J. Pharm. Pharmacol. 1993, 45: 166–170
Received February 11, 1992

Structural Requirements for the Direct and Cytochrome P450-dependent Reaction of Cyclic α,β-Unsaturated Carbonyl Compounds with Glutathione: a Study with Coumarin and Related Compounds

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Abstract—The interaction of glutathione (GSH) with coumarin, or one of a series of compounds related to coumarin, was assessed in the absence and presence of liver microsomes (direct reaction and indirect reaction, respectively) to determine the structural requirements for direct and mono-oxygenase-mediated reaction of cyclic α,β-unsaturated carbonyls with GSH. Acrolein was used as a positive control for the direct reaction, and produced complete or nearly complete depletion of GSH under all assay conditions. 5,6-Dihydro-2H-pyran-2-one and 2-cyclohexen-1-one also produced substantial depletion of GSH in the direct reaction, which was not increased by the addition of liver microsomes. Coumarin, 2H-pyran-2-one and precocene I (a substituted pyran lacking the 2-one structure) were not substrates for the direct reaction but did cause depletion of GSH when incubated in the presence of rat or human liver microsomes. These depletions were dependent on a functioning mono-oxygenase system as judged by the effects of omission of cofactors, addition of competitive or inactivating inhibitors of cytochrome P450, and induction. Dihydrocoumarin, ∂-valerolactone, cyclohexanone and 4H-pyran-4-one were not substrates for either the direct or indirect reaction. These findings are rationalized on the basis of a direct nucleophilic attack of GSH on the α,β-centre of the α,β-unsaturated carbonyl compounds, which is hindered by benzenoid resonance in coumarin and 2H-pyran-2-one, for which enzyme-mediated reaction with GSH, probably via a 3,4-epoxide, is the favoured mechanism.

Coumarin (2H-benzopyran-2-one) is a naturally-occurring constitutent of many plants, which is added to perfumes, toilet soaps and tobacco products (Opdyke 1974; International Agency for Research on Cancer 1976). It was banned from use as a food flavouring agent in the USA in 1954 on the finding of hepatotoxicity in rats (Cohen 1979), but is currently undergoing clinical trials for the treatment of cancer (Marshall et al 1989, 1990; Dexeus et al 1990), high-protein lymphoedema (Jamal et al 1989), chronic infections and disorders of the immune system (Egan et al 1990).

The hepatotoxicity of coumarin is preceded by depletion of liver glutathione (GSH) (Lake 1984). This finding, together with that of covalent binding to protein of radioactivity derived from [^{14}C]coumarin following microsomal metabolism (Lake 1984), has been taken to indicate that the hepatotoxicity is mediated by a reactive metabolite produced by the microsomal cytochrome P450-dependent mono-oxygenase (MMO) system, possibly a 3,4-epoxide (Lake et al 1989).

Coumarin is a substituted cyclic α,β-unsaturated carbonyl compound. It has been established that linear and cyclic α,β-unsaturated carbonyls may combine directly with GSH in a Michael-type 1,4-addition reaction, either non-enzymically (Esterbauer et al 1975) or mediated by a liver cytosol glutathione *S*-transferase (Boyland & Chasseaud 1967). There is, therefore, the possibility of both a direct and an indirect (i.e. MMO-mediated) reaction of cyclic α,β-unsaturated carbonyls with GSH. In this study we have investigated these two reactions with a variety of chemicals structurally related to coumarin to determine the structural basis for the discrimination of the two reactions. The formulae of the chemicals used in this study are presented in Fig. 1.

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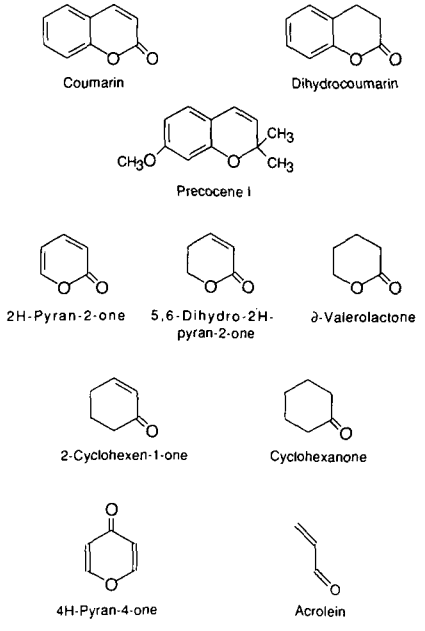

FIG. 1. Structures of the compounds used in this study.

