The potency of inducers of NAD(P)H:(quinone-acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases

Structural and electronic correlations

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Induction of glutathione transferases (EC. 2.5.1.18), NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2; quinone reductase) and other detoxification enzymes is a major mechanism for protecting cells against the toxicities of electrophiles, including many carcinogens. Although inducers of these two enzymes belong to many different chemical classes, they nevertheless contain (or acquire by metabolism) electrophilic centres that appear to be essential for inductive activity, and many inducers are Michael reaction acceptors [Talalay, De Long & Prochaska (1988) *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8261–8265]. The inducers therefore share structural and electronic features with glutathione transferase substrates. To define these features more precisely, we examined the inductive potencies (by measuring quinone reductase in murine hepatoma cells) of two types of glutathione transferase substrates: a series of 1-chloro-2-nitrobenzenes bearing *para*-oriented electron-donating or -withdrawing substituents and a wide variety of other commonly used and structurally unrelated glutathione transferase substrates. We conclude that virtually all glutathione transferase substrates are inducers, and their potencies in the nitrobenzene series correlate linearly with the Hammett σ or σ^- values of the aromatic substituents, precisely as previously reported for their efficiencies as glutathione transferase substrates. More detailed information on the electronic requirements for inductive activity was obtained with a series of methyl *trans*-cinnamates bearing electron-withdrawing or -donating substituents on the aromatic ring, and in which the electronic densities at the olefinic and adjacent carbon atoms were measured by ^{13}C n.m.r. Electron-withdrawing *meta*-substituents markedly enhance inductive potency in parallel with their increased non-enzymic reactivity with GSH. Thus, methyl 3-bromo-, 3-nitro- and 3-chloro-cinnamates are 21, 14 and 8 times more potent inducers than the parent methyl cinnamate. This finding permits the design of more potent inducers, which are important for elucidation of the molecular mechanisms of induction.

INTRODUCTION

Induction of glutathione transferases, glucuronosyltransferases and NAD(P)H:(quinone-acceptor) oxidoreductase (quinone reductase) is a major and critical mechanism whereby animals and their cells can be protected against the toxicity and carcinogenicity of many potentially harmful xenobiotics (Talalay *et al.*, 1987). Understanding of the mechanisms of these inductions is therefore of both theoretical interest and practical importance in devising chemoprotective strategies. The structural diversity of these inducers of chemoprotective enzymes is quite remarkable. They belong to many chemical classes, including diphenols (e.g. phenolic antioxidants), azo dyes, coumarins, cinnamates, 1,2-dithiol-3-thiones, aromatic isothiocyanates, thiocarbamates and many others (Wattenberg, 1985; Talalay *et al.*, 1987). Protective enzymes are often increased co-ordinately in cells and tissues, and such elevations are associated with markedly raised levels of cognate mRNAs and enhanced rates of synthesis of these enzymes (Pearson *et al.*, 1983; Benson *et al.*, 1984).

Recent evidence suggests that most of the inducers contain, or acquire by cellular metabolism, distinctive and previously unrecognized electrophilic centres, and many are classical Michael reaction acceptors (Prochaska *et al.*, 1985; Talalay *et al.*, 1988).

[In 1887 A. Michael reported that olefins conjugated with electron-withdrawing groups (Z) are susceptible to attack by nucleophiles. The 'Michael reaction acceptors' may have the following structures: $\text{CH}_2=\text{CH}-\text{Z}$, $\text{Z}'-\text{CH}=\text{CH}-\text{Z}$ (including quinones) or $\text{R}-\text{C}\equiv\text{C}-\text{Z}$ (acetylenes), in which Z is an electron-withdrawing group. The usual order of decreasing reactivity with standard nucleophiles such as morpholine or pyrrolidine is: $\text{Z} = \text{NO}_2 > \text{COAr} > \text{CO} > \text{COR} > \text{CN} > \text{CO}_2\text{R} > \text{CONH}_2 > \text{CONR}_2$ (Shenhav *et al.*, 1970; March, 1985).] Furthermore, the potencies of inducers (of the Michael acceptor type) in elevating quinone reductase levels in the Hepa 1c1c7 mouse hepatoma cell line correlated closely with their predicted efficiencies of reaction with standard nucleophiles (Shenhav *et al.*, 1970). A detailed structure-activity survey led to the prediction of inductive activity among many new types of compounds. Inductions were observed when cells were exposed to several classes of cyclic and acyclic olefins, including vinyl ketones, vinyl sulphone, acrylates, crotonates, fumarates, maleates, coumarins and cinnamates (Talalay *et al.*, 1988). The inductive activity of 1,2- and 1,4-, but not of 1,3-diphenols, and of the corresponding phenylenediamines, could then be explained by their intracellular oxidation to quinones or quinoneimines, which cannot be formed from the *meta*-substituted compounds (Prochaska *et al.*, 1985).

