The acetaminophen metabolite N-acetyl-p-benzoquinone imine (NAPQI) inhibits glutathione synthetase *in vitro*; a clue to the mechanism of 5-oxoprolinuric acidosis?

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Abbreviations: NAPQI, N-acetyl-p-benzoquinone imine; GS, glutathione synthetase; γ-GCS, γ-glutamylcysteine synthetase; γ-GCT, γ-glutamyl cyclotransferase; γ-GACT γ-glutamylamine cyclotransferase; GAB, glutamyl-L-α-aminobutyric acid; APAP-Cys, acetaminophen-cysteine

Abstract

1. Metabolic acidosis due to accumulation of L-5-oxoproline is a rare, poorly understood, disorder associated with acetaminophen treatment in malnourished patients with chronic morbidity. L-5-oxoprolinuria signals abnormal functioning of the γ -glutamyl cycle which recycles and synthesises glutathione. Inhibition of glutathione synthetase (GS) by N-acetyl-p-benzoquinone imine (NAPQI) could contribute to 5-oxoprolinuric acidosis in such patients. We investigated the interaction of NAPQI with GS *in vitro*.
2. Peptide mapping of co-incubated NAPQI and GS using mass spectrometry demonstrated binding of NAPQI with cysteine-422 of GS which is known to be essential for GS activity. Computational docking shows that NAPQI is properly positioned for covalent bonding with cysteine-422 via Michael addition and hence supports adduct formation.
3. Co-incubation of 0.77 μ M of GS with NAPQI (25 to 400 μ M) decreased enzyme activity by 16% to 89%. Inhibition correlated strongly with the concentration of NAPQI and was irreversible.
4. NAPQI binds covalently to GS causing irreversible enzyme inhibition *in vitro*. This is an important novel biochemical observation. It is the first indication that NAPQI may inhibit glutathione synthesis, which is pivotal in NAPQI detoxification. Further studies are required to investigate its biological significance and its role in 5-oxoprolinuric acidosis.

Introduction

High anion gap metabolic acidosis (HAGMA) due to accumulation of L-5-oxoproline (pyroglutamic acid) is a rare, serious, biochemical disturbance which has been attributed to treatment with acetaminophen. It develops acutely in patients receiving regular treatment with the drug, generally in therapeutic doses and with non-toxic plasma concentrations. The disturbance usually resolves within days of acetaminophen withdrawal and seldom recurs. Among 50 patients reported to 2014 (Abkur et al., 2014; Brohan et al., 2014; Liss et al., 2013; Pitt and Hauser, 1998) the majority were poorly nourished, had one or more chronic morbidities requiring pain relief, and often on-going sepsis. Some were alcohol abusers, had renal impairment, and/or post-operative infection and three were pregnant. The median age was 54 y (range 1-84 y) and 84 % were women. None had a reported relevant family history. The diagnosis is made by analysis of organic acids in blood and/or urine by specialist paediatric metabolic laboratories. Because these tests are not routinely available, and few adult clinicians are aware of them, the condition has almost certainly been under-diagnosed (Emmett, 2014; Liss et al., 2013; Pitt and Hauser, 1998). 5-Oxoprolinuria was only introduced into diagnostic mnemonics for HAGMA in 2008 (Mehta et al., 2008).

Evidence supporting an association with acetaminophen is the observation that rats treated chronically with 1 mmol of acetaminophen daily develop progressive 5-oxoprolinuria after three weeks (Ghuri et al., 1993). Further, human hepatocytes transfected with cytochrome P450 2E1 produce large amounts of 5-oxoproline when exposed to acetaminophen (Geenen et al., 2011; Geenen et al., 2013). However, the mechanisms leading to 5-oxoprolinuria are poorly understood.

L-5-oxoproline is generated endogenously by the action of γ -glutamyl cyclotransferase (γ -GCT) on γ -glutamyl amino acids, and of γ -glutamylamine cyclotransferase (γ -GACT) on γ -glutamyl amines. These include γ -glutamyl- ϵ -lysine released during degradation of fibrin and other proteins cross-linked by transglutaminase (Oakley et al., 2010; Williamson and Meister, 1982). In addition, it is a by-product of glutathione synthesis and is also released from modified terminal amino acids of peptides and proteins. 5-

Oxoproline is hydrolysed to L-glutamate by 5-oxoprolinase. γ -GCT, γ -GACT and 5-oxoprolinase are constituents of the γ -glutamyl cycle (Larsson and Anderson, 2005) which co-ordinates the activities of the enzymes involved in glutathione catabolism and the two required for glutathione synthesis (glutathione synthetase (GS) and γ -glutamylcysteine synthetase (γ -GCS; γ -glutamylcysteine ligase). γ -GCS is rate-limiting for the cycle and is regulated by glutathione through feedback inhibition (Figure 1a).

Figure 1. 5-Oxoproline metabolism via the γ -glutamyl cycle (a) with normal GS activity; (b) with genetic GS deficiency. γ -GCS = γ -glutamylcysteine synthetase; GS = glutathione synthetase; γ -GCT = γ -glutamyl cyclotransferase; γ -GACT= γ -glutamylamine cyclotransferase.