Materials and Methods

Animals and treatments

Male Wistar rats, starting weight approximately 120 g, were obtained from the University of Nottingham Medical School Animal Unit. They were housed at a room temperature of 22°C on a 12-h light/dark cycle, relative humidity 50 ± 10%, and allowed free access to drinking fluid and a standard diet (Diet 41B, Heygates (Pilsbury) Ltd, York, UK) throughout the treatment period. Phenobarbitone sodium was administered in the drinking water at 0·1% (w/v) for 7 days, whilst β-naphthoflavone was administered intraperitoneally in arachis oil once daily for three days at a dose of 80 mg kg^{-1}. Control rats were not treated.

Human liver sample

A sample of histologically-normal human liver was obtained from a 66-year old female patient undergoing lobectomy for a well-differentiated adenocarcinoma.

Chemicals

Coumarin, precocene I, metyrapone and equine glutathione *S*-transferase (G6522) were purchased from the Sigma Chemical Co., Poole, Dorset, UK. 2-Cyclohexen-1-one and cyclohexanone were purchased from Lancaster Synthesis Ltd, Morecambe, Lancs, UK. Dihydrocoumarin, 2H-pyran-2-one, 5,6-dihydro-2H-pyran-2-one, ∂-valerolactone and 4H-pyran-4-one were purchased from the Aldrich Chemical Co. Ltd, Poole, Dorset, UK. These chemicals were used without any further purification.

1-Aminobenzotriazole (1-ABT) was synthesized from diethyl(benzotriazol-1-yl)-iminomalonate (Lancaster Synthesis) by the method of Campbell & Rees (1969). The sources of the other chemicals used in the microsomal incubations and the GSH assays have been described previously (Garle & Fry 1989).

Microsome isolation and assays

Microsomes were isolated from the pooled livers of each group of rats, and from the human liver sample, by Ca^{2+}-aggregation as described previously (Garle & Fry 1989), and stored at −196°C until use. The success of the induction treatments with phenobarbitone and β-naphthoflavone were confirmed by the observations of significant increases in the cytochrome P450 content and 7-ethoxycoumarin *O*-deethylase activity of the isolated microsomes (data not shown), the magnitudes of which were similar to those previously reported from this laboratory (Garle & Fry 1989). Assays for the measurement of microsomal P450 and protein content, and enzyme activity have been described previously (Garle & Fry 1989).

GSH depletion assay

Substrate-mediated loss of GSH was determined essentially as described by Garle & Fry (1989). In brief, the incubation mixture (1 mL) contained liver microsomes (final concentration 0·75–1·00 mg protein mL^{-1}), 0·1 M phosphate buffer (pH 7·4), 5 mM $MgSO_4$, 0·5 mM NADP, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (1 unit mL^{-1}), 200 μM GSH, and substrate, added in methanol at a final concentration of 5 μL mL^{-1}. In some experiments with coumarin, glutathione *S*-transferase (final concentration 1 unit mL^{-1}) was also incorporated into the incubation mixture. The incubation was carried out for 30 min at 37°C, after which the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid, and the amount of GSH remaining in the deproteinized supernatant was determined by the method of Sedlack & Lindsay (1968) after correction for the depletion of GSH that occurred in the absence of substrate.

Measurement of the direct reaction of substrate with GSH was carried out as described above with the modification that either the microsomes or the cofactors (NADP + glucose 6-phosphate) were omitted from the incubation. Preliminary experiments confirmed that microsome or cofactor omissions produced essentially identical results.

The effect of metyrapone in the microsome-supported reactions was determined by performing the assay as described above, with metyrapone added at a final concentration of 1 mM. The effect of 1-ABT, a mechanism-based inactivator of MMO activity, on the microsome-supported reactions was determined by incubating rat liver microsomes, cofactors and 1-ABT (final concentration of 1 mM) at 37°C for 15 min before addition of fresh cofactors, GSH and

Table 1. Depletion of GSH mediated by coumarin and other compounds.

