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IRREVERSIBLE INHIBITION OF HUMAN GLUTATHIONE *S*-TRANSFERASE ISOENZYMES BY TETRACHLORO-1,4-BENZOQUINONE AND ITS GLUTATHIONE CONJUGATE

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Abstract—The quinones tetrachloro-1,4-benzoquinone (1,4-TCBQ) and its glutathione conjugate (GS-1,4-TCBQ) are potent irreversible inhibitors of most human glutathione *S*-transferase (GST) isoenzymes. Human π, ψ, and μ are almost completely inhibited at a molar ratio 1,4-TCBQ/GST = 2/1. The isoenzyme B1B1 was inhibited up to 75%, and higher concentrations (1,4-TCBQ/GST = 6/1) were needed to reach this maximum effect. For these isoenzymes 75–85% of the maximal amount of inhibition was already reached on incubation of equimolar ratios of 1,4-TCBQ and subunit GST, while approximately 1 nmol (0.82–0.95) 1,4-[U-^{14}C]TCBQ per nmol subunit GST could be covalently bound. These results suggest that these GST isoenzymes possess only one cysteine in or near the active site of GST, which is completely responsible for the inhibition. In agreement, human isoenzyme B2B2 which possesses no cysteine, was not inhibited and no 1,4-TCBQ was bound to it. The rate of inhibition was studied at 0°: 1,4-TCBQ, trichloro-1,4-benzoquinone and GS-1,4-TCBQ all inhibit GST very fast. Especially for B1B1, the inhibition by the glutathione conjugate is significantly faster than inhibition by 1,4-TCBQ: the glutathione moiety seems to target the quinone to the enzyme. For the other isoenzymes only minor differences are observed between 1,4-TCBQ and its glutathione conjugate under the conditions used.

The glutathione *S*-transferases (GSTs†) are a group of isoenzymes that catalyse the reaction of electrophilic compounds with glutathione [1]. Among the broad substrate spectrum are α,β-unsaturated carbonyl derivates, epoxides, quinones and a range of other alkylating agents [1]. In addition, GSTs also detoxify electrophilic metabolites by serving as targets for alkylation or arylation [2–5]. However, the effect of this reaction on the activity of GSTs has not been studied in detail.

The GSTs of rat, mouse and man are divided in three distinct classes: the α-, μ-, and π-class. They vary widely in tissue distribution. The highest concentrations of GSTs are found in the liver, with the exception of the π-class which is most abundant in the kidney and placenta. The μ-class GSTs are expressed in only 50% of human individuals [6].

Inhibitors of GSTs are of considerable interest: firstly, it has been proposed that inhibition of GSTs could overcome the resistance to some antineoplastic drugs that certain tumor cells display [7, 8]. Secondly, GSTs are involved in the biosynthesis of leukotrienes and prostaglandins [9]. Modulation of the biosynthesis of these compounds by inhibition of GSTs could be of potential therapeutic benefit in the treatment of related disorders [9].

A large number of inhibitors of GSTs are known [6]. Most of these however act in a competitive manner, i.e. their effects are reversible. Recently, tetrachloro-1,4-benzoquinone (1,4-TCBQ) has been shown to inhibit rat glutathione *S*-transferase very strongly in an irreversible fashion [10]. This compound has been shown to react with cysteine residues in the vicinity of the active site [11]. Using rat isoenzymes, several characteristics of the reaction were studied. Most importantly, the glutathione conjugate of 1,4-TCBQ (GS-1,4-TCBQ) has been shown to inhibit even more strongly: the glutathione moiety seems to target the conjugate to the active site of the enzyme. The corresponding β-mercaptoethanol conjugate, showed a much slower rate of inhibition [10]. Lastly, in contrast with other reagents [11], the reaction of the quinone as well as the quinone conjugate with the first cysteine of GST, has the major effect on the inhibition [12].

The present study has been designed to determine the inhibition of the *human* isoenzymes by 1,4-TCBQ, the rate of inhibition by 1,4-TCBQ, trichloro-1,4-benzoquinone, and GS-1,4-TCBQ, as well to determine the number of cysteine residues that react with 1,4-TCBQ.

