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DIFFERENTIAL UPTAKE OF ISOMERIC 2-BROMOHYDROQUINONE-GLUTATHIONE CONJUGATES INTO KIDNEY SLICES

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2-Bromo-(diglutathion-Syl)hydroquinone (2-Br-[diGSyl]HQ) is a more potent nephrotoxicant than any of three mono-substituted isomers. The reason for this differential toxicity is unknown. We now report that the rate of uptake of 2-Br-(diGSyl)HQ , 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)-HQ and 2-Br-6(GSyl)HQ by kidney slices was 2.4, 1.2, 1.0 and 0.3 nmoles/mg/10 min, respectively. AT-125 (0.5 mM) inhibited γ-glutamyl transpeptidase (GGT) in intact and homogenized kidney slices by 47% and 92%, respectively and decreased the accumulation of the isomeric [^{35}S]-conjugates by 49%, 21%,25% and 30%, respectively. The data suggest that the accumulation of 2-Br-(GSyl)HQ conjugates into isolated kidney slices may in part be mediated by GGT and that the more extensive renal uptake of the di-substituted conjugate may be partially responsible for its enhanced nephrotoxicity. In addition, 2-Br-(diGSyl)HQ gave rise to the most covalently bound material of the different isomers studied suggesting that both physiological and biochemical factors contribute to the potent and selective nephrotoxicity of this compound.

Conjugation with glutathione (GSH) has been implicated in the activation of chemicals to mutagenic and carcinogenic electrophiles and evidence is accumulating that GSH conjugates of a variety of compounds and/or their corresponding cysteine conjugates are nephrotoxic (1,2). The kidney possesses relatively high activities of the enzymes involved in mercapturic acid formation; GSH S-transferase(s), GGT, cysteinyl glycine dipeptidase and N-acetyl transferases (3,4). In addition, the recent elucidation of the "thiomethyl shunt" pathway provides a biochemical basis upon which to rationalize the mechanism(s) of GSH S-conjugate mediated nephrotoxicity (5,6). Thus, because of is biochemical profile the kidney may be particularly susceptible to the toxic effects of S-conjugates formed during mercapturic acid synthesis.

However, in addition to biochemical factors, another major determinant of target-organ toxicity is the manner in which the substrate gains access to its target. The kidneys comprise 0.4% of the body weight in most mammals but receive 25% of cardiac output. This high blood flow is in part responsible for the large amount of xenobiotics in the systemic circulation which are delivered to the kidneys, in particular the renal cortex, which receives over 90% of the renal blood flow (7). In addition, the ability of the kidney to concentrate tubular fluid may enhance toxicity as a result of

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increased xenobiotic concentrations. Thus the specialized functions of the proximal tubular cells may enhance toxicity in several ways, all of which result in high intracellular concentrations of potentially toxic xenobiotics. The kidneys also remove a large proportion of plasma GSH (8-10) and as much as 70% of plasma GSH clearance is renal (7). Thus, the physiological functions of the kidney may also predispose this organ to GSH S-conjugate mediated toxicity. One mechanism for the delivery of GSH and GSH S-conjugates to the kidney is glomerular filtration, although while 70% of the GSH in plasma that enters the kidney is removed, only 25% of this is removed by the glomeruli. A significant amount of circulating GSH is also removed by a non-filtration mechanism (9,10) which involves transport into renal cells across the basal-lateral membrane (11-13). Both a sodium-coupled and a GGT mediated transport system may be responsible for the basal-lateral mediated transport of GSH, the relative contributions of which are unclear.

We have recently shown that in contrast to three mono-GSH substituted conjugates of 2-BrHQ, 2-Br-(diGSyl)HQ is a potent and selective proximal tubular toxicant (14). Little information exists on the renal handling of GSH S-conjugates and none with respect to disubstituted GSH conjugates. In the present study we report the uptake of the isomeric [^{35}S]-2-Br-(GSyl)HQ conjugates into freshly isolated rat kidney slices. The data suggest that the transport of 2-Br-(GSyl)HQ conjugates into isolated kidney slices may in part be mediated by GGT and that the more extensive renal uptake of the 2-Br-(diGSyl)HQ may contribute to its enhanced nephrotoxicity.