Abbreviations used: quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), also known as menadione reductase and DT-diaphorase; CD, concentration of an inducer that doubles the specific activity of quinone reductase.

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Another striking correlation suggested by these structure-activity investigations was that the inducers of quinone reductase and other protective enzymes might also be substrates for glutathione transferases (Talalay *et al.*, 1988). Boyland and Chasseaud (Boyland & Chasseaud, 1967; Chasseaud, 1979) pointed out that a characteristic property of glutathione transferases was their ability to catalyse the addition of GSH to electrophilic olefins by enhancing the nucleophilicity of GSH under physiological conditions. These observations raised the question whether the capacity of compounds to act as protective enzyme inducers and to serve as glutathione transferase substrates merely reflected a common requirement for electrophilicity, or whether interactions of the inducers with glutathione transferases (and/or glutathione) were part of the mechanism for the signalling of enhanced enzyme transcription.

The present study was designed to provide detailed information on the relationship between proficiency of compounds to induce quinone reductase (a prototype for protective enzymes) and their ability to serve as glutathione transferase substrates, and specifically to relate the electronic requirements for both activities. Three classes of chemical compounds were studied. First, we synthesized a series of methyl esters of *trans*-cinnamic acid substituted with electron-donating or -withdrawing groups on the aromatic ring, and examined the effects of such substitution on the distribution of electron density in the molecule and on potency of enzyme induction. Secondly, we measured the inductive potency of a series of 4-substituted 1-chloro-2-nitrobenzenes and related the Hammett σ values of the substituents to the potencies of these compounds as inducers and their efficiencies as glutathione transferase substrates. Thirdly, we tested a wide variety of other commonly used and structurally unrelated glutathione transferase substrates for their ability to act as inducers.

We found not only a consistent correlation between the electronic requirements for induction and capacity to serve as glutathione transferase substrates, but also that *meta*-substitution of methyl cinnamate could provide markedly more potent enzyme inducers, thus paving the way for development of even more potent agents and providing further insight into the molecular mechanisms of induction.

A preliminary account of this work has been published (Spencer *et al.*, 1989).

EXPERIMENTAL PROCEDURES

Compounds

Methyl cinnamate and all substituted *trans*-cinnamic acids were obtained from Aldrich (Milwaukee, WI, U.S.A.). Methyl cinnamate was distilled in a vacuum (95–95.5 °C, 0.5 mmHg). All acids were converted into their methyl esters (5 g scale) by dissolution in a 10-fold excess of dried methanol and refluxing for 24 h with an equimolar amount of boron trifluoride etherate (Aldrich) (Kadaba, 1971). The solvent was removed under reduced pressure, and the product was dissolved in ethyl acetate or chloroform. The organic phase was washed with 5% (w/v) Na₂CO₃, dried over MgSO₄ or Na₂SO₄, and the solvent was evaporated under reduced pressure. Solid compounds were recrystallized at least twice from ethyl acetate and/or methanol. Methyl 3-methoxycinnamate (compound 9, see the Appendix) was distilled twice (125–130 °C; 2.2 mmHg), and could then be crystallized from hexane. The following compounds were purified by two distillations in a vacuum: methyl 2-chlorocinnamate (3) at 155 °C, 4.3 mmHg; methyl 2-fluorocinnamate (4) at 125–127 °C, 3 mmHg; methyl 2-methoxycinnamate (5) at 165–170 °C, 3 mmHg. The identity and purity of the compounds were established by ¹H n.m.r. and ¹³C n.m.r. and by i.r. and u.v.

spectroscopy. The spectroscopic constants for these compounds are given in the Appendix to this paper. The coupling constants between the protons on the double bond ($J_{\alpha\beta}$) were 16.0–16.8 Hz, establishing that the methyl cinnamates were pure *trans*-isomers.

All 4-substituted 1-chloro-2-nitrobenzenes were purchased from Aldrich, except for 4-chloro-3-nitrobenzamide, which was obtained from Pfaltz and Bauer (Waterbury, CT, U.S.A.).

Quinone reductase assays

The specific activities of quinone reductase in Hepa lcl7 cells were determined on cells (10000 per well) grown in 96-well micro-titre plates for 24 h, according to Prochaska & Santamaria (1988) with the following modification. The foetal calf serum was mixed with 1% (w/v) of HCl-washed activated charcoal (Sigma Chemical Co., St. Louis, MO, U.S.A.) and heated for 90 min at 55 °C. The serum was then filtered twice through a 0.22 μ m-pore-size filter before addition to the alpha minimal essential medium at a final concentration of 10% (v/v). The specific activities were obtained from the ratio of the quinone reductase activity to the absorbance of a parallel plate treated with the same inducers but stained with Crystal Violet. The specific activities of inducer-treated cells were related to those of control cells receiving only the vehicle used to dissolve the inducers [0.1% v/v dimethyl sulphoxide or 0.1% v/v acetonitrile]. The period of induction was 24 h or 48 h, as specified in the experiments. Each compound was tested at a series of dilutions, and the CD values were determined from plots of the ratio of the specific activities of treated to control cells with respect to concentration of inducer. All experiments included parallel control inductions with a series of concentrations of β -naphthoflavone. The CD values for β -naphthoflavone ranged from 20 to 60 nM (mean 37 nM) in different experiments.