Figure 1a

Figure 1b

Significant accumulation of L-5-oxoproline occurs rarely and indicates a mismatch between L-5-oxoproline production and its degradation. This occurs in two very rare inherited enzyme disorders of the γ -glutamyl cycle with low activities of either GS or 5-oxoprolinase (Njålsson et al., 2005; Ristoff and Larsson, 2007). In GS deficiency, intracellular glutathione is reduced to 5% to 21%. Inhibition of γ -GCS is lifted, and excessive amounts of γ -glutamylcysteine are synthesised and diverted to 5-oxoproline, overwhelming the capacity of 5-oxoprolinase (Figure 1b). Individuals with moderate or severe forms of the disorder present neonatally or in early infancy with metabolic acidosis and haemolytic anaemia (Njålsson et al., 2005). In 5-oxoprolinase deficiency, there is massive L-5-oxoprolinuria, but no acidosis, glutathione deficiency or haemolysis (Larsson and Anderson, 2005; Ristoff and Larsson, 2007). A significant proportion of the L-5-oxoproline in this disorder probably derives from renal metabolism.

There is no published evidence that any of the reported patients with 5-oxoprolinuric HAGMA had either of these inherited defects. However, they are likely to have had low glutathione reserves because of their chronic morbidity, and hence increased γ -glutamylcysteine production. Glutathione depletion also reduces the capacity to detoxify N-acetyl-p-benzoquinone imine (NAPQI), the toxic metabolite of acetaminophen (Bessems and Vermeulen, 2001; Cohen and Khairallah, 1997; Mitchell et al., 1973). Pitt & Hauser (1998) suggested that in such patients GS becomes rate limiting or is inhibited, leading to γ -glutamylcysteine accumulation.

NAPQI is a highly reactive electrophile which forms adducts selectively with liver proteins, most often by reaction with cysteine residues (Bessems and Vermeulen, 2001; Chen et al., 1999; Cohen and Khairallah, 1997). Twenty nine proteins which bind NAPQI have been identified (Dietze et al., 1997; Qiu et al., 1998). In the majority, the functional effects of binding were not investigated. However, NAPQI binding has been associated with reduced activity of seven hepatic enzymes (glutamine synthetase (Bulera et al., 1995; Gupta et al., 1997), glutamate dehydrogenase (Halmes et al., 1996), mitochondrial aldehyde dehydrogenase (Landin et al., 1996), glyceraldehyde-3-phosphate dehydrogenase (Dietze et al., 1997), N-

10-formyl-tetrahydrofolate dehydrogenase (Pumford et al., 1997), carbamyl phosphate synthetase-1 (Gupta et al., 1997), and glutathione S-transferase pi (Jenkins et al., 2008). Although not previously reported to form a NAPQI adduct, human GS has three cysteine residues C294, C409 and C422 (Gali and Board, 1995) and is a potential candidate for NAPQI binding. C422 is essential for enzyme activity (Gali and Board, 1997).

We investigated the interaction of NAPQI with GS *in vitro* in order to ascertain whether inhibition of GS could be a tenable explanation for acetaminophen associated 5-oxoprolinuric HAGMA.

Materials and methods

Chemicals

N-terminal His-tagged recombinant human GS (hGS) expressed in *E. coli* (purity > 95% by SDS-PAGE) used in the NAPQI/GS binding studies was from Novus Biologicals Ltd. (Cambridge, UK). His-tagged recombinant hGS used in the enzyme kinetic studies was expressed in *E. coli* and purified as reported previously (~105 kDa; purity > 99% by SDS-PAGE (Dinescu et al., 2004)). L-γ-glutamyl-L-α-aminobutyric acid (GAB) > 90% pure was custom-made by Peptide Protein Research Ltd. (Fareham, UK). NAPQI was from Dalta Pharma Services (Toronto, Canada). Phospho(enol) pyruvic acid monosodium salt (97% pure), lactate dehydrogenase from rabbit muscle type II, pyruvate kinase from rabbit muscle type III, adenosine 5'-triphosphate disodium salt hydrate, reduced nicotinamide adenine dinucleotide disodium salt, Trisma hydrochloride (Tris-HCl) and all other chemical reagents were from Sigma-Aldrich (Poole, UK).

NAPQI/GS binding studies

A previously published procedure (Parkinson et al., 2014) was modified to analyse NAPQI/GS adducts. From preliminary studies, 5 µg (0.048 nmol) of enzyme was the smallest amount of enzyme needed to produce informative spectra of the modified peptide. 5 µL of GS (1 g/L in 100 mM ammonium bicarbonate pH 8.0)