METHODS

Reagents. [^{35}S]-Glutathione (112.5 Ci/mmole; >98.5% pure) was purchased from New England Nuclear. Glutathione, lactic acid, p-aminohippurate (PAH) and tetraethylammonium (TEA) were obtained from Sigma. 2-BrHQ and silver oxide were products of ICN Pharmaceuticals. AT-125 (L-(α-5S)-α-amino-S-chloro-4,5-dihydro-5-isoxazoleacetic acid) was generously provided by the National Cancer institute. All other reagents were of the highest grade commercially available.

Animals. Male Sprague Dawley rats (Taconic Farms, Germantown, NY; 150-200 g) were used for all experiments and were allowed food and water *ad libitum* prior to the experiments.

Preparation of Kidney slices. Animals were killed by cervical dislocation . The kidneys were removed and placed in ice-cold saline. Slices were prepared by carefully cutting the kidney with a series of single sided razor blades fixed in series by a clamp. Slices were precision cut to a uniform thickness of 0.4-0.5 mm and placed in phosphate buffered medium (pH 7.4) on ice until a sufficient number for experimentation were prepared.

Synthesis of 2-Bromohydroquinone ^{35}S-GSH conjugates. GSH conjugates of 2-BrHQ were synthesized according to our established method (15) with the following modification. 2-Bromoquinone was synthesized by mixing 1 g 2-BrHQ and 1.8 g silver oxide in 250 ml methylene chloride with stirring at room temperature (25°C) for 4 hr. The mixture was filtered under vacuum and the majority of the solvent was removed by rotor-evaporation under vacuum. The remaining solvent was evaporated to dryness under nitrogen. 2-Bromoquinone (10 mM in 0.5 ml methanol) was added dropwise to 50 mM ^{35}[S]-GSH (4400 dpm/nmole) in 10 ml 100 mM Tris-HCl buffer (pH 7.4) and the content was stirred for 2 hrs. Aliquots of the mixture of 2-Br-([^{35}S]-GSyl)HQ conjugates were injected onto a Partisil M9 10/25 ODS 3 reverse-phase semipreparative column to isolate individual isomers. The column was eluted with water/methanol /acetic acid (90:9:1; v/v/v) at a flow rate of 3 ml/min. HPLC was performed with a model 6000A chromatograph (Water Associates) equipped with a UV absorbance detector (254 nm) and Waters model U6K injector. Individual peaks corresponding to the synthesized reference compounds (15) were collected from several injections of the mixture of 2-Br-([^{35}S]-GSyl)HQ conjugates. The methanol was then evaporated under nitrogen and the remaining aqueous fractions frozen in dry ice/acetone and lyophilized. This procedure yielded ^{35}S-labelled 2-Br-(diGSyl)HQ, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ and 2-Br-6-(GSyl)HQ.

Organic ion accumulation. The ability of kidney slices to accumulate organic ions was studied by the method of Smith *et al.* (16) under established conditions. Briefly, renal slices (100-200 mg)

were incubated in 4 ml of 7.4 mM phosphate buffered medium (pH 7.4) containing 96.7 mM NaCl, 40 mM KCl , 0.74 mM $CaCl_2$, 10 mM lactate and 74 µM PAH or 10 µM TEA (2 mCi/20 ml ^{14}C-TEA). The mixtures were incubated at 25°C for 90 minutes under a 100 % oxygen atmosphere and agitated at 100 cycles/min. The pH remained constant throughout the time of incubation. At the end of the incubation, the kidney slices were removed and gently dried on filter paper. The slices were weighed and transferred into 15 ml conical tubes containing 3 ml of 10% trichloroacetic acid (TCA), homogenized and the volume adjusted to 10 ml with distilled H_2O. The mixtures were then centrifuged at 2000 rpm for 10 minutes. For the determination of radioactivity remaining in the incubation medium, 1 ml of the medium was added to 4 ml of 3.75% TCA and the mixture vortexed. For the determination of TEA uptake, 1 ml aliquots from both the TCA treated kidney slices and the TCA treated incubation medium were removed and radioactivity determined by liquid scintillation spectroscopy. For the determination of PAH uptake 2 ml aliquots of supernatant from the TCA treated kidney slices and 1 ml aliquots from the TCA treated incubation medium were assayed using the method of Smith *et al.* (17). PAH or TEA accumulation was expressed as the slice to medium (S/M) ratio. In some experiments, kidney slices were pretreated with various concentrations (0.25-1.0 mM) of a mixture of 2-Br-(GSyl)HQ isomers for varying periods of time (0-100 minutes), after which the slices were removed and quickly rinsed with fresh incubation medium. Organic ion accumulation into these pretreated kidney slices was determined as described above.