Spectroscopic methods

The u.v. spectra were obtained on solutions of compounds in acetonitrile on a Beckman DU-7 recording spectrophotometer. The ¹³C-n.m.r. spectra (100.6 MHz) were obtained on a Varian XL 400 spectrometer (upgraded to VXR 400), and ¹H-n.m.r. spectra on an IBM FT-80 spectrometer. All samples were dissolved in [²H]chloroform. I.r. spectra (on KBr pellets or neat liquids) were recorded on a Perkin-Elmer model 1600 FT-IR instrument. We are grateful to Charles Long, Department of Chemistry, Johns Hopkins University, for making the n.m.r. and i.r. facilities available for these experiments.

RESULTS AND DISCUSSION

All enzyme induction studies were made with cultured Hepa lcl7 mouse hepatoma cells, which respond to nearly all known chemoprotective enzyme inducers by large elevation of the specific activity of quinone reductase (Prochaska *et al.*, 1985; De Long *et al.*, 1986a,b, 1987; Talalay *et al.*, 1988).

Effects of electron-donating and electron-withdrawing substituents on the potency of methyl cinnamate as an inducer of quinone reductase

Methyl cinnamate is a Michael reaction acceptor and a moderately potent inducer (CD = 82 μ M) of quinone reductase in Hepa lcl7 cells. It is therefore an ideal parent compound for the study of the effects of electron-donating and -withdrawing groups on inducer potency. In confirmation of prior studies (Talalay *et al.*, 1988), cinnamic acid and cinnamitrile were inactive and cinnamamide was less active than methyl cinnamate. Cinnamaldehyde (CD = 133 μ M) was somewhat less potent than the methyl ester (Table 1). In contrast, β -nitrostyrene (C₆H₅CH=CH-NO₂, CD = 15.5 μ M) and *trans*-4-phenylbut-3-en-2-one

Table 1. Effect of various terminal electron-withdrawing groups on the potency of styrene derivatives as inducers of quinone reductase in Hepa lc1c7 murine hepatoma cells

Hepa lc1c7 cells were plated at a density of 10000 cells per well, grown for 24 h, and then exposed to a range of concentrations of the inducing compounds for 48 h.

Inducer	Substituent Z*	Concentration (μM) required to double specific activity of quinone reductase (CD)
Cinnamic acid	CO_2H	Inactive
Methyl cinnamate	CO_2CH_3	82
Cinnamaldehyde	CHO	133
<i>trans</i> -4-Phenylbut-3-en-2-one	COCH_3	17
Cinnamamide	CONH_2	268
Cinnamionitrile	CN	Inactive
β -Nitrostyrene	NO_2	15.5

* Z is the substituent in the structure:

($\text{C}_6\text{H}_5\text{CH}=\text{CH}-\text{COCH}_3$; $\text{CD} = 17 \mu\text{M}$) were considerably more potent than the parent methyl cinnamate, as would be expected from their greater Michael reactivity. The unexpectedly low potency of the aldehyde in comparison with the ketone may result from more rapid metabolism of the aldehyde and the fact that the equilibrium of reduction of aldehydes to primary alcohols (poorer Michael acceptors) is much more favourable than reduction of ketones to secondary alcohols. Otherwise, the potencies of induction generally paralleled the expected efficiencies of these compounds as Michael reaction acceptors (Shenhav *et al.*, 1970; March, 1985).

Determination of the potency of ring-substituted methyl cinnamates to induce quinone reductase showed that *meta*-substitution with electron-withdrawing groups strikingly increased the inductive potency ($\text{CD} = 4\text{--}10 \mu\text{M}$) in comparison with the parent methyl cinnamate ($\text{CD} = 82 \mu\text{M}$) (Table 2). Although *ortho*-substitution with electron-withdrawing groups also increased the potency of induction ($\text{CD} = 25\text{--}31 \mu\text{M}$), the electronic properties of these groups did not appear to be important. In contrast, *para*-substitution invariably decreased

Table 2. Potency of ring-substituted methyl *trans*-cinnamates as inducers of quinone reductase in Hepa lc1c7 murine hepatoma cells: comparison with Hammett σ values

Hepa lc1c7 cells were plated at a density of 10000 cells per well, grown for 24 h, and then exposed to a range of concentrations of the inducing compounds for 48 h. The concentration required to double (CD) the quinone reductase specific activity was determined. For the parent methyl *trans*-cinnamate $\text{CD} = 82 \mu\text{M}$. Abbreviation: N.D., not determined.