MATERIALS AND METHODS

Chemicals and radiochemicals. Pentachloro-[U-^{14}C]phenol was from CEA (Gif sur Yvette, France, 36.9 μCi/μmol). 1,4-TCBQ was from Merck (Darmstadt, F.R.G.), its glutathione conjugate was synthesized as described elsewhere [10]. Trichloro-1,4-benzoquinone (1,4-TriClBQ) was a generous gift of B. Spenkelink (Agricultural University Wageningen, The Netherlands). 1,4-[U-^{14}C]TCBQ

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† Abbreviations: 1,4-TCBQ, tetrachloro-1,4-benzoquinone; 1,4-TCHQ, tetrachloro-1,4-hydroquinone; 1,4-TriClBQ, trichloro-1,4-benzoquinone; GS-1,4-TCBQ, 2-*S*-glutathionyl-3,5,6-trichloro-1,4-benzoquinone; CDNB, 1-chloro-2,4-dinitrobenzene; HPLC, high performance liquid chromatography; GST, glutathione *S*-transferase.

was prepared from pentachloro-[U-^{14}C]phenol by microsomes from dexamethasone-induced male rats, as described previously [13]. The conditions used were: incubation for 1 hr at 37° with 1 mg/mL microsomal protein, 1 mM NADPH, with after 30 min and extra 1 mM addition, 2 μmol pentachloro-[U-^{14}C]phenol (36.9 μCi/μmol) in 1.5 mL of acetone, 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$ and 2 mM ascorbic acid. The total volume was 100 mL. The reaction was stopped with 1 mL of 6 N HCl and extra ascorbic acid was added to 10 mM. 1,4-[U-^{14}C]TCHQ and pentachloro-[U-^{14}C]phenol were extracted [three times with 140 mL of acetone : ethylacetate (1 : 2)], and H_2O was removed with Na_2SO_4. The solvent was removed by evaporation. 1,4-[U-^{14}C]TCHQ was purified by preparative HPLC (Zorbax ODS, Dupont, 21.2 mm × 25 cm) using a gradient of 50–100% methanol against 0.05% formic acid in 45 min, followed for 45 min at 100% methanol, with a flow of 3 mL/min, and UV detection at 296 nm. The conversion was approximately 37%. 1,4-[U-^{14}C]TCBQ was freshly prepared for each experiment by quantitative oxidation for 5 min at 25° with an excess of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in methanol and purified by HPLC as described above, and collected on ice (k': 1.9, 2.4, and 3.5, respectively, for 1,4-TCHQ, 1,4-TCBQ and pentachlorophenol).

Purification. GST isoenzymes were purified from liver and placenta using *S*-hexylglutathione affinity chromatography. Separation of the GST isoenzymes was achieved with chromatofocusing with a mono P-column (Pharmacia, The Netherlands), as previously described [14]. The purity was confirmed by HPLC analysis [15] and isoelectric focusing [14]. Specific activities with CDNB as second substrate (see below) were: 37.4, 23.8, 89.8, 85.4 and 69.9, respectively, for human B1B1, B2B2, μ, ψ and π.

All enzyme concentrations are expressed as the concentration of the subunit.

Enzyme assays. To determine the amount of inhibition at different molar ratios of 1,4-TCBQ/GST, incubations of 25 pmol enzyme (M_r: 25,900, 25,900, 26,700, 26,600 and 24,800, respectively, for human B1B1, B2B2, μ, ψ and π [16]) with 6.25 to 250 pmol 1,4-TCBQ were performed for 15 min at 25°, in 0.1 M potassium phosphate buffer (pH 6.5), supplemented with 1 mM EDTA, after which GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25° (pH 6.5), according to Habig *et al.* [17].

In order to detect a time-dependent inhibition of GST by 1,4-TCBQ, 1,4-TriClBQ and GS-1,4-TCBQ, 25 pmol of enzyme was incubated with 75 pmol of quinone in a cuvette in 110 μL at 0°. At various time intervals 850 μL, containing glutathione (1 μmol) and potassium phosphate buffer (final concentration: 0.1 M, with 1 mM EDTA, pH 6.5) at 25° was added, whereafter 40 μL CDNB (1 μmol) was added and the inhibition of enzymatic CDNB conjugation was measured at 340 nm at 25°.

All enzyme assays were performed in duplicate, while controls were treated in the same way.

