate (Compound **35**) and maleate (Compound **38**) might result from the lower electrophilicity of the double bond or from steric effects. The relatively low inductive potency of dimethyl acetylenedicarboxylate (Compound **41**; CD = 87 μM) in comparison to the acetylenic monocarboxylates (Compounds **21** and **26**) and the olefinic dicarboxylates (Compounds **34** and **37**), correlates with decreased Michael acceptor efficiency, possibly because of the tendency of one carbonyl group to destabilize the generation of a positive center by the other carbonyl function.

These observations are of potential importance since fumarate and itaconate occur naturally and are used as food additives. Furthermore, fumaric acid is the active principle responsible for some of the many pharmacological effects of extracts of shepherd's purse (*Capsella bursa-pastoris*), a crucifer widely used for medicinal purposes in Asia. Such extracts, or fumaric acid itself, protect against chemical carcinogenesis in liver of rats and in forestomach and lungs of mice (17, 18).

Induction of QR and GST in Mouse Tissues. The identification of a number of Michael reaction acceptors that elevated QR levels in Hepa 1c1c7 cells raised the question whether such compounds were (*i*) active when administered to mice *in vivo*, (*ii*) active in tissues other than liver, and (*iii*) whether GSTs (which are normally not significantly induced in Hepa cells) were also induced. Hence, we administered several of these newly recognized inducers—dimethyl maleate (Compound **37**), dimethyl itaconate (Compound **40**), methyl acrylate (Compound **13**), 5,6-dihydro-2*H*-pyran-2-one (Compound **4**), and 2-methylene-4-butyrolactone (Compound **5**)—by gavage (25–75 μmol daily for 5 days) to female CD1 mice and measured the specific activities of QR and GST activities with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) in the cytosols of liver, forestomach, and glandular stomach (Table 4) according to a standard protocol (9, 10). Mice treated with BHA and *tert*-butylhydroquinone were used as positive controls and gave inductions similar to those reported previously (see ref. 9; values not shown in Table 4).

The results in mice *in vivo* are similar to the findings obtained with Hepa 1c1c7 cells. With few exceptions, all compounds induced all three enzyme activities and, for the most part, coordinately. Furthermore, several of the new electrophiles were more potent and more effective than BHA and *tert*-butylhydroquinone. For example, in the glandular stomach, phase II enzymes are only slightly responsive to BHA and *tert*-butylhydroquinone (9, 10), whereas five doses of 25 μmol of dimethyl itaconate raised the enzyme specific activities 2.63- to 6.73-fold. Examination of the least toxic of these compounds with widest tissue specificity will, therefore, be of great interest in cancer protection experiments *in vivo*. Chemoprotection by these new compounds would further strengthen the view that enzyme induction is a central mechanism of chemoprotection.

Sulfur Compounds: Isothiocyanates, 1,2-Dithiol-3-thiones, Thiocarbamates, and Allyl Sulfides

These sulfur-containing compounds: (*i*) protect rodents against the toxic and neoplastic effects of carcinogens; and (*ii*) induce QR and GST in rodent tissues and QR in the Hepa 1c1c7 cell line. Moreover, many sulfur compounds are present in commonly consumed vegetables that protect against cancer (1, 13, 14, 19–28). We therefore considered whether electrophilic centers might likewise be responsible for the enzyme inducing activity of these sulfur-containing compounds.

Isothiocyanates. The inductive ability of various alkyl and aromatic isothiocyanates depended on the presence of at least one hydrogen on the carbon adjacent to the isothiocya-

Table 4. Induction patterns of QR and GSTs measured with CDNB and DCNB in mouse tissues