Uptake of 2-Br-([^{35}S]-GSyl)HQ conjugates into rat kidney slices. Kidney slices were incubated in 1 ml of 7.4 mM phosphate buffered medium at pH7.4 containing 96.7 mM NaCl, 40 mM KCl, 10mM lactate and 0.74 mM $CaCl_2$. The incubation was initiated by the addition of 100 µM of the various 2-Br-(GSyl)HQ conjugates and the contents were incubated at 25°C under 100% oxygen. The incubates were agitated at 100 cycles/min and at various times 3 kidney slices were removed and rinsed twice in ice-cold saline. The tissues were then dried gently on filter papers, weighed and transferred to tubes containing 2 ml of 1N NaOH, homogenized using a polytron and the contents heated at 80°C for 20 minutes or until all the protein was solubilized. A 1 ml aliquot of the alkaline protein solution was removed for radioactivity determination and an aliquot used for protein determination (Bio-Rad protein Assay; Bio-Rad Laboratories. Richmond, Calif.). For covalent binding studies, experiments were carried out as described above and at various time points, slices were removed and transferred to tubes containing 2 ml of 10% TCA and the tissues homogenized with a polytron. The protein precipitates were then washed with 3 ml 10% TCA followed by exhaustive washing with ether/methanol (80:20, v/v) until no significant radioactivity was detected in the washes. The pellets were then dissolved in 2 ml of 1N NaOH, a 1 ml aliquot used for radioactivity determination and a 1 ml aliquot for protein determination. In some experiments, slices were preincubated with AT-125 (0.5 mM) for 20 minutes prior to the addition of the various 2-Br-(GSyl)HQ conjugates.

γ-Glutamyl transpeptidase activity. GGT activity was assayed with a Sigma Kit 545. The amount of tissue used (both homogenates and slices) and the incubation times were adjusted so that the generation of the end product, p-nitroaniline, was within the standard curve. For the determination of GGT in kidney slices, slices were incubated with the GGT substrate γ-glutamyl-p-nitroaniline. An aliquot of the incubation medium was removed, and p-nitroaniline production assayed. The kidney slices were then dissolved in 1 ml 1N NaOH and an aliquot used for protein determination. For the determination of GGT in kidney slice homogenates, the kidney slices were homogenized in 0.05M phosphate buffer, pH 7.4 (1:10; w/v). This homogenate was further diluted by 40 fold and a 10 µl aliquot of this dilution was used to assay GGT activity. GGT activity is expressed as U/mg protein.

RESULTS

In Vitro Accumulation of 2-Bromohydroquinone-Glutathione Conjugates into Kidney Slices. In order to demonstrate the viability and functional integrity of the rat kidney slices prepared for these experiments, their ability to accumulate both organic anions (PAH) and organic cations (TEA) was determined. The high slice to medium ratio of these preparations indicate the functional integrity of the slices. The effect of 2-Br-(GSyl)HQ conjugates on both PAH and TEA accumulation by rat renal slices is shown in figure 1. The 2-Br-(GSyl)HQ conjugates caused a preferential inhibition of PAH uptake and TEA uptake was essentially unaffected. Maximum inhibition of both PAH (65%) and TEA (12%) uptake occurred at a concentration of 0.5mM 2-Br-(GSyl)HQ. The inhibition of PAH uptake further confirms the metabolic capability of these

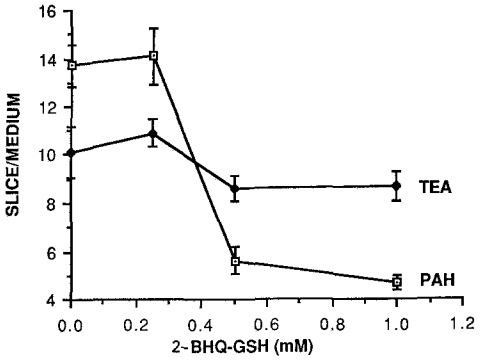

Figure 1. Effect of 2-Br-(GSyl)HQ conjugates on PAH and TEA accumulation by rat renal slices.