Substituent X*	For substituents oriented				
	<i>ortho</i>		<i>meta</i>		<i>para</i>
	CD (μM)	CD (μM)	Hammett σ	CD (μM)	Hammett σ
NO_2	30.0	6.00	+0.71	157	+0.78
Br	N.D.	4.00	+0.39	165	+0.23
Cl	25.0	9.80	+0.37	180	+0.23
F	31.0	N.D.	+0.34	300	+0.06
CH_3O	57.0	110	+0.12	218	-0.27
CH_3	N.D.	N.D.	-0.07	170	-0.17

* Substituents X in the structure:

inductive potency, possibly because steric factors may have interfered with the participation of these compounds in intracellular Michael reactions. Such *para*-substitution was also relatively insensitive to the Hammett values of the substituents, suggesting that electron-withdrawing or electron-donating effects were overshadowed by other factors. The most striking observation is the profound increase in potency obtained when the parent methyl cinnamate ($\text{CD} = 82 \mu\text{M}$) was *meta*-substituted with electron-withdrawing groups: Br ($\text{CD} = 4.0 \mu\text{M}$), NO_2 ($\text{CD} = 6.0 \mu\text{M}$), or Cl ($\text{CD} = 9.8 \mu\text{M}$). In contrast, the electron-donating *meta*-methoxyl group ($\text{CD} = 110 \mu\text{M}$) weakened the inductive potency slightly relative to the parent compound.

The non-enzymic rates of reactions of the substituted methyl cinnamates with GSH have also been measured spectrophotometrically and the bimolecular rate constants (k_2) determined (see the Appendix). The k_2 values for *meta*-substituted methyl

Table 3. Inductive potencies, Hammett σ constants, bimolecular rate constants (k_2) for reaction with GSH, and spectroscopic data for *meta*-substituted methyl *trans*-cinnamates

The concentrations required to double the quinone reductase specific activities of Hepa lc1c7 murine hepatoma cells were taken from Table 2 in the text. ^{13}C -n.m.r. chemical shifts are taken from Table 2 in the Appendix; bimolecular rate constants (k_2) are taken from Table 3 in the Appendix.

<i>meta</i> -Substituent	CD (μM)	Hammett σ	k_2 ($\text{M}^{-1} \cdot \text{min}^{-1}$)	^{13}C -n.m.r. chemical shifts (p.p.m.)			I.r. (cm^{-1}) C=O
				C- α	C- β	C=O	
H	82.0	0	0.97	117.8	144.8	167.4	1716.3 (broad)
NO_2	6.0	+0.71	1.13	120.9	141.9	166.5	1707.5
Br	4.0	+0.39	1.41	119.2	143.1	166.9	1715.5
Cl	9.8	+0.37	1.45	119.2	143.2	167.0	1715.7
CH_3O	110	+0.12	0.76	118.0	144.7	167.3	1719.8 (broad)

cinnamates are shown in Table 3 for comparison with inductive potencies of these compounds and the Hammett σ values of the substituent groups. The linear correlation coefficient between k_2 and CD values is -0.80 (and rises to -0.93 if the anomalous *meta*-nitro derivative is excluded). Thus, there is a reasonably good correlation between these properties. Moreover, the linear correlation coefficient between the CD and Hammett σ values is -0.81 (and rises to -0.84 if the anomalous determination for the *meta*-nitro derivative is excluded).

In interpreting the effects of aromatic substitution of the reactivity of the side chain, and particularly the reactivity of the β -carbon of the olefin as a Michael acceptor, both resonance and inductive effects require consideration (Bromilow *et al.*, 1981). In this respect, *meta*-substituents are instructive since they do not produce resonance effects, and are therefore probes for purely inductive influences. ^{13}C -n.m.r. spectra (Table 3) showed that in the presence of *meta*-oriented electron-withdrawing substituents (NO_2 , Br or Cl) which increased the potency of enzyme induction, the resonances of the α -carbon of the double bond are shifted downfield by 1.5–3.2 p.p.m. (deshielding), whereas the resonances of the β -carbon are shifted upfield by 1.8–2.9 p.p.m. (shielding) in comparison with the unsubstituted methyl cinnamate. The i.r. frequencies for the carbonyl group also showed a tendency towards lower values in the presence of *meta*-oriented electron-withdrawing ring substituents, indicating an overall decreased electron density of the carbonyl double bond, although the electron density on the carbonyl carbon was increased as shown by the ^{13}C -n.m.r. chemical shift. These findings suggest that electron-withdrawing substituents on the phenyl ring produced a redistribution of electron density on the side chain of the following type:

Since the attack by the unidentified intracellular nucleophile occurs on the β -carbon, the efficiency of this reaction must be controlled by the total charge distribution and electron density of the side chain, rather than that of the β -carbon atom alone.