was added to 5-50 μL of NAPQI (1 g/L in 5% acetonitrile/ammonium bicarbonate) and incubated at 37°C for 1 h. The reaction mixture was lyophilised using a centrifugal evaporator, and resuspended in 15 μL of NuPAGE LDS sample buffer without dithiothreitol (Novex; Life Technologies, Paisley, UK). The enzyme was separated by electrophoresis at 200 mV for approximately 45 min using a Nu-Page 4-12% Bis-Tris gel (Novex; Life Technologies) with unmodified GS as a control. Following staining with Coomassie Blue, the protein band was excised and *in-gel* digestion undertaken as described by Shevchenko et al. (1996). Peptide extracts containing 500-1000 ng of peptide were resuspended in solvent A (0.1% formic acid in water (v/v)), loaded onto a reversed-phase trap column (Xbridge BEH C_{18} NanoEase column, 5 μm particle size, 300 μm x 50 mm (Waters; Elstree, UK) at a trapping flow rate of 5 $\mu\text{L}/\text{min}$, and washed for 10 min with solvent A prior to analytical separation using a C_{18} reversed-phase column (BEH C_{18} , 1.7 μm particle size, 75 μm x 150 mm, Waters). The peptides were eluted over a 37 min continuous gradient from 1% acetonitrile/0.1% formic acid up to 80% solvent B (acetonitrile/0.1% formic acid), at a flow rate of 300 nL/min. The eluates were sprayed directly into a G2-S Synapt Q-ToF mass spectrometer (Waters, Manchester, UK) fitted with a nano-lockspray source and operating using the data independent mode of acquisition, MS^E . Data were acquired from $m/z = 50$ -1990 using alternate low and elevated collision energy (CE) scans with the low collision energy set to 5 V and an elevated collisional energy ramp from 15-40 V. The lock mass Glu-fibrinopeptide, $m/z = 785.8426$ ($\text{M}+2\text{H}$) $^{2+}$ was infused at a concentration of 100 fmol/ μL with a flow rate of 300 nL/min and data acquired every 60 s. The raw mass spectra were processed using ProteinLynx Global Server Version 2.4 (Waters) and the processed data were used to generate reduced charge state and deisotoped precursor and associated product ion peak lists. These peak lists were searched against the human database (obtained from UniProt 01/2013). A maximum of one missed cleavage was allowed for tryptic digestion and the variable modification was set to contain oxidation of methionine, carboxyamidomethylation of cysteine, deamidation of glutamine and asparagine and NAPQI modification of cysteine. Searches were performed with a false positive rate set at 2%.

Analysis of GS activity

hGS was reconstituted in 10 mM Tris-HCl buffer pH 8.2 (protein concentration 162 mg/L) and stored at 4 °C. GS activity was measured at 37 °C using a published spectrophotometric procedure (Dinescu et al., 2004) adapted for use on a Konelab 20 spectrophotometric analyser (Labmedics; Manchester, UK). This procedure couples ADP production from ATP during the GS reaction to NADH oxidation by a reporter enzyme system [phospho(enol) pyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH) and reduced NAD (NADH)]. Oxidation of NADH was monitored at 340 nm. Because the natural enzyme substrate γ -glutamylcysteine would conjugate with NAPQI, the synthetic dipeptide GAB was used as an alternative. GS activity is the same with both substrates (Oppenheimer et al., 1979). In brief, for a final volume of 200 μ L per test sample, 10 μ L of GS, GS/NAPQI co-incubate or water was added to a pre-warmed mixture of assay buffer (100 mM Tris-Cl buffer pH 8.2, 50 mM KCl, 20 mM MgCl_2), 0.34 mM NADH_2 , 10 mM glycine, 10 mM GAB, 10 mM ATP, 5 mM PEP, 10 units of PK, and 4 units of LDH. Absorbance at 340 nm was measured at 28 s intervals for 168 s (7 recordings). One unit of GS activity was defined as the amount of GS that catalysed 1 μ mol of product formation/min (i.e. NADH oxidized/min) at 37°C.

NAPQI/GS co-incubation

Five mg of NAPQI in 672 μ L acetonitrile (50 mM NAPQI) was diluted freshly in water to concentrations of 50-2000 μ M. The solutions were kept on water ice, protected from light. Equal volumes of diluted NAPQI and GS (130 mg/L in preliminary experiments and 162 mg/L subsequently), were mixed, co-incubated at 37 °C for 28 min, cooled rapidly in water ice and transferred to the spectrophotometer to be sampled. The enzyme reaction was started exactly 30 min after the commencement of co-incubation.

Effect of NAPQI on the coupled reporter system used to measure GS activity

Possible inhibition/inactivation of one or more components of the reporter pathway was investigated in two experiments by monitoring the change in absorbance at 340 nm after adding ADP as substrate to the reporter system in the presence or absence of NAPQI.

Experiment 1: Ten μL of water (control) or 10 μL of 200 μM NAPQI (test) was equilibrated at 37 °C with the routine assay mixture containing all the components of the reporter system but without addition of GAB, ATP or GS. After recording baseline absorbance, 10 μL of water (control) or 0.84 mM ADP was added to start the enzyme reaction sequence. The absorbance was recorded again at 1 min.

Experiment 2: The effects of NAPQI on the activity of the full reporter system (PK, LDH and NADH) and on NADH alone were investigated. By comparison, it was possible to differentiate effects of NAPQI on NADH from those on PK + LDH. Ten μL of water, 200 μM or 400 μM of NAPQI were equilibrated at 37 °C for 3 min with the routine assay mix containing all the reporter system components (PEP, PK, LDH and NADH) but without GAB, ATP and GS (series a), or containing only PEP and NADH (series b). After recording baseline absorbance, 10 μL of 0.84 mM ADP or water was added to start the reaction and the absorbance was recorded again at 1 min.