renal slices. The time course of accumulation of the isomeric 2-Br([^{35}S]-GSyl)HQ conjugates into kidney slices is illustrated in figure 2. Thus the initial rate of uptake of 2-Br-(diGSyl)HQ, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ and 2-Br-6-(GSyl)HQ by kidney slices was 2.4 ± 0.2, 1.2 ± 0.1, 1.0 ± 0.1 and 0.3 ± 0.03 nmoles/mg protein/10 min respectively. The initial rate of uptake of the most potent nephrotoxicant, 2-Br-(diGSyl)HQ was therefore at least 2 fold faster than either of the three mono-substituted GSH conjugates. In addition, the extent of 2-Br-(diGSyl)HQ uptake was greater than either of the three mono-substituted isomers.

γ-Glutamyl Transpeptidase Activity in Kidney Slices. GGT activity was measured in both kidney slices and in subsequently homogenized slices (Table 1). Enzyme activity in intact kidney slices accounted for about 15% of that assayed in subsequently homogenized kidney slices.

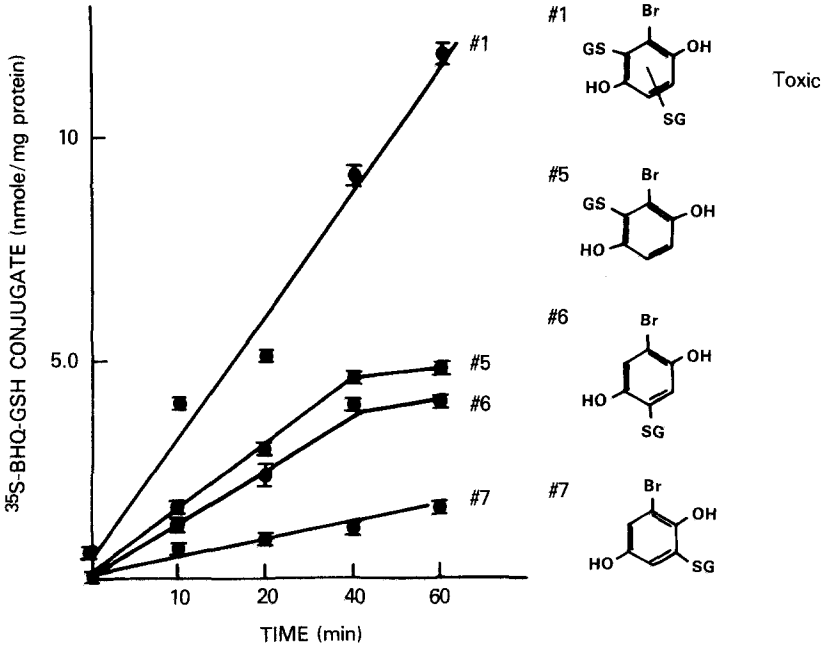

Figure 2. Uptake of isomeric 2-Br-([^{35}S]GSyl)HQ conjugates into kidney slices.

Table 1

The Effect of AT-125 on γ-Glutamyl transpeptidase Activity in Rat Kidney Slices and Homogenate

Tissue preparation	γ-Glutamyl transpeptidase (unit/μg protein)	
	-AT-125	+AT-125 (0.5mM)
Kidney slice	0.55 ± 0.06	0.29 ± 0.05[a] (47%)
Kidney slice homogenate	3.79 ± 0.48	0.29 ± 0.01[b] (92%)

The figures represent the mean ± S.D. of triplicate incubations. Value in parentheses represent the per cent inhibition. Values were statistically significantly different when compared to incubations in the absence of AT-125 at the following confidence levels [a]=$p<0.05$, [b]=$p<0.01$.

AT-125 (0.5mM), an inhibitor of several glutamine utilizing enzymes (18) including GGT (19-21) inhibited GGT in intact and homogenized kidney slices by 47% and 92%, respectively. In addition, AT-125 decreased the accumulation of 2-Br-(diGSyl)HQ, 2-Br-3-(GSyl)HQ, 2-Br-5-(GSyl)HQ and 2-Br-6-(GSyl)HQ by 49%, 21%, 25% and 30%, respectively (Table 2). The covalent binding of the 2-Br-([^{35}S]-GSyl)HQ conjugates to kidney slice protein was also inhibited by pretreatment with AT-125 (Table 2).