Common substrates for glutathione S-transferases as inducers of quinone reductase

The glutathione transferases are isofunctional enzymes that belong to several gene families, and promote the conjugation of mostly hydrophobic substrates with glutathione. Individual glutathione transferases show distinctive but overlapping substrate specificities, and efforts to identify the multiplicity of isoenzymes in different tissues have depended in part on surveys of substrate specificities (Mannervik, 1988). In preliminary studies (Talalay *et al.*, 1988) we reported that several common substrates used for the assay of glutathione transferases were also inducers of quinone reductase. We have now carried out a systematic survey of a broader range of glutathione transferase substrates (Table 4). 1-Chloro-2,4-dinitrobenzene, which is usually a very efficient inducer of quinone reductase, with a CD value of $6.4 \mu\text{M}$, extrapolated from the value of $2.4 \mu\text{M}$ in Table 4. (Since many glutathione transferase substrates were quite toxic to the cells, doubling of quinone reductase specific activities could not always be attained, and in Table 4 we report concentrations required to raise quinone reductase activities to 1.5 times control values.) Virtually all substrates for glutathione transferases are also

Table 4. Potencies of glutathione transferase substrates as inducers of quinone reductase in Hepa 1c1c7 murine hepatoma cells

All compounds were dissolved in acetonitrile, except sulphobromophthalein which was dissolved in 0.9% NaCl. Hepa 1c1c7 cells were plated at a density of 10000 cells per well, grown for 24 h, then exposed to a range of concentrations of the inducing compound for 48 h.

Inducer	Concentration (μM) required to increase quinone reductase specific activity 1.5-fold
(a) 1-Chloro-2,4-dinitrobenzene	2.4
(b) <i>trans</i> -4-Phenylbut-3-en-2-one	5.6
(c) 4-Nitrobenzyl chloride	7.6
(d) 1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	9.6
(e) Methyl iodide	18.0
(f) Nitropyridine <i>N</i> -oxide	23.0
(g) 1,4-Dichloro-2-nitrobenzene	25.0
(h) Ethacrynic acid	31.0
(i) Cumene hydroperoxide	34.0
(j) 4-Nitrophenethyl bromide	46.0
(k) Sulphobromophthalein	47.0
(l) <i>t</i> -Butyl hydroperoxide	60.0
(m) <i>trans</i> -Stilbene oxide	150
(n) 4-Nitrophenyl acetate	Inactive (> 250)

inducers of quinone reductase. The only substrate tested that was inactive was 4-nitrophenyl acetate ($\text{CD} > 250 \mu\text{M}$). It is difficult to make quantitative comparisons among compounds of such structural diversity with respect to their efficiencies as substrates and inducers since k_{cat} and K_m values are determined on isolated enzymes, whereas induction potencies also reflect rates of inactivation, entry into cells and competing reactions. The extent of competing reactions is especially important since many of the electrophilic inducers listed in Table 4 are highly reactive with both small and macromolecular nucleophiles.

Fig. 1. Rank order of potency of a series of *para*-substituted (R) 1-chloro-2-nitrobenzenes as inducers of quinone reductase in Hepa lc1c7 cells, plotted as a function of Hammett σ^- values

The cells (10000) were grown in micro-titre plate wells for 24 h and then exposed for 24 h to a series of concentrations of ten *para*-substituted 1-chloro-2-nitrobenzenes. The CD values for the compounds were as follows: *p*-COCH₃, 19 μ M; *p*-NO₂, 23 μ M; *p*-CONH₂, 25 μ M; *p*-CHO, 33 μ M; *p*-Cl, 48 μ M; *p*-CO₂H, 220 μ M; *p*-SO₃H, 425 μ M; *p*-NH₂, 668 μ M; *p*-CH₃, 6.8 mM; *p*-OCH₃, 9.5 mM. Each value was based on eight determinations at each concentration, in two separate experiments. The linear correlation coefficient is $r = 0.85$, corresponding to $\geq 99.8\%$ confidence limits, with eight degrees of freedom.

Effects of *para*-substituents on the potency of induction of 1-chloro-2-nitrobenzenes

Substitution of 1-chloro-2-nitrobenzene with electron-donating or electron-withdrawing groups at the *para*-position caused changes in the inductive potency. A linear correlation ($r = 0.83$) was found between rank order of inductive potency and Hammett σ values. A similar correlation ($r = 0.85$) was observed when Hammett resonance σ^- values were used (Fig. 1). Consequently, the presence of electron-withdrawing groups at a position *para* to that undergoing conjugation with GSH increased the potency of induction.