The possibility that doubling the amounts of PK and LDH in the standard GS assay might restore activity of GS co-incubated with NAPQI was also investigated. After pre-incubating water or NAPQI (200 μM) with GS for 30 min at 37 °C, 10 μL was transferred to the standard assay mixture, but with addition of normal or twice the normal amounts of PK or LDH. GS activity was measured by the standard procedure.

Protein concentration

GS protein was analysed with an automated pyrogallol red/molybdate procedure calibrated with human serum albumin (Beckman Coulter Inc., High Wycombe, UK).

Computations

Kinetic data were recorded and plotted in Excel (Microsoft Office Word, 2007). K_m and V_{max} were calculated using non-linear regression (curve fit) enzyme kinetics programs of Graph Pad Prism 5 version 5.04 (GraphPad; La Jolla, CA, USA). Graph Pad Prism was used for statistical analyses. Statistical significance was < 0.05 .

Computer modeling of NAPQI interaction with hGS

Utilizing the crystal structure of hGS (PDB ID = HGS) as a starting structure, a molecular dynamics (MD) simulation of free dimeric wild-type hGS (Polekhina et al., 1999) was conducted. GROMACS was used for the 10 ns run with the AMBER99sb force field and the simple point charge water model, as described in previous work (Berendsen et al., 1981; Cornell et al., 1995; De Jesus et al., 2014; Hess et al, 2008; Hornak et al., 2006; Wang et al., 2000). The lowest energy frame was then extracted from the last ns of the simulation for further analysis. The structure of NAPQI was optimised in Gaussian '09 with the B3LYP functional and 6-31+G(d) basis set (Andersson and Uvdal, 2005; Becke, 1988; Becke, 1993; Stephens et al., 1994). The Molecular Operating Environment (MOE) 2013.08 software (Chemical Computing Group Inc.; Montreal, Canada, 2014) was then used to dock NAPQI near C409 and C422 with an AMBER99 force field (Cornell et al., 1995; Molecular Operating Environment, 2014; Wang et al., 2000). The induced fit docking method was coupled with the London and GBVI/WSA scoring functions (Corbeil et al., 2012; Labute, 2008).

Results

Binding of NAPQI to GS

NAPQI binding studies confidently identified a single modified peptide (419 to 434). This 16 amino acid sequence (VVQCISELGIFGVYVR) had a molecular mass (1989 Da) consistent with deamidation at position 421, carboamidomethylation and modification by NAPQI (Appendix 1, Figure 5, a to d). Fragmentation mass spectra were unable to isolate the exact amino site of modification, but could pinpoint the modifications to

within the amino acids 421-423 (Gln, Cys, Ile). In light of the excess iodoacetamide used to block the remaining cysteine residues after reaction and removal of excess NAPQI, it is probable that the deamidated glutamine residue (421) was carboamidomethylated and Cys 422 modified by NAPQI. The modified peptide was observed in two independent experiments, but not in control samples incubated in the absence of NAPQI. The analytical procedure provided good coverage of the GS protein, with > 80% of the peptides consistently matched correctly with the primary amino acid sequence of the protein. Peptide modification was evident with mixtures of 5 μg (0.048 nmol) of GS and 5 μg (33.6 nmol) of NAPQI. However, the mass spectrometer signals were more intense with a higher NAPQI concentration and spectral quality was improved at 15 μg .

Activity of GS

Activity of GS without NAPQI

GS is an allosteric enzyme with two identical subunits which show negative cooperativity to the γ -glutamyl substrate: the affinity of GS for this substrate falls with increased availability of γ - glutamyl substrate (Dinescu et al., 2004; Luo et al., 2000; Njalsson et al., 2001; Oppenheimer et al., 1979). Because we worked with high GAB substrate concentrations, we only observed activity at the high K_m : double reciprocal plots of substrate concentration versus enzyme activity were linear. From two experiments, with GAB concentrations ranging from 0.625 mM to 10 mM the calculated K_m (95% confidence intervals, CI) was 1.12 (0.95, 1.30) mM and 1.22 (0.89, 1.55) mM, V_{max} was 7.75 (7.41, 8.09) and 7.89 (7.26, 8.52) $\mu\text{mol/mg/min}$ and k_{cat} was 13.6 (13.0, 14.2) and 13.8 (12.7, 14.9) s^{-1} . Others, who extended their observations to lower, more physiological, substrate concentrations (< 0.4 mM) found non-linear plots indicative of allosteric activity, and estimated separate approximate K_m values for the active sites of the enzyme (Luo et al., 2000; Njalsson et al., 2001; Oppenheimer et al., 1979). Our K_m is higher than estimates for the initial K_m for a comparable GS preparation and assay, but similar to K_m estimates for the second binding site ($K_{m2} = 1.50$ mM; Dinescu et al., 2004), and to a reported value of 0.99 ± 0.07 mM reported for GS purified from

human blood haemolysates (Njålsson et al., 2000). K_{cat} was higher than a value of 6.5 s^{-1} reported for a similar GS preparation analysed with 20 mM GAB as substrate at pH 8.2 (Dinescu et al., 2004), and closer to a more recently reported value of 19.5 s^{-1} (Slavens et al., 2011). The enzyme was stable for at least 7 days after reconstitution.