	GSH depletion[2]			
	Direct	Indirect		
Substrate[1]		Control	Phenobarbitone	β-Naphthoflavone
Coumarin	1 ± 0	11 ± 1	23 ± 1	25 ± 1
Dihydrocoumarin	< 1	< 1	< 1	< 1
2H-Pyran-2-one	< 1	16 ± 1	12 ± 2	12 ± 1
4H-Pyran-4-one	< 1	2 ± 1	< 1	< 1
5,6-Dihydro-2H-pyran-2-one	60 ± 2	38 ± 1	58 ± 1	47 ± 1
∂-Valerolactone	< 1	< 1	< 1	< 1
2-Cylcohexen-1-one	70 ± 3	69 ± 1	46 ± 2	64 ± 2
Cyclohexanone	< 1	< 1	< 1	< 1
Precocene I	< 1	3 ± 1	13 ± 1	14 ± 1
Acrolein	100 ± 1	100 ± 2	77 ± 5	100 ± 5

Values are the mean ± s.e.m. of at least 4 determinations. The enzyme-mediated reaction was performed in the presence of liver microsomes obtained from untreated rats (control), or from rats treated with phenobarbitone or β-naphthoflavone. [1] 1mM concentration, with the exception of precocene I (0·1 mM). [2] Expressed as % depletion/30 min (direct reaction) or as % depletion (mg protein)$^{-1}$/30 min (indirect reaction).

Table 2. Effect of metyrapone (1 mM) and 1-ABT (1 mM) on the depletion of GSH mediated by coumarin, precocene I and 2H-pyran-2-one in the presence of liver microsomes isolated from phenobarbitone-treated rats.

Substrate	Control	GSH depletion[1] + Metyrapone	+ 1-ABT
Coumarin (1 mM)			
Experiment 1	32±1	17±1	ND[2]
Experiment 2	23±2	ND	1±0
Precocene I (0·1 mM)	12±0	4±1	ND
2H-Pyran-2-one (1 mM)			
Experiment 1	21±1	33±1	ND
Experiment 2	13±1	ND	2±1

Values are mean±s.e.m. of at least 4 determinations. [1] Reported as % GSH depletion (mg protein)$^{-1}$ 30 min. [2] ND = not determined.

test chemical; preliminary studies demonstrated that this treatment with 1-ABT produced >80% loss of spectrally-detectable P450.

Percent GSH depletion was calculated, and the data are reported as the mean±s.e.m. of at least four determinations carried out on the same batch of microsomes.

Results

The results obtained for the direct reaction and the reaction mediated by rat liver microsomes are presented in Table 1. Acrolein was used as a positive control for the direct reaction, and, as expected, this compound produced complete or nearly complete depletion of GSH in the absence or presence of liver microsomes.

Coumarin did not significantly deplete GSH in the direct reaction, but was a substrate for metabolism-mediated depletion, the magnitude of which was increased following induction of the microsomal mono-oxygenase system by phenobarbitone or β-naphthoflavone. A direct reaction of coumarin with microsomal glutathione *S*-transferase could be ruled out by a lack of GSH depletion in the presence of coumarin with liver microsomes but without cofactors (data not shown). Furthermore, the extent of GSH depletion mediated by coumarin in the presence of liver microsomes from control or induced rats was not altered by co-incubation with glutathione *S*-transferase (data not shown). Essentially identical results were obtained with precocene I, which needed to be used at a concentration of 0·1 mM because of marked inhibition of mono-oxygenase activity at higher concentrations. Depletion of GSH mediated by coumarin and precocene I was significantly reduced in the presence of 1-ABT or metyrapone (Table 2). Dihydrocoumarin produced no depletion of GSH under any assay condition.

2H-Pyran-2-one behaved similarly to coumarin in that GSH depletion occurred only in the presence of liver microsomes, required the presence of cofactors and was inhibited by 1-ABT, although in this case the extent of depletion was not modified by use of liver microsomes from inducer-treated rats, neither did metyrapone have any effect. 4H-Pyran-4-one produced only trivial levels of GSH depletion in the direct and the enzyme-mediated reaction. 5,6-Dihydro-2H-pyran-2-one was a potent depletor in the direct reaction, and the presence of liver microsomes did not enhance, but rather decreased, the reaction. ∂-Valerolactone produced no depletion of GSH under any of the assay conditions.