DISCUSSION

The target organ and site-specific nephrotoxicity of a variety of GSH/cysteine S-conjugates has primarily been attributed to biochemical factors responsible for the bioactivation of these compounds to toxic metabolites. In contrast, little attention has been paid to the physiological function of the

Table 2

The Effect of AT-125 on ^{35}S-2-Br(GSyl)HQ-GSH Conjugates Uptake and Covalent Binding into Kidney Slices

2-BHQ-GSH Conjugates	Uptake(nmol/mg/60min)		Covalent Binding(nmol/mg/60min)	
	-AT-125	+AT-125	-AT-125	+AT-125
2-Br-(diGSyl)HQ	7.5 ± 0.5	3.8 ± 0.3[a] (49)	6.5 ± 0.7	3.6 ± 0.2[b] (45)
2-Br-3-(GSyl)HQ	2.8 ± 0.4	2.2 ± 0.2 (21)	2.1 ± 0.3	1.8 ± 0.2 (14)
2-Br-5-(GSyl)HQ	2.4 ± 0.3	1.8 ± 0.2 (25)	2.1 ± 0.3	1.5 ± 0.1 (29)
2-Br-6-(GSyl)HQ	1.0 ± 0.2	0.7 ± 0.1 (30)	0.7 ± 0.1	0.5 ± 0.1 (29)

Kidney slices were incubated with 100 μM of various 2-Br-(GSyl)HQ conjugates for 60 minutes at 25°C with and without preincubation of AT-125 (0.5 mM). Accumulation or covalent binding of the radiolabelled compounds was determined as described under "Methods". The figures represent the mean ± S.D. of triplicate incubations. Values in parentheses represent % inhibition in the presence of AT-125. Value was statistically significantly different when compared to incubations in the absence of AT-125 at the following confidence levels of [a]=$p<0.01$ and [b]=$p<0.02$.

kidney in predisposing this organ to S-conjugate mediated toxicity. The present data demonstrate that the rate of uptake of 2-Br-(diGSyl)HQ into kidney slices was between 2 to 7 fold greater than that of the 2-Br-(monoGSyl)HQ conjugates (figure 2). We have shown that 2-Br-(diGSyl)HQ is a more potent nephrotoxicant than either of the three mono-substituted isomers (14). Thus, the more extensive renal uptake of the disubstituted conjugate may be partially responsible for its enhanced nephrotoxicity.

The accumulation of 2-Br-(GSyl)HQ into renal slices cannot be diffusion driven since it is well established that GSH and GSH conjugates do not passively cross biological membranes. One mechanism for the delivery of GSH and GSH S-conjugates to the kidneys is glomerular filtration. However, while 70% of the GSH in plasma that enters the kidney is removed, only 25% of this is removed by the glomeruli. The fraction that enters the tubular lumen is hydrolyzed by GGT and cysteinylglycine dipeptidase. AT-125 is an inhibitor of several glutamine utilizing enzymes (18) including GGT (19-21) and it has been used to probe the role of GGT in the nephrotoxicity of a number of GSH conjugates. In the present study, the accumulation of the isomeric 2-Br-([^{35}S]-GSyl)HQ conjugates into kidney slices was inhibited by AT-125 (Table 2) which suggests that their accumulation may in part be mediated by GGT. These data are also consistent with previous observations in which it was demonstrated that AT-125 protected against the *in vivo* nephrotoxicity of both 2-BrHQ (15) and 2-Br-(diGSyl)HQ (14). AT-125 pretreatment has also been shown to protect against S-(1,2-dichlorovinyl)GSH (DCVG)-mediated elevations in urine glucose excretion (2). In contrast, AT-125 failed to protect against S-(2-chloroethyl)GSH induced nephrotoxicity (22).