The correlation of inductive potency with Hammett σ values closely resembles the findings that substitution of 1-chloro-2-nitrobenzene with electron-withdrawing groups also increased the catalytic constants of both rat liver cytosolic glutathione transferases B and C (Keen *et al.*, 1976) and rat liver microsomal glutathione transferases (Morgenstern *et al.*, 1988) toward these substrates, thus providing evidence for the importance of the degree of electrophilicity at the reaction centre for both substrate and inducer activities. Furthermore these authors (Morgenstern *et al.*, 1988; Keen *et al.*, 1976) have determined the second-order rate constants for the non-enzymic reactions of these substituted 1-chloro-2-nitrobenzenes and obtained good correlations with the abilities of these compounds to serve as substrates for various glutathione transferases.

Conclusions

Glutathione transferases catalyse the addition of GSH to compounds of the type $R_1R_2C=CR_3Z$, wherein Z is an electron-withdrawing group that renders the olefin electrophilic. The reactivity of such substrates depends on the electron-withdrawing capacity of the Z group, and on the electronic and steric effects of R_1 , R_2 , and R_3 (Boyland & Chasseaud, 1967; Chasseaud, 1979). Glutathione transferases also promote nucleophilic attack by GSH on many other types of electrophilic centres. The

function of these enzymes is to bind GSH and the second substrate, and to increase the nucleophilicity of GSH (Chasseaud, 1979; Jakoby & Habig, 1980; Mannervik, 1988).

An obvious and close correlation therefore exists between the structural features required for induction of quinone reductase and glutathione transferases, and those required for compounds to serve as substrates for glutathione transferases. This correlation is further strengthened by the finding that nearly all of the commonly used substrates for glutathione transferases (e.g. electrophilic olefins, halogenated aromatic rings substituted with electron-withdrawing groups, epoxides, quinones and hydroperoxides) are inducers of quinone reductase. Even more remotely related chemoprotective enzyme inducers such as organic isothiocyanates and thiocarbamates are also conjugated biologically with GSH (reviewed in Sies & Ketterer, 1988).

The detailed molecular mechanisms involved in the induction of enzymes by monofunctional inducers probably do not involve participation of a classical receptor protein (Prochaska & Talalay, 1988). All inducers are electrophilic and might be expected to react with intracellular thiol groups. However, preliminary experiments clearly indicate that changes in GSH concentrations are unlikely to play a role in the regulation of glutathione transferase and quinone reductase activities, since manipulation of GSH concentrations by various techniques has no effect on enzyme induction (S. R. Spencer & P. Talalay, unpublished work). The central issue raised by the findings reported in this paper, i.e. that glutathione transferase substrates are inducers and that inducers of quinone reductase are glutathione transferase substrates, is whether this relation merely indicates that both processes require the presence of electrophilic centres, or whether perhaps glutathione transferases, or their subunits, participate in the mechanism of signalling enhanced transcription of the genes for detoxification enzymes of the type discussed in this paper. This question is now susceptible to experimental analysis.

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APPENDIX

The potency of inducers of NAD(P)H:(quinone acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases: structural and electronic correlations

Table 1. ¹H-n.m.r. and u.v. spectroscopic constants of methyl *trans*-cinnamates

The n.m.r. measurements were made on solutions in [2H]chloroform on an 80 MHz spectrometer. U.v. measurements were made in acetonitrile.