Measurement of covalent binding of 1,4-*TCBQ to human GST.* The specific activity of freshly prepared

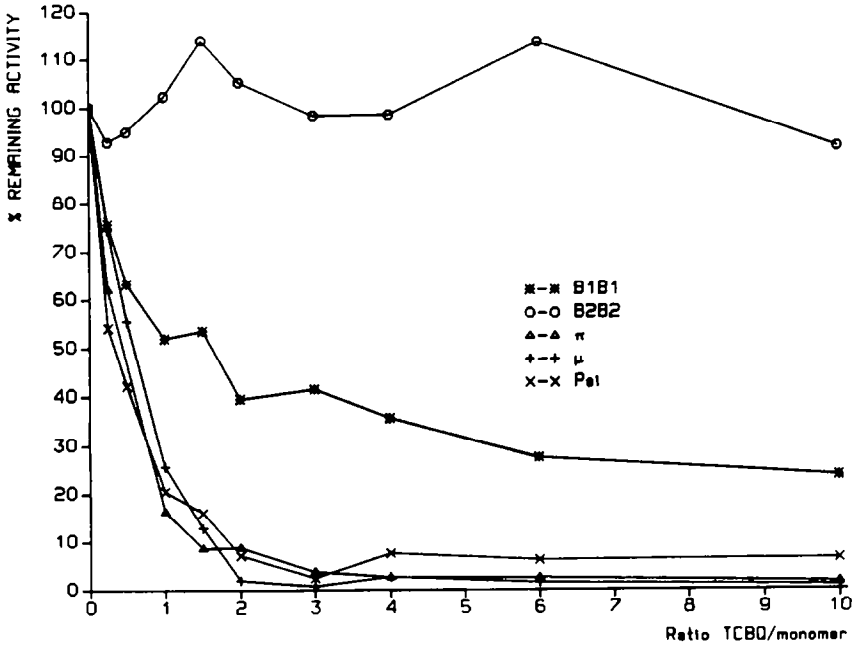

Fig. 1. Remaining activity at different molar ratios of 1,4-TCBQ/enzyme, after incubations of 25 nM subunit human GST B1B1, B2B2, μ, ψ and π, with 6.25–250 nM 1,4-TCBQ for 15 min at 25°, in 0.1 M potassium phosphate buffer (pH 6.5), supplemented with 1 mM EDTA, after which GST activity towards CDNB was measured at 25°. The results are the average of two incubations.

1,4-[U-^{14}C]TCBQ was decreased to 10,000 dpm/nmol. One nanomole of enzyme was incubated in a microconcentration tube (centricon TM 10, Amicon, U.S.A.) with 10 nmol 1,4-[U-^{14}C]TCBQ for 30 min at 25° in 1 mL of 0.1 M potassium phosphate (pH 6.5) with 1 mM EDTA, followed by incubation for 5 min with 2 mM ascorbic acid. Solvent containing unreacted (hydro)quinone was removed by centrifugation for 30 min at 5000 *g* (at 10°), followed by three washing steps: addition of 0.5 mL of methanol/H_2O (1:1) and centrifugation for 40 min at 5000 *g*. The dry filter was dissolved overnight in 1 mL of soluene 350 (Packard, U.S.A.) at 37° and the sample was screened for radioactivity in 15 mL of scintillation liquid (Hionic fluor, Packard).

Statistical methods. Nonlinear regression analysis was performed with the statistical package Genstat5. Parallel curve analysis was performed using the equation of the curve: $Y = a_i + b_i e^{-k_i t}$.

RESULTS

Extent of inhibition

The inhibition of human GSTs by 1,4-TCBQ is shown in Fig. 1. Almost complete inhibition (up to 98%) for the isoenzymes of the μ- and π-classes was observed at a molar ratio 1,4-TCBQ/GST = 2/1. Inhibition of the representatives of the α-class was not complete: the isoenzyme B1B1 was inhibited up