2-Cyclohexen-1-one produced a substantial depletion of GSH in the direct reaction, which was not increased by incubation with liver microsomes, whilst cyclohexanone produced only trivial levels of GSH depletion in all of the assay conditions.

Coumarin and 2H-pyran-2-one produced GSH depletion in incubations containing human liver microsomes (Table 3), the levels of which were similar to those found with rat liver microsomes. Again, dihydrocoumarin and ∂-valerolactone produced, at most, only trivial depletion of GSH in the presence of human liver microsomes.

Table 3. GSH depletion mediated by coumarin, dihydrocoumarin, 2H-pyran-2-one and ∂-valerolactone in human liver microsomal incubations.

Compound	GSH depletion[1]
Coumarin	10±2
Dihydrocoumarin	<1
2H-Pyran-2-one	13±2
∂-Valerolactone	1±1

Values are means±s.e.m. of at least 4 determinations. Each compound was used at a final concentration of 1 mM. [1] Reported as % GSH depletion (mg protein)$^{-1}$/30 min.

Discussion

Coumarin and related compounds may be classified into three groups on the basis of their reactions with GSH: those that react directly with GSH and for which metabolism has been demonstrated or inferred not to enhance reaction with GSH (acrolein, 5,6-dihydro-2H-pyran-2-one, 2-cyclohexen-1-one); those that react with GSH only in the presence of the liver MMO system (coumarin, precocene I, 2H-pyran-2-one); and those that do not react with GSH either directly or in the presence of liver microsomes (dihydrocoumarin, ∂-valerolactone, 4H-pyran-4-one, cyclohexanone). Evidence for the involvement of the MMO system was provided by studying the effects of omission of cofactors, addition of metyrapone (a competitive inhibitor) or 1-ABT (a mechanism-based inactivator), and induction. In comparison with coumarin and precocene I, 2H-pyran-2-one-mediated indirect depletion of GSH was not modified by addition of metyrapone or by use of liver microsomes isolated from induced rats, thus possibly suggesting the involvement of different cytochrome P450 forms in metabolism of 2H-pyran-2-one and coumarin/precocene I.

Acrolein reacted directly with GSH, causing complete depletion of GSH in the experimental conditions employed. This finding agreed with that of Esterbauer et al (1975) and is consistent with a direct nucleophilic attack of GSH on an α,β-unsaturated carbonyl in a Michael-type 1,4-addition. Direct reaction with GSH also occurred with 2-cyclohexen-1-one, although the extent of reaction was less than that recorded for acrolein. These findings are also in agreement with those of Esterbauer et al (1975), who ascribed these differences to differences in rate of adduct formation. Insertion of an oxygen atom (to produce 5,6-dihydro-2H-

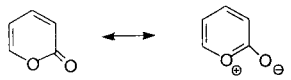

FIG. 2. Resonance forms of 2H-pyran-2-one.

pyran-2-one) had little effect on the extent of direct reaction with GSH. Morphinone, a metabolite of morphine which is a substituted 2-cyclohexen-1-one, has also been reported to react directly with GSH (Nagamatsu et al 1982). Nucleophilic attack by GSH on the α,β-double bond occurs because the latter is rendered electron-deficient by the electron-withdrawing carbonyl group. Substituents which can donate electrons to the double bond will cause diminution or abolition of reaction with GSH.

Desaturation of the 5,6-position (to produce 2H-pyran-2-one) abolished the direct conjugation with GSH observed with 5,6-dihydro-2H-pyran-2-one. Coumarin, which is a substituted 2H-pyran-2-one, behaved similarly to 2H-pyran-2-one in this respect. These 2H-pyran-2-ones are aromatic in character as indicated by their benzene-like resonance forms (as shown for 2H-pyran-2-one in Fig. 2). Such stabilized systems are relatively unreactive towards nucleophiles such as GSH, in accordance with the observed lack of direct reaction. Similar arguments are proposed to explain the lack of direct reaction of 4H-pyran-4-one with GSH.