Activity of glutathione synthetase co-incubated with NAPQI

NAPQI was pre-incubated in concentrations ranging from 25 to 400 μM with 81 mg/L (0.77 μM ; two experiments) or 65 mg/L (0.62 μM ; one experiment) of GS at for 30 min at 37 °C. Residual enzyme activity was measured with GAB substrate concentrations ranging from 0.625 mM to 10 mM. Figures 2a and 2b show the results for one experiment in which the full range of NAPQI concentrations was investigated at five concentrations of GAB. Similar results were obtained in a second experiment which examined the effects of NAPQI at the lower end of the range (0 to 100 μM) with GAB concentrations of 2.5 mM and 10 mM (Appendix 2, Figures 6a and 6b).

Figure 2. Inhibition of activity of GS (81 mg/L; 0.77 μM) pre-incubated with NAPQI (0-400 μM) for 30 min at 37°C. Enzyme activity was measured by the described procedure, with GAB substrate concentrations ranging from 0.625 mM to 10 mM. NAP = NAPQI; concentrations 0-400 μM .

Figure 2a: GS activity, reported as μmol of NADH oxidized in the reporter reaction/min/mg of GS;

Figure 2b: time course of GS activity after 30 min of preincubation of NAPQI with GS. GS activity was monitored at 28 s intervals for 168 s under standard assay conditions with 10 mM GAB as substrate. Activity reported as μmol of NADH oxidized/mg of GS.

As shown in Figures 2a and 2b, pre-incubation with NAPQI decreased GS activity and the enzyme inhibition was dose-dependent. The residual activity of GS pre-incubated with NAPQI was calculated as a percentage of uninhibited activity (pre-incubation without NAPQI) at each concentration of GAB used in the enzyme assay. The combined results of all three experiments undertaken showed a highly significant inverse correlation between the NAPQI concentration and the residual activity (Spearman correlation coefficient $r = -0.98$ (95% CI - 0.99, - 0.96, $n = 38$, $P < 0.001$). Increasing the concentration of GAB used as substrate in the GS assay did not reverse the enzyme inhibition caused by pre-incubation with NAPQI. In the most comprehensive experiment (Figure 2), the median (range) of residual GS activity for five concentrations of GAB was: 83.2% (82.1-85.1%) with 25 μM NAPQI; 56.0 % (53.7-63.0%) with 100 μM NAPQI; 38.2% (35.4-45.2%) with 200 μM NAPQI and 10.8% (8.0-16.4%) with 400 μM NAPQI. The ratio of μmol of NAPQI to μmol GS in the co-incubates ranged from approximately 30:1 with 25 μM NAPQI to 500:1 with 400 μM NAPQI. Comparison of residual GS activity with different concentrations of NAPQI and GAB using the one-way Anova test confirmed a lack of association between residual enzyme activity and substrate concentration

(Kruskal-Wallis statistic $K_w = 0.97$, $P = 0.965$, $n = 38$, 6 groups; combined results from all three experiments).

These findings indicate that NAPQI inhibits GS irreversibly.

Effect of NAPQI on the coupled reporter system used to measure GS activity

NAPQI is reduced to acetaminophen by NAD(P)H by direct chemical reaction (Dahlin et al., 1984). Oxidation of large amounts of NADH by interaction with NAPQI might deplete NADH in the reporter system and produce spuriously low results for GS activity. This was investigated, as well as the possibility that NAPQI might inhibit PK and LDH used in the coupled reporter system. However, this seemed unlikely since, despite the large abundance of these enzymes in liver, to date they have not been reported to form adducts with NAPQI.

In the first experiment to investigate these issues, there was no difference in the change in absorbance after adding ADP as substrate to the reporter system incubated without NAPQI (-0.195 units/min) or with 10 μ L of 200 μ M NAPQI for 8 min (-0.196 units/min) or 38 min (-0.195 units/min). However, NAPQI reduced the baseline absorbance, prior to adding ADP, by 6% and 5% respectively. It was possible that this was due to oxidation of NADH by NAPQI. This would only be relevant to the the GS analyses if it occurred very rapidly because, in the standard procedure, NADH and the reporter enzymes were not exposed to NAPQI until GS/NAPQI co-incubates were added to the assay mix to start the GS reaction.

The second experiment investigated the effects of exposure to high concentrations of NAPQI for 3 min on the activity of the full reporter system (PEP, PK, LDH and NADH) with ADP as substrate and on NADH without PK and LDH. From our other studies, pre-incubation of GS with NAPQI at these concentrations reduced measured GS activity by around 65% and 89%, respectively. From Table 1 it is clear that NAPQI did not inhibit the reporter reaction under the assay conditions. With the complete reporter system, NAPQI actually increased the rate of fall of absorbance. This was explained by chemical oxidation of NADH by NAPQI (see series (b)) which, however, was insufficient to limit the activity of LDH. In a follow-up

experiment addition of 10 μL of 400 μM NAPQI to NADH in assay buffer without ADP or the reporter enzymes led to an immediate small initial decrease in absorbance at 340 nm (2%), followed by a gradual increase back towards the baseline value.