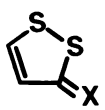
Inducing agent	Dose, μmol per mouse per day	Enzyme	Ratio of specific activities (treated/control) Liver	Forestomach	Glandular stomach
Dimethyl maleate (**37**)	25	QR	2.42 ± 0.18	2.44 ± 0.13	2.68 ± 0.14
		CDNB	1.44 ± 0.08	3.61 ± 0.12	3.42 ± 0.17
		DCNB	1.11 ± 0.02	3.96 ± 0.16	6.35 ± 0.76
	75	QR	2.43 ± 0.10	2.89 ± 0.23	2.82 ± 0.20
		CDNB	3.61 ± 0.09	4.36 ± 0.36	6.15 ± 0.52
		DCNB	2.09 ± 0.03	5.34 ± 0.49	5.05 ± 0.84
Dimethyl itaconate (**40**)	25	QR	2.37 ± 0.22	2.74 ± 0.11	2.63 ± 0.13
		CDNB	1.79 ± 0.06	3.47 ± 0.06	5.07 ± 0.53
		DCNB	0.90 ± 0.06	4.34 ± 0.30	6.73 ± 0.71
	75	QR	3.30 ± 0.05	2.73 ± 0.15	2.90 ± 0.07
		CDNB	2.79 ± 0.07	4.27 ± 0.28	6.71 ± 0.30
		DCNB	1.71 ± 0.08	4.90 ± 0.22	7.75 ± 0.57
Methyl acrylate (**13**)	25	QR	2.43 ± 0.10	2.11 ± 0.07	2.94 ± 0.10
		CDNB	1.64 ± 0.10	2.78 ± 0.07	3.65 ± 0.29
		DCNB	0.93 ± 0.03	2.98 ± 0.17	4.72 ± 0.22
5,6-Dihydro-2*H*-pyran-2-one (**4**)	50	QR	3.40 ± 0.12	2.11 ± 0.23	2.55 ± 0.03
		CDNB	2.55 ± 0.03	2.58 ± 0.35	2.50 ± 0.20
		DCNB	1.51 ± 0.06	2.04 ± 0.39	4.37 ± 0.24
2-Methylene-4-butyrolactone (**5**)	50	QR	7.93 ± 0.33	1.04 ± 0.05	2.39 ± 0.06
		CDNB	2.30 ± 0.06	2.29 ± 0.03	3.11 ± 0.16
		DCNB	1.32 ± 0.11	1.61 ± 0.09	3.30 ± 0.13

The SEM values of the treated/control ratios were obtained by dividing the SEM of the mean of each treated group by the control value. The enzyme specific activities (nmol/min/mg of protein ± SEM) of vehicle-treated controls were as follows. Liver: QR, 126 ± 3.3; CDNB, 1800 ± 30; DCNB, 37.9 ± 3.9. Forestomach: QR, 1454 ± 88; CDNB, 1130 ± 69; DCNB, 15.6 ± 0.8. Glandular stomach: QR, 3955 ± 187; CDNB, 684 ± 40; DCNB, 8.35 ± 1.06. All compounds were administered to 6-week-old female CD1 mice (three to six mice per group) by gavage in indicated single daily doses in 0.1 ml of Emulphor EL620P for 5 days. Cytosols were prepared from the tissues 24 hr after the last treatment and assayed for enzyme activities as described (9, 10).

nate group (i.e., R_1R_2CH—N=C=S). Thus, benzyl (CD = 1.8 μM), phenethyl (CD = 2.0 μM), ethyl (CD = 30 μM), *n*-propyl (CD = 14 μM), *n*-butyl (CD = 15 μM), allyl (CD = 4 μM), and cyclohexyl (CD = 14 μM) isothiocyanates were potent enzyme inducers. In contrast, *tert*-butyl, phenyl, 2-fluorophenyl, 3-fluorophenyl, 4-fluorophenyl, 4-chlorophenyl, 4-tolylphenyl, and α-naphthyl isothiocyanates did not induce. Since an α-hydrogen is essential for inductive activity, it is tempting to speculate that tautomerization of the methylene-isothiocyanate moiety to a structure resembling an α,β-unsaturated thioketone may be important for inductive activity:

$$R_1R_2CH-N=C=S \rightleftharpoons R_1R_2C=N-\overset{H}{\underset{|}{C}}=S$$

1,2-Dithiol-3-thiones. Substituted 1,2-dithiol-3-thiones were initially recognized to be potential anticarcinogens because of their ability to induce phase II enzymes in mice (25, 29). Since unsubstituted 1,2-dithiol-3-thione (Compound **42**) and 1,2-dithiol-3-one (Compound **43**) are efficient inducers of phase II enzymes, signaling of phase II enzyme induction does not require ring substitution (26, 27). The 1,2-dithiol-3-thione nucleus contains an olefin conjugated to an electron-withdrawing thioketone or conventional ketone, a structural feature common to all the inducers discussed here:



X = S (Compound **42**)
X = O (Compound **43**)

Allyl Mono-, Di-, and Trisulfides. Recently, Wattenberg and colleagues (28) have described the anticarcinogenic effects of allyl mono-, di-, and trisulfides and their ability to induce GST. An unsaturated allylic moiety is essential for both enzyme inductive and anticarcinogenic effects. Although these compounds do not appear to be Michael acceptors *per se*, nucleophilic attack on the electrophilic terminal carbon adjacent to the sulfur, with the resultant elimination of a thiol, may be a mechanism by which these compounds are active:

Indeed, we found that allyl bromide (CD = 200 μM) is approximately equiactive with allyl disulfide (CD = 150 μM) as an inducer of QR.