2H-Pyran-2-one and coumarin were, however, substrates for an hepatic microsome-mediated reaction with GSH, as was precocene I. These findings with coumarin are in agreement with those recently reported by Peters et al (1991). Whilst it is possible that the presence of the hepatic microsomal protein stabilized the 2H-pyran-2-ones in the pyran structure, so enabling the reaction with GSH to occur, it is more likely that the reaction of these 2H-pyran-2-ones with GSH in the presence of liver microsomes was mediated by the production of a reactive epoxide at the $\alpha,\beta(3,4)$-site, as has been proposed by Lake et al (1989). This assertion is based on the following evidence. No reaction of 2H-pyran-2-one or coumarin with GSH occurred in the presence of liver microsomes but in the absence of cofactors (data not shown), thus arguing against a purely stabilizing role for the liver microsomes. Precocene I, which lacks the 2-one structure, was also a substrate for the indirect reaction with GSH; the major rat liver microsomal metabolite of precocene I has been identified as a 3,4-dihydrodiol, which most likely arises by hydration of a reactive 3,4-epoxide (Halpin et al 1984). 4H-Pyran-4-one, which possesses an α,β-unsaturated carbonyl structure but which lacks a double bond in the 3,4-position, was not a substrate for the indirect reaction with GSH.

Cytochrome P450-mediated epoxidation is known to proceed well with electron-rich double bond systems and it is thus chemically rational that precocene I and the 2H-pyran-2-ones react in this fashion, the intermediate epoxides then being subject to attack by the nucleophilic GSH. Indeed, during preparation of this paper the presence of a coumarin mercapturic acid (*N*-acetyl-*S*-(3-coumarinyl)cysteine) in the urine of coumarin-dosed rats has been reported, which was considered to be produced from a 3,4-epoxide (Huwer et al 1991).

4H-Pyran-4-one might also be expected to show some

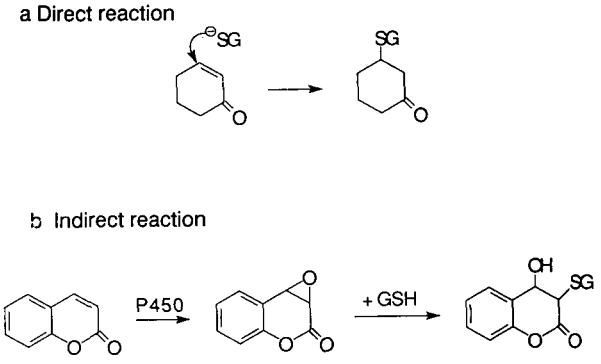

FIG. 3. Examples of direct and indirect reaction of cyclic α,β-unsaturated carbonyl compounds with GSH, using 2-cyclohexen-1-one and coumarin as models.

degree of reactivity in cytochrome P450-mediated oxidations, on simple chemical grounds, although 4-pyrones are generally more stable than 2-pyrones. Other selectivity and reactivity factors may supervene.

The lack of reactivity of the saturated analogues of 2-cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one and coumarin in either the direct or enzymic reaction is not unexpected given the reaction mechanisms proposed above.

It is interesting to note that human liver microsomes also have the capability to convert coumarin and 2H-pyran-2-one into metabolites which deplete GSH (Table 3), but further studies are required to determine if such metabolism poses a toxicological hazard to man.

In conclusion, the data presented in this paper have helped to define the structural details which allow discrimination of direct vs cytochrome P450-dependent reaction of cyclic α,β-unsaturated carbonyl compounds with GSH. Aromatic resonance appears to be particularly important. The direct reaction with GSH involves nucleophilic attack on the α,β-unsaturated centre, while the indirect reaction probably involves epoxidation at the same centre; examples of these reactions are given in Fig. 3. The order of reactivity of acrolein, 2-cyclohexen-1-one and coumarin with GSH in the direct reaction reported in this paper follows that observed in the transferase-mediated reaction (Boyland & Chasseaud 1967), so it is likely that similar structural details will be important in the discrimination of transferase- vs cytochrome P450-mediated reactions involving cyclic α,β-unsaturated carbonyls.

Acknowledgements

The assistance of Mr J. Doran, of the Department of Surgery, University Hospital, Queen's Medical Centre in obtaining the sample of human liver is gratefully acknowledged, as is the financial assistance of the industrial sponsors of the Fund for Replacement of Animals in Medical Experimentation. The authors greatly appreciate the advice given by Professor C. W. Rees in regard to the synthesis of 1-ABT.

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