Methyl cinnamate (1): δ (p.p.m.) 3.80 (3H, s), 6.45 (1H, d, $J_{\alpha\beta}$ = 16.1 Hz), 7.3–7.6 (5H, m, arom), 7.71 (1H, d, $J_{\beta\alpha}$ = 16.1 Hz). U.v.: $\lambda_{\max}$ = 274.2 nm (a_m = 18 800 M ⁻¹ ·cm ⁻¹).
Methyl 2-nitrocinnamate (2): δ (p.p.m.) 3.83 (3H, s), 6.35 (1H, d, $J_{\alpha\beta}$ = 16.3 Hz), 7.4–8.1 (4H, m, arom), 8.13 (1H, d, $J_{\beta\alpha}$ = 16.3 Hz). U.v.: $\lambda_{\max}$ = 242.5 nm (a_m = 12 800 M ⁻¹ ·cm ⁻¹); sh 270 nm.
Methyl 2-chlorocinnamate (3): δ (p.p.m.) 3.79 (3H, s), 6.38 (1H, d, $J_{\alpha\beta}$ = 16.0 Hz), 7.1–7.7 (4H, m, arom), 8.05 (1H, d, $J_{\beta\alpha}$ = 16.0 Hz). U.v.: $\lambda_{\max}$ = 273.0 nm (a_m = 15 400 M ⁻¹ ·cm ⁻¹).
Methyl 2-fluorocinnamate (4): δ (p.p.m.) 3.79 (3H, s), 6.51 (1H, d, $J_{\alpha\beta}$ = 16.6 Hz), 6.9–7.6 (4H, m, arom), 7.80 (1H, d, $J_{\beta\alpha}$ = 16.6 Hz). U.v.: $\lambda_{\max}$ = 268.2 nm (a_m = 19 200 M ⁻¹ ·cm ⁻¹).
Methyl 2-methoxycinnamate (5): δ (p.p.m.) 3.76 (3H, s), 3.83 (3H, s), 6.50 (1H, d, $J_{\alpha\beta}$ = 16.4 Hz), 6.8–7.6 (4H, m, arom), 8.00 (1H, d, $J_{\beta\alpha}$ = 16.4 Hz). U.v.: $\lambda_{\max}$ = 273.7 nm (a_m = 18 900 M ⁻¹ ·cm ⁻¹); 322 nm (a_m = 10, 400 M ⁻¹ ·cm ⁻¹).
Methyl 3-nitrocinnamate (6): δ (p.p.m.) 3.81 (3H, s), 6.53 (1H, d, $J_{\alpha\beta}$ = 16.2 Hz), 7.5–8.4 (4H, m, arom), 7.72 (1H, d, $J_{\beta\alpha}$ = 16.2 Hz). U.v.: $\lambda_{\max}$ = 260.5 nm (a_m = 24 100 M ⁻¹ ·cm ⁻¹).
Methyl 3-bromocinnamate (7): δ (p.p.m.) 3.80 (3H, s), 6.42 (1H, d, $J_{\alpha\beta}$ = 16.0 Hz), 7.1–7.7 (4H, m, arom), 7.61 (1H, d, $J_{\beta\alpha}$ = 16.0 Hz). U.v.: $\lambda_{\max}$ = 271.2 nm (a_m = 11 900 M ⁻¹ ·cm ⁻¹).
Methyl 3-chlorocinnamate (8): δ (p.p.m.) 3.81 (3H, s), 6.42 (1H, d, $J_{\alpha\beta}$ = 16.8 Hz), 7.2–7.6 (4H, m, arom), 7.63 (1H, d, $J_{\beta\alpha}$ = 16.8 Hz). U.v.: $\lambda_{\max}$ = 270.5 nm (a_m = 17 200 M ⁻¹ ·cm ⁻¹).
Methyl 3-methoxycinnamate (9): δ (p.p.m.) 3.82 (3H, s), 6.40 (1H, d, $J_{\alpha\beta}$ = 16.0 Hz), 6.8–7.4 (4H, m, arom), 7.66 (1H, d, $J_{\beta\alpha}$ = 16.0 Hz). U.v.: $\lambda_{\max}$ = 276 nm (a_m = 20 800 M ⁻¹ ·cm ⁻¹); sh 310 nm.
Methyl 4-nitrocinnamate (10): δ (p.p.m.) 3.83 (3H, s), 6.45 (1H, d, $J_{\alpha\beta}$ = 16.6 Hz), 7.4–8.3 (4H, dd, arom), 7.73 (1H, d, $J_{\beta\alpha}$ = 16.6 Hz). U.v.: $\lambda_{\max}$ = 301.7 nm (a_m = 17 900 M ⁻¹ ·cm ⁻¹).
Methyl 4-bromocinnamate (11): δ (p.p.m.) 3.78 (3H, s), 6.40 (1H, d, $J_{\alpha\beta}$ = 16.1 Hz), 7.3–7.6 (4H, dd, arom), 7.62 (1H, d, $J_{\beta\alpha}$ = 16.1 Hz). U.v.: $\lambda_{\max}$ = 283.2 nm (a_m = 15 200 M ⁻¹ ·cm ⁻¹); sh 298 nm and sh 305 nm.
Methyl 4-chlorocinnamate (12): δ (p.p.m.) 3.78 (3H, s), 6.37 (1H, d, $J_{\alpha\beta}$ = 16.0 Hz), 7.3–7.5 (4H, dd, arom), 7.63 (1H, d, $J_{\beta\alpha}$ = 16.0 Hz). U.v.: $\lambda_{\max}$ = 279.7 nm (a_m = 16 300 M ⁻¹ ·cm ⁻¹); sh 296 nm and sh 302 nm.
Methyl 4-fluorocinnamate (13): δ (p.p.m.) 3.82 (3H, s), 6.34 (1H, d, $J_{\alpha\beta}$ = 16.6 Hz), 6.8–7.6 (4H, dd, arom), 7.66 (1H, d, $J_{\beta\alpha}$ = 16.6 Hz). U.v.: $\lambda_{\max}$ = 272.5 nm (a_m = 18 000 M ⁻¹ ·cm ⁻¹); sh 293 nm and sh 299 nm.
Methyl 4-methoxycinnamate (14): δ (p.p.m.) 3.82 (3H, s), 6.30 (1H, d, $J_{\alpha\beta}$ = 16.0 Hz), 6.8–7.6 (4H, dd, arom), 7.65 (1H, d, $J_{\beta\alpha}$ = 16.0 Hz). U.v.: $\lambda_{\max}$ = double peak 296 and 307 nm (a_m = 17 500 M ⁻¹ ·cm ⁻¹).
Methyl 4-methylcinnamate (15): δ (p.p.m.) 3.81 (3H, s), 6.39 (1H, d, $J_{\alpha\beta}$ = 16.2 Hz), 7.1–7.6 (4H, dd, arom), 7.68 (1H, d, $J_{\beta\alpha}$ = 16.2 Hz). U.v.: $\lambda_{\max}$ = 284.2 nm (a_m = 17 600 M ⁻¹ ·cm ⁻¹); sh 295 nm and sh 305 nm.