Table 1. Effects of NAPQI on the reporter enzyme system in the glutathione synthetase assay. Oxidation of NADH with the full reporter system (PEP, NADH, PK, LDH) and the incomplete system (PEP and NADH only) with and without NAPQI was measured by the decrease in absorbance at 340 nm one min after addition of 0.84 mM ADP as substrate; [†]10 μL NAPQI added to 190 μL of assay mix.

NAPQI $\mu\text{mol/L}^{\dagger}$	Full reporter system: PEP, NADH, PK, LDH	Incomplete reporter system: PEP, NADH ; no PK or LDH	Decrease in absorbance due to PK + LDH activity	Decrease in absorbance not due to PK + LDH activity
	Decrease in absorbance (a) (units/min)	Decrease in absorbance (b) (units/min)	(a-b) (units/min)	(b/a x100) (%)
0	-0.197	-0.004	-0.193	2.0%
200	-0.212	-0.021	-0.191	9.9%
400	-0.225	-0.034	-0.191	15.1%

For supportive evidence that NAPQI did not inhibit PK or LDH, the effect of increasing the amounts of PK and LDH in the standard GS assay procedure was investigated. Doubling the amounts of reporter enzymes had no effect on the measured activity of GS pre-incubated with NAPQI. The mean (range) of GS activities ($\mu\text{mol/mg/min}$) of four replicates for each experimental condition were: normal versus double PK/LDH: *without NAPQI*: 6.7 (6.7-7.1) versus 6.7 (6.7-6.7); *with 200 μM NAPQI*: 2.4 (2.0-2.8) versus 2.4 (2.0-2.8).

Collectively, the findings show that under the assay conditions used, NAPQI added in concentrations up to 400 μM did not inhibit PK or LDH. At the higher concentrations it probably oxidised NADH to a small extent but this did not limit the reporter reaction. It might have caused a small spurious increase in measured GS activity, but not a decrease. Thus the assay was valid.

Computer modeling

A preliminary docking study of NAPQI into a low energy structure of free hGS indicated that C422 was a better binding site than C409 (Figure 3). The Michael acceptor of NAPQI (C4') bound 4.3-10.2 Å from the

C409 sulphur. The poses near C409 exhibited a high variation in orientation with several poses on the exterior of the protein, indicating that C409 was not an ideal binding site for NAPQI. In contrast, all poses near C422 nestled deeply into the binding pocket with the Michael acceptor on NAPQI 3.0-3.8 Å from the C422 sulphur.

Figure 3. Docked poses near C409 and C422 show the specificity of the C422 binding site, relative to the non-specific binding near C409.

The binding site near C422 was between the anti-parallel β -sheets at the C terminus of hGS (Fig. 4).

The two dominate binding orientations at C422 had equivalent Michael acceptor positions. The ring of both orientations bound in the same position, but the acetamide side chains pointed in opposite directions. In both poses the ring of NAPQI was centred above the S of C422 with the guanidyl of R418 and the isopropyl of L410 on the opposite side of the ring. In the first group of poses, the acetamide of NAPQI pointed into the pocket near S55, N408, and T41. The acetamide of NAPQI pointed towards V420 and the surface of the protein in the second type of pose. Computational models of hGS indicated that while NAPQI did not exhibit specific binding near C409, NAPQI was properly positioned for covalent bonding with C422 via Michael addition by either dominate binding orientation.

Figure 4. NAPQI binding near Cys422 of hGS. Backbone of hGS shown in ribbon format with relevant residues shown as sticks. Representative poses of NAPQI shown in green and purple.

4. Discussion

Glutathione has an essential role in detoxifying NAPQI (Bessemers and Vermeulen, 2001; Geenen et al., 2013; Mitchell et al., 1973). Despite this, the possibility that NAPQI might inhibit glutathione synthesis, and thus reduce the protection against toxicity, has not been explored. Pitt and Hauser (1998) suggested that inhibition of GS might contribute to the development of acetaminophen-associated 5-oxoprolinuric acidosis. We investigated the interaction of NAPQI with GS *in vitro* in order to ascertain whether this could be a tenable explanation. The findings demonstrated clearly that NAPQI binds covalently to GS causing irreversible inhibition of enzyme activity. This is a novel biochemical observation.

Peptides produced by trypsin digestion of the enzyme were analysed in order to identify NAPQI-modified amino acids using mass spectrometry, as reported for studies of other liver proteins (Dietze et al., 1997; Qiu et al., 1998). NAPQI was shown to bind irreversibly to C422, the cysteine residue which is essential for GS activity (Gali and Board, 1997). This could result from a Michael-type addition reaction to the cysteinyl SH group to form a C-4' thiohemiketal followed by aromatisation to 3'-proteinS-acetaminophen. Alternatively, the cysteinyl SH could be added to the imine double bond at C1 of NAPQI, forming an *ipso* adduct which then rearranges to 2'-proteinS-acetaminophen (Bessemers and Vermeulen, 2001; Chen et al., 1999; Dietze et

al., 1997). Human GS has two identical subunits, each with a central active site surrounded by mobile loops that control activity and bind the substrates (Dinescu et al., 2007; Gali and Board, 1995; Polekhina et al., 1999). While C422 is not directly involved with the active site, it is located within a mini-barrel which contributes one wall to the ATP-binding site and it may have a structural role (Polekhina et al., 1999). The modeling studies showed that NAPQI can nestle deeply in the molecular pocket containing C422. Covalent binding of NAPQI at this site might interfere with catalytic loop motions and block the active site, thus reducing GS activity.