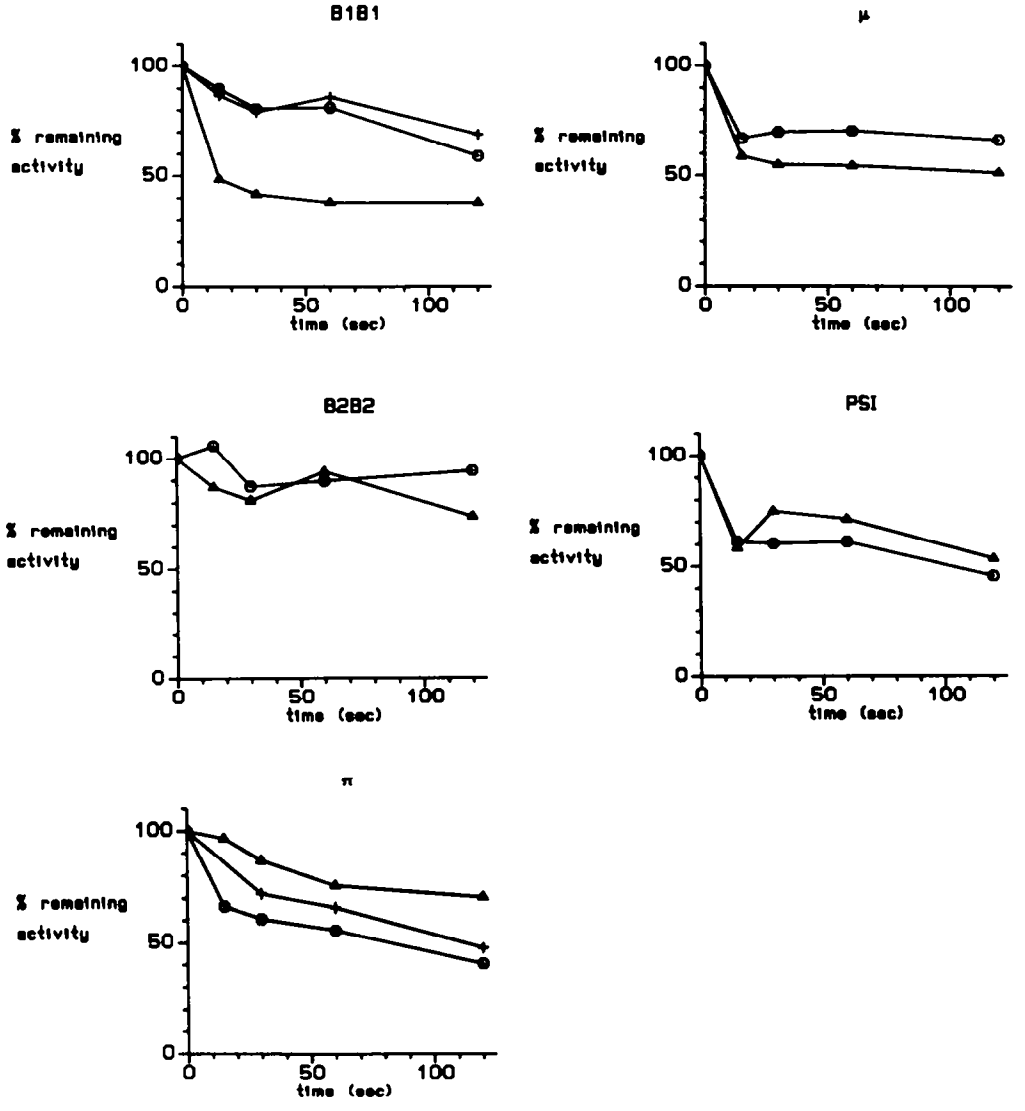

Fig. 2. Time-dependent inhibition of human GST by 1,4-TCBQ (○), 1,4-TriClBQ (+), and its glutathione conjugate (△), 25 pmol enzyme was incubated with 75 pmol quinone in a cuvette in 110 μL at 0°. At various time intervals 850 μL, containing glutathione (1 μmol) and potassium phosphate buffer (final concentration: 0.1 M, with 1 mM EDTA, pH 6.5) at 25° was added, whereafter 40 μL CDNB (1 μmol) was added and the inhibition of enzymatic CDNB conjugation was measured at 340 nm. The results are the average of two incubations.

to 75%, and a higher concentration was needed to reach the maximum effect (1,4-TCBQ/GST = 6/1). Human B2B2 was not inhibited at all. Interestingly, for all these isoenzymes (with the exception of B2B2), the major part of the inhibition (about 75 to 85%) has been reached at 1,4-TCBQ/GST = 1/1.

Timecourse of inhibition

The timecourse of inhibition with 1,4-TCBQ and GS-1,4-TCBQ for all isoenzymes is shown in Fig. 2. In order to slow down the reaction, the incubations were performed at 0°. Both 1,4-TCBQ and GS-1,4-TCBQ inhibited the GST isoenzymes B1B1, π, μ, and ψ very quickly. Some interesting differences were observed: the rate of inhibition by GS-1,4-TCBQ was significantly higher than the rate of inhibition by 1-4-TCBQ, only for the human B1B1 (t-test, $\alpha = 0.10$). Under the conditions used only minor differences between 1,4-TCBQ and the glutathione conjugate were observed for μ and ψ, while for human π the parent quinone without the glutathione moiety inhibited faster. B2B2 was again not inhibited (Fig. 2). The inhibitory effect of 1,4-TriClBQ was also investigated for the isoenzymes B1B1 and π. 1,4-TriClBQ still inhibited GST B1B1 and π very quickly, but somewhat slower than 1,4-TCBQ. The targeting effect of the glutathione moiety for the isoenzyme B1B1 was somewhat more obvious, while 1,4-TriClBQ still inhibits GST π somewhat faster than the glutathione conjugate.