GGT present on the plasma membrane side of the proximal tubular cells (basal-lateral membrane) is more susceptible to inhibition by AT-125 than that within the brush border membrane (lumenal side) (23,24). In the present studies AT-125 inhibited GGT in kidney slices by 50% and in subsequently homogenized slices by 92% (Table 1). Preparation of kidney slices results in collapse of the brush border membrane which requires the hydrostatic pressure of tubular fluid to maintain its rigidity. Thus, GGT within the brush border membrane is essentially inaccessible to substrate in kidney slice preparations and the majority of the activity measured in such slices is probably due to the enzyme within the basal-lateral membrane. Homogenization of the kidney slices results in ready accessibility of the substrate to GGT. Consequently, GGT activity in kidney slices represents only 15% of that in the subsequently homogenized slice. Interestingly, while GGT is inhibited 50%, the uptake of 2-Br-(diGSyl) is similarly inhibited by 49% (Table 2). However, under similar conditions, uptake of the three mono-substituted 2-Br-([^{35}S]-GSyl)HQ conjugates into kidney slices is inhibited by only 21-30% (Table 2). This may indicate that different transport mechanisms contribute differentially to the transport of di- and mono-substituted GSH conjugates. Indeed, it is becoming increasingly apparent that multiple transport processes may exist for the proximal tubular accumulation of GSH S-conjugates (1). The proportion of 2-Br-(GSyl)HQ accumulated by renal slices as the intact conjugates or via absorption of the corresponding cysteine conjugates subsequent to metabolism by GGT is not known and is currently under investigation.

The residual GGT activity in kidney slices in the presence of AT-125 may be sufficient to account for the substantial uptake of 2-Br-(GSyl)HQ conjugates. Alternatively, a significant amount of circulating GSH is also removed by a non-filtration mechanism (9,10) which involves transport

into renal cells across the basal-lateral membrane (11-13). Lash and Jones (13) have characterized an electrogenic, sodium-coupled and probenecid-sensitive transport system for the uptake of intact GSH into renal basal-lateral membrane vesicles. A similar mechanism for the delivery of GSH S-conjugates to the kidneys, in addition to glomerular filtration, may also be operating. For example, GSH and GSH S-conjugates undergo a similar pattern of interorgan metabolism (2,25,26) and the same enzymes are responsible for the conversion of both GSH and GSH S-conjugates to cysteine and cysteine S-conjugates respectively (27). In addition, the sodium-coupled, probenecid sensitive basal-lateral membrane GSH transport system exhibits a broad substrate specificity for γ-glutamyl compounds (13). Thus, the AT-125 insensitive component of 2-Br-(GSyl)HQ uptake into renal slices may be due to the system described by Lash and Jones (13) for the uptake of intact GSH into renal basal-lateral membrane vesicles. For example, the transport of DCVG has been investigated in renal basal-lateral membrane vesicles (28) and vesicular uptake was demonstrated to be both sodium-dependent and to be inhibited by probenecid. Whether a similar sodium-dependent transport system is responsible for 2-Br-(GSyl)HQ uptake into renal slices is not known but it should be noted that pretreatment of rats *in vivo* with probenecid failed to protect them against 2-Br-(diGSyl)HQ mediated elevations in BUN and against increases in the urinary excretion of LDH, protein and glucose (14). However, since GGT is also found in the basal-lateral membrane of kidney epithelia (29), the relative contribution of sodium-coupled and GGT mediated GSH transport to total renal plasma GSH clearance is debatable. In this respect, *in vivo* evidence suggests that the ability of the kidney to utilize GSH is due to GSH breakdown mediated by GGT both in the tubule and basal-laterally (30,31). In either case, it is clear that basal-lateral mediated uptake accounts for the ability of the kidney to extract most of the GSH passing through the renal circulation.

Isolated kidney slices also possess the necessary enzyme(s) for the activation of 2-Br-(GSyl)HQ conjugates to reactive metabolites that covalently bind to tissue macromolecules (Table 2). Interestingly, the most potent nephrotoxicant, 2-Br-(diGSyl)HQ, was the most extensively covalently bound of the different isomers studied. Thus, in addition to the more extensive uptake of 2-Br-(diGSyl)HQ into renal slices additional biochemical factors may also be responsible for its potent nephrotoxicity.

In conclusion, we have demonstrated that the more potent nephrotoxicity exhibited by 2-Br-(diGSyl)HQ as compared to three mono-GSH substituted isomers may, in part, be due to its more extensive renal uptake. In this respect, GGT mediated transport may play an important role in the concentration of nephrotoxic GSH conjugates in proximal tubular cells. Thus, the physiological function of the kidney, in addition to its biochemical profile might also predispose this organ to the toxicity of various sulphur conjugates (1).

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