Table 2. ^{13}C -n.m.r. chemical shifts (p.p.m.) of 2-, 3- and 4-substituted methyl *trans*-cinnamates

The ^{13}C -n.m.r. spectra were obtained at 25 °C on a Varian XL 400 FT-NMR instrument. Compounds were dissolved in 2.0 ml of $[\text{2H}]\text{chloroform}$ in 10 mm n.m.r. tubes, at about 0.1 M concentration and the centre of the solvent peak (non-deuterated chloroform contaminant) was used as internal standard ($\delta = 77.00$ p.p.m.).

Compound no.	X =	C- α	C- β	C=O	Me (ester)	Me'	C-1	C-2	C-3	C-4	C-2'	C-3'
1	H	117.75	144.83	167.39	51.68		134.33	128.03	128.85	130.26		
<i>ortho</i> -Substituents (C-2)												
2	2-NO ₂	122.85	140.12	166.17	52.01		130.54	148.28	124.86	130.28	129.10	133.50
3	2-Cl	120.43	140.58	166.86	51.82		132.63	134.91	130.13	131.01	127.57	127.04
4	2-F	120.34	137.42	167.20	51.75		122.40	161.30	116.16	131.66	129.01	124.39
5	2-MeO	118.26	140.22	167.88	51.54	55.42	123.31	158.29	111.08	131.43	128.86	120.64
<i>meta</i> -Substituents (C-3)												
6	3-NO ₂	120.93	141.90	166.50	51.98		136.06	122.38	148.61	124.50	133.57	129.93
7	3-Br	119.24	143.08	166.91	51.81		136.42	130.69	122.98	133.01	126.62	130.34
8	3-Cl	119.23	143.19	166.95	51.81		136.15	127.75	134.88	130.10	126.20	130.10
9	3-MeO	118.04	144.73	167.31	51.68	55.25	135.69	112.92	159.83	116.08	120.71	129.84
<i>para</i> -Substituents (C-4)												
10	4-NO ₂	122.03	141.83	166.39	52.03		140.42	128.60	124.12	148.45		
11	4-Br	118.44	143.42	167.09	51.77		133.23	129.39	132.09	124.50		
12	4-Cl	118.33	143.36	167.11	51.76		132.81	129.13	129.18	136.16		
13	4-F	117.51	143.51	167.26	51.72		130.62	129.90	116.01	163.86		
14	4-MeO	115.22	144.48	167.72	51.55	55.34	127.07	129.68	114.28	161.34		
15	4-Me	116.65	144.83	167.58	51.60	21.43	131.60	128.02	129.57	140.68		

Table 3. Second-order rate constants (k_2) for the non-enzymic reaction of ring-substituted methyl *trans*-cinnamates with glutathione

Rate constants were determined by incubation of a 0.1 mM concentration of each methyl *trans*-cinnamate with 10 mM-GSH, 0.2 mM-EDTA, 1% (v/v) acetonitrile and 0.1 M-sodium carbonate buffer, pH 9.0, at 50 °C. The rates were determined spectrophotometrically at the wavelengths indicated with use of the molar difference absorbance coefficients (Δa_m) determined as described by Keen *et al.* (1976).

Substituent	Wavelength of measurement (nm)	Δa_m (M ⁻¹ ·cm ⁻¹)	k_2 (M ⁻¹ ·min ⁻¹)	$\log k_2$
H	300	5700	0.97	-0.013
2-Cl	300	7400	1.49	0.17
2-F	300	10600	1.19	0.08
2-CH ₃ O	320	9900	0.84	-0.08
3-NO ₂	280	16500	1.13	0.05
3-Br	300	5600	1.41	0.15
3-Cl	290	11700	1.45	0.16
3-F	300	9800	1.39	0.14
3-CH ₃ O	300	9700	0.76	-0.12
4-NO ₂	320	8000	2.74	0.44
4-Br	300	12800	0.75	-0.12
4-Cl	300	14600	1.35	0.13
4-F	300	9800	1.38	0.14
4-CH ₃ O	300	15000	0.51	-0.29
4-CH ₃	300	15000	0.55	0.26

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