Thiocarbamates. There is no obvious Michael-based mechanism for phase II enzyme induction by chemoprotective anticarcinogenic thiocarbamates such as disulfiram [$(C_2H_5)_2NC(=S)S]_2$, diethyldithiocarbamate [$(C_2H_5)_2NC(=S)SH$], and bisethylxanthogen [$(C_2H_5OC(=S)S)_2$]. Possibly, thiocarbamates require metabolic activation to form the required electrophiles since at least some thiocarbamates are conjugated with glutathione (see ref. 30).

Inducers Are Substrates for GSTs. GSTs catalyze the conjugation of glutathione (GSH) with compounds of the type $R_AR_BC=CR_CZ$, in which Z is an electron-withdrawing group. The enzymatic reactivity of such compounds depends on the electron-attracting power of the Z group and on the electronic (attraction or repulsion) and steric effects of the R_A, R_B, and R_C groups (30–32). GSTs catalyze Michael additions of GSH to hydrophobic electrophiles by increasing the nucleophilicity of GSH (30, 33).

A remarkable similarity is immediately apparent between the structural features of substrates for GSTs and those required for phase II enzyme induction. Many α,β-unsaturated enzyme inducers such as esters, aldehydes, ketones, lactones, nitriles, nitroalkenes, and sulfones, share these properties (30–32). Thus, the ethyl esters of maleate, fumarate, and acrylate, and dimethyl itaconate share the capacity to induce and to serve as substrates for GSTs. Furthermore, the very active nitroalkene inducer, β-

nitrostyrene, is also an excellent substrate for GST, as are methyl vinyl sulfone (Compound **18**) and ethyl vinyl ketone (related to Compound **17**). Whereas methyl acrylate (Compound **13**) is active in both systems, the closely related methyl methacrylate (Compound **14**) is not. Moreover, free fumaric (Compound **33**), cinnamic (Compound **27**), and crotonic (Compound **22**) acids, and α,β-dimethyl acrylate (Compound **15**) are inactive in both systems (30–32).

In a series of aromatic and alkyl isothiocyanates, only analogues bearing a free hydrogen on the carbon adjacent to the isothiocyanate group were inducers of QR in Hepa 1c1c7 cells (see above). It is striking that the ability of these compounds to form GSH conjugates also required the presence of an α-hydrogen (34, 35).

Are members of other classes of inducers also conjugated with glutathione in animal tissues? Quinones are good Michael acceptors and their reaction with GSH is enzyme promoted. Some organic isothiocyanates are metabolized to products that suggest an initial conjugation with GSH (34, 35), and some thiocarbamates appear also to undergo initial sulfoxidation followed by conjugation with GSH (30).

Furthermore, several common substrates used to assay GST were found to be inducers. Thus, the CD for QR induction by DCNB was 150 μM. CDNB produced a 1.6-fold induction at 10 μM but was toxic at higher concentrations. Two other substrates for GST were also inducers: ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid] (CD = 30 μM) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (CD = 68 μM).

It is perhaps not surprising that inducers of phase II xenobiotic metabolizing enzymes should prove to be substrates for GST (and vice versa) since many xenobiotics (or even endobiotics) induce enzymes for their own metabolism. Both induction and substrate activity require the presence of an electrophilic Michael acceptor function. Whether these processes are related through a requirement for electrophilicity, or whether there is a more fundamental causal relationship between them is presently unclear.

In conclusion, it is gratifying that the capacity of an extraordinary variety of seemingly unrelated anticarcinogens to induce protective enzymes can be attributed to the presence, or acquisition by metabolism, of a simple and hitherto unrecognized chemical property: that of a Michael reaction acceptor.

We are grateful for many valuable and pleasurable discussions with our colleagues: Jih Ru Hwu, Gary H. Posner, and Cecil H. Robinson. We thank Annette B. Santamaria for expert technical assistance. These studies were supported by grants from the National Institutes of Health (CA 44530), the American Cancer Society (SIG-3 and RDP-30), and the American Institute for Cancer Research. H.J.P. was supported by National Institutes of Health Training Grant CA 09243.

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