NAPQI was shown to inhibit GS *in vitro* through an irreversible interaction. From the combined results of three experiments, NAPQI in concentrations ranging from 25 μ M to 400 μ M inhibited the activity of 81 mg/L (0.77 μ M) of GS by 16 % to 89%. Inhibition was significantly dose-related. Other proteins and glutathione were not included in the assays, and we do not know whether these concentrations are biologically relevant. The amount of free NAPQI in hepatocytes cannot be quantified directly because of its reactivity. Exposure of mouse hepatocytes *in vitro* to 250 μ M and 500 μ M of NAPQI, but not to 100 μ M, caused toxic changes (cell surface blebs) (Moore et al., 1985). Intracellular concentrations are probably much lower *in vivo*. Protein derived acetaminophen (APAP)-cysteine is measured in plasma as an indicator of the amount of NAPQI-protein adduct formed in liver. Plasma concentrations range from <1 to 27 μ M after overdose (Heard et al., 2011; Muldrew et al., 2002; McGill et al., 2013). However, these probably under-estimate the liver concentrations. In experimental animals given acetaminophen, damage to the liver by ischaemia/reperfusion increased blood levels of APAP-cysteine significantly (McGill et al., 2013).

Despite speculation, there is no published evidence that any of the 50 patients reported to 2014 with acetaminophen-associated 5-oxoprolinuric HAGMA had a genetic defect of the γ -glutamyl cycle. GS was measured in fibroblasts or red cells in only four and was normal. One also had normal 5-oxoprolinase activity (Brohan et al., 2014; Liss et al., 2013; Pitt and Hauser 1998). In addition, we measured red cell GS in one of our own patients and found a normal value (not reported). Heterozygotes for inherited GS

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deficiency have GS activity of 55 +/- 13% (mean [SD]) of normal and are asymptomatic (Njålsson et al., 2005). Inhibition of the activity of a *normal* GS enzyme by NAPQI could cause 5-oxoprolinuria, but why would this only occur in such a small sub-group of the world population who consume acetaminophen?

We suggest that reduced detoxification of NAPQI resulting from glutathione depletion, and its constant production from repeated chronic ingestion of acetaminophen, could increase intracellular NAPQI to levels which inhibit GS. This could contribute to 5-oxoprolinuria.

The patients reported were at high risk of glutathione depletion because of a poor intake of cysteine, chronic liver disease, or on-going glutathione losses through degradation during sepsis-induced oxidative and nitrosative stress, often in combination (Emmett, 2014; Malmezat et al., 2000; Wu et al., 2004). Glutathione depletion increases the risk for acetaminophen toxicity (Mitchell et al., 1973). This was shown when glutathione synthesis was reduced in γ GCS-deficient rats (Akai et al., 2007) and mice (McConnachie et al., 2007; Slitt et al. 2005), and in lymphocytes of a human patient with inherited GS deficiency (Spielberg and Gordon, 1981). In early studies in mice, covalent binding of NAPQI to proteins did not start until 30-45 min after exposure to large toxic doses of acetaminophen (≥ 350 mg/kg) which reduced liver glutathione by 75%. However, this still left a significant amount of glutathione (1 mM) (Mitchell et al., 1973). In more recent studies, protein adducts formed within 15 min (Muldrew et al., 2002), and were detectable even after non-toxic acetaminophen doses as low as 15 mg/kg, with only a minimal decrease in glutathione (McGill et al., 2013). These later studies suggest that NAPQI binding to proteins and glutathione occurs simultaneously (and independently) from the onset of NAPQI production, and that severe glutathione depletion is not a pre-requisite for protein binding. In fact, protein adducts were detectable in serum of healthy adults taking therapeutic doses of acetaminophen (Heard et al., 2011). However, glutathione depletion does increase the *amount* of adduct formed because less glutathione is available to scavenge NAPQI (McGill et al., 2013; Mitchell et al., 1973).

By releasing γ -GCS from feedback inhibition, glutathione depletion increases γ -glutamylcysteine synthesis. Expression and activity of γ -GCS are increased further by oxidative and nitrosative stress, inflammation and inflammatory cytokines (Malmezat et al., 2000; Wu et al., 2004). Compared with γ -GCT, GS has a much higher activity and affinity for γ -glutamylcysteine. Normally therefore, the flood of increased γ -glutamylcysteine is directed towards glutathione synthesis to replenish stores (Wu et al., 2004). It could be diverted to 5-oxoproline if GS activity was inhibited or over-whelmed.