Covalent binding

GST isoenzymes were incubated with radiolabeled 1,4-TCBQ to study the extent it covalently binds. The maximal binding is presented in Table 1. All GST isoenzymes bind about 1 nmol (0.82–0.95) per nmol GST, with the exception of B2B2, where no binding was observed.

DISCUSSION

In the present study it has been shown that human GST B1B1, μ, ψ and π are inhibited strongly as a result of covalent modification. Quinones are known to react very rapidly with sulfhydryl groups [10]. As expected, the human B2B2 which possesses no cysteine residues [18], is thus not inhibited by 1,4-TCBQ, and only 0.08 nmol 1,4-TCBQ could be bound per nmol subunit B2B2. The low amount of binding observed presumably reflects the relatively slow reaction of quinones with amino groups [19]. The isoenzymes B1B1, μ, ψ and π have been shown to be inhibited to about 75–85% of the maximum amount at a molar ratio 1,4-TCBQ/GST = 1/1, while approximately one cysteine could be modified by 1,4-[U-^{14}C]TCBQ. This suggests that human GST B1B1, μ, ψ and π possess one cysteine residue in or near the vicinity of the active site, which is completely responsible for the inhibition. These phenomena have already been reported for some isoenzymes of rat and human GST: the modification of only one cysteine of rat isoenzyme 4-4 and 7-7 by respectively 1,4-TCBQ or *N*-ethylmaleimide, and of only one cysteine of human π by *N*-(4-anilino-1-naphthyl) maleimide or CDNB, resulted in complete inhibition [2, 12, 20, 21]. However, the quinones differ in their inhibitory characteristics from these compounds: lower concentrations are needed and in contrast to *N*-ethylmaleimide, they also inhibit the human α- and μ-classes [2]. The total amount of cysteine residues per GST subunit ranges from 1 (B1) to 4 (μ, π) [22–24]. Thus, the binding of only one nmol 1,4-TCBQ per nmol GST further indicates that complete modification of all cysteine residues does not occur for human μ and π. In accordance with this result, it has been shown that *N*-(4-anilino-1-naphthyl) maleimide reacts only with one cysteine residue per human π subunit [20], the other three cysteines are probably located inside the hydrophobic core, and/or form disulfide bonds. Using rat isoenzyme 4-4, all three available cysteine residues could be modified by 1,4-TCBQ [12], thus the cysteine residues of rat isoenzyme 4-4 (μ-class) are better accessible than those of human μ.

The glutathione conjugate of 1,4-TCBQ, which retains its oxidized structure [10], inhibited a mixture of rat GST isoenzymes at a much higher rate than the corresponding β-mercaptoethanol conjugate. The glutathione moiety seems to target the quinone to the enzyme. In the present study, the rate of inhibition by 1,4-TCBQ and its glutathione conjugate were mutually compared. In general, only minor differences were observed. Thus, intracellularly formed GS-1,4-TCBQ does not slow the rate of inhibition. In the case of the isoenzyme B1B1, this glutathione conjugate even accelerated the inhibition.

In conclusion, *in vivo* the cysteine residues of human GST can become a target for quinones, as well as for other alkylating and arylating agents such as ethacrynic acid, acrolein, maleimide derivates and metabolites of bromobenzene [2–5], thereby providing a second detoxification pathway by GST. However, with the exception of B2B2, all these subunits possess one cysteine residue that is important for the conjugating activity. If that residue is modified, the enzyme will be inhibited.

Table 1. Covalent binding of 1,4-TCBQ to human glutathione *S*-transferases

Isoenzyme	Covalent binding (nmol TCBQ/nmol GST subunit)
B1B1	0.83 ± 0.07
B2B2	0.08 ± 0.00
π	0.89 ± 0.27
μ	0.95 ± 0.12
ψ	0.82 ± 0.12

One nanomole (subunit) enzyme was incubated with 10 nmol 1,4-[U-^{14}C]TCBQ for 30 min at 25°, for experimental details see Materials and Methods. Values are the average ± SD of duplicate incubations.

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