In humans who survive an acute acetaminophen overdose, exposure of hepatocytes to NAPQI is curtailed rapidly. Data for 5-oxoproline are lacking since organic acid analyses are not part of the routine work-up of such patients. However, two (possibly three) of the 50 cases reported with acetaminophen-induced 5-oxoprolinuria described above had acute acetaminophen toxicity. Hence it is possible that 5-oxoprolinuria may occur after acute overdose but resolves and passes undetected. The situation is different in patients with low glutathione because of an underlying chronic morbidity. Their glutathione reserves are drained constantly by repeated challenge from acetaminophen ingestion. In this chronic situation, NAPQI could accumulate when the capacity to replace the glutathione losses becomes inadequate. Continuation of acetaminophen, even in therapeutic doses, could then cause toxicity and increase adduct formation.

In such patients with complex morbidities, other factors, for example accelerated protein catabolism, could increase 5-oxoproline production (Figure 1a). Cysteine deficiency arising from an inadequate intake of cysteine and its precursor methionine, and urinary losses of sulphated acetaminophen metabolites, could also contribute. Cysteine is the rate-limiting substrate for glutathione synthesis. *In vitro*, incubation of γ -GCS with L-glutamate without cysteine resulted in production of phosphorylated glutamate which readily converted to 5-oxoproline (Orlowski and Meister 1971). Emmett (2014) proposed that through this process cysteine depletion, together with increased activity of γ -GCS due to low glutathione levels, would lead to accumulation of 5-oxoproline. Demonstration of low hepatocyte concentrations of γ -glutamylcysteine would support this proposal, but there are no published data.

Although not investigated, an alternative explanation could be that NAPQI inhibits 5-oxoprolinase. This enzyme has essential cysteine residues and is rapidly inactivated by the sulfhydryl group inhibitor, N-ethylmaleimide (Williamson and Meister, 1982). Acidosis is not a feature of *inherited* 5-oxoprolinase deficiency (Ristoff and Larsson, 2007), but might be provoked by simultaneous GS inhibition or renal failure. Other possible explanations for 5-oxoprolinuric acidosis are highly unlikely in the majority of cases. Glycine deficiency may contribute to mild 5-oxoprolinuria in children with severe protein-energy malnutrition, but deficiency of this non-essential amino is otherwise exceptionally rare (Emmett, 2014). Foods rich in glutamine (notably tomato juice) which cyclises to 5-oxoproline are unlikely sources. Flucloxacillin was proposed in one case report (Croal et al., 1998), and listed as a cause since, but this is still unproven. The D-isomer probably accounts for 5-oxoprolinuria reported during treatment with the γ -aminobutyric acid (GABA) antagonist vigabatrin (Larsson et al., 2005).

Conclusions

NAPQI binds covalently to GS causing irreversible inhibition of enzyme activity *in vitro*. These novel preliminary findings are the first indication that NAPQI may inhibit glutathione production. This has not been explored so far, despite the pivotal role of glutathione in protection against acetaminophen toxicity. The biological relevance of GS inhibition should be investigated in cell cultures. The possibility that NAPQI may also inhibit other enzymes in the glutathione and supportive methionine transsulphuration pathways should be explored. The hypothesis that inhibition of GS by NAPQI could contribute to the development of acetaminophen associated 5-oxoprolinuric HAGMA merits further investigation.

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Declaration of interest

The authors report no declarations of interest

Appendices

Appendix 1. Mass spectra and error plots of fragment ions of the GS peptide modified by NAPQI after co-incubation of 5 µg of hGS with 15 µg of NAPQI for 60 min at 37 °C. Results for two independent experiments.

Figure 5

Figure 5a. Mass spectrum of the modified peptide VVQCISELGIFGVYVR (MW = 1989.026) from experiment 1. NAPQI ($\Delta\text{mass} = 149.0477$ Da), carboamidomethylation ($\Delta\text{mass} = 57.021$ Da) and deamidation are isolate to fragment ions from y16 to y11 corresponding to the amino acids VVQCI.

Figure 5b. Error plot of fragment ions from the modified peptide VVQCISELGIFGVYVR (MW = 1989.026) from experiment 1. NAPQI, carboamidomethylation and deamidation are isolate to fragment ions from y16 to y11 corresponding to the amino acids VVQCI.

Figure 5c. Mass spectrum of the modified peptide VVQCISELGIFGVYVR (MW = 1989.026) from experiment 2. NAPQI, carboamidomethylation and deamidation are isolate to fragment ions from y16 to y10 corresponding to the amino acids VVQCIS.

Figure 5d. Error plot of fragment ions from the modified peptide VVQCISELGIFGVYVR (MW = 1989.026) from experiment 2. NAPQI, carboamidomethylation and deamidation are isolate to fragment ions from y16 to y10 corresponding to the amino acids VVQCI.

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Appendix 2. Inhibition of activity of GS (81 mg/L; 0.77 μ M) pre-incubated with NAPQI (0-100 μ M) for 30 min at 37 $^{\circ}$ C. Enzyme activity was measured by the described procedure, with GAB substrate concentrations 2.5 mM and 10.0 mM. NAP = NAPQI; concentrations 0-100 μ M.

Figure 6a. GS activity, reported as μ mol of NADH oxidized in the reporter reaction/min/mg of GS.

Figure 6b. Time course of GS activity after 30 min of preincubation with NAPQI. GS activity was monitored at 28 s intervals for 168 s under standard assay conditions with 10 mM GAB as substrate. Activity reported as μmol of NADH oxidized/ mg of GS.

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