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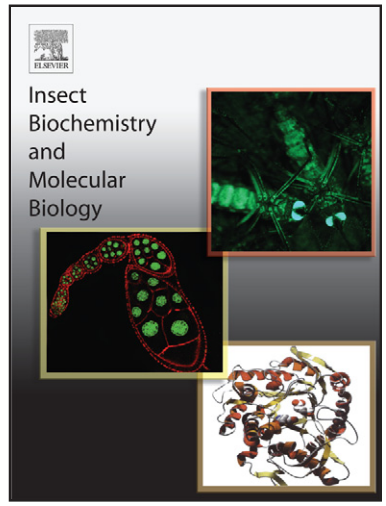

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The *Aedes aegypti* glutathione transferase family

Nongkran Lumjuan[1,2], Bradley J. Stevenson[1], La-aied Prapanthadara[2], Pradya Somboon[3], Peter M. Brophy[4] Brendan J. Loftus[5], David W. Severson[6] and Hilary Ranson[1]*

[1]Vector Group, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK

[2]Research Institute for Health Sciences, Chiang Mai University, PO Box 80, Chiang Mai, 50202, Thailand

[3]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand

[4]Institute of Biological Sciences, University of Wales, Aberystwyth SY23 3DA

[5] Department of Parasite Genomics, The Institute for Genomic Research, 9712 Medical Center Dr., Rockville, MD 20850, USA

[6]Center for Tropical Disease Research and Training, Department of Biological Sciences, University of Notre Dame, Indiana, 46556, USA

***Corresponding author:** Tel: +44 151 705 3310, Fax: +44 151 705 3369 E-mail address: hranson@liverpool.ac.uk, Vector Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

Abstract

In this report we describe the glutathione transferase (GST) gene family in the dengue vector *Aedes aegypti* and suggest a novel role for a new class of mosquito GSTs. Twenty six GST genes are present in *Ae. aegypti*, two of which are alternatively spliced to give a total of 29 transcripts for cytosolic GSTs. The six classes identified in other insect species are all represented and, as in *Anopheles gambiae*, the majority of the mosquito GSTs belong to the insect specific Delta and Epsilon classes with eight members each. Sixteen secure 1:1 orthologs were identified between GSTs in *Ae. aegypti* and *An. gambiae*, but only four of these have recognisable orthologs in *Drosophila melanogaster*. Three mosquito-specific GSTs were identified which did not belong to any previously recognised GST classes. One of these, *GSTx2*, has been previously implicated in conferring DDT resistance in *Ae. aegypti* from South America. However, we found no evidence for increased levels of this GST protein in DDT/pyrethroid resistant populations from Thailand. Furthermore, we show that the recombinant GSTX2-2 protein is unable to metabolise DDT. Interestingly, GSTX2-2 showed an affinity for hematin, and this, together with the restricted distribution of this class to haematophagous insects, may indicate a role for these enzymes in protecting mosquitoes against heme toxicity during blood feeding.

Keywords: glutathione transferase, insecticide resistance, *Aedes aegypti*, *Anopheles gambiae*, DDT, heme

1. Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are major phase II detoxification enzymes found in most organisms. They metabolise a wide range of hydrophobic toxic compounds such as drugs, insecticides and toxic endogenous substrates by catalysing the conjugation of glutathione to the hydrophilic centre of the toxic substances. In general the consequence of this reaction is to increase the solubility of the compounds, thereby aiding excretion. GSTs can also bind hydrophobic compounds that are not their substrates. This non-substrate binding is possibly associated with the sequestration, storage and transportation of drugs, hormones and other metabolites, such as bilirubin, fatty acid and heme (Hayes *et al.*, 1995). GSTs can be divided into three main groups: cytosolic, microsomal (also known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)) and mitochondrial GSTs (Jakobsson *et al.*, 1996; Robinson *et al.*, 2004; Sheehan *et al.*, 2001). Mosquitoes contain both cytosolic and microsomal GSTs but the mitochondrial class of GSTs have not been found in any insect species to date. This report focuses on the cytosolic GST family from the major dengue and yellow fever mosquito vector, *Aedes aegypti.*

Cytosolic GSTs consist of two subunits forming homodimers or heterodimers. Each GST subunit contains a specific glutathione (GSH)-binding site (G-site) next to a nonspecific electrophilic ligand-binding site (H-site). The G-site is found at the N-terminal of the protein which is highly conserved. The residues in the H-site, which interact with the hydrophobic substrate, are mainly found in the C-terminus. The high level of diversity in this region is responsible for the differences in substrate specificities (Mannervik *et al.*, 1988; Ranson *et al.*, 1998).

Insect cytosolic GSTs have been assigned to at least six classes: Delta, Epsilon, Omega, Sigma, Theta and Zeta (Ranson *et al.*, 2001). Members of these six classes have been identified in the malaria vector *Anopheles gambiae* and the fruitfly *Drosophila melanogaster* (Ranson *et al.*, 2002). The Delta and Epsilon classes, both specific to insects, are the largest classes comprising over 65 % of the total complement of cytosolic GSTs in these two Diptera (Ranson *et al.*, 2002). The majority of GSTs implicated in xenobiotic metabolism in insects belong to these classes. The Omega, Sigma, Theta and Zeta classes have a much wider taxonomic distribution and likely play essential housekeeping roles (Board *et al.*, 2000; Wildenburg *et al.*, 1998).

Insect GSTs have been implicated in resistance to insecticides through direct metabolism of the insecticide (Wei *et al.*, 2001), sequestration (Kostaropoulos *et al.*, 2001) or by protecting against secondary toxic effects, such as increases in lipid peroxidation, induced by insecticide exposure (Vontas *et al.*, 2001). GST-mediated detoxification has been reported for both organophosphate (OP) insecticides (Motoyama *et al.*, 1975) and 1,1,1-trichloro-2,2-*bis*(p-chlorophenyl)ethylene (DDT) (Prapanthadara *et al.*, 2002; Ranson *et al.*, 1997; Tang *et al.*, 1994). The major role of GSTs in OP resistance is the detoxification of the insecticide by a conjugation reaction with GSH resulting in *O*-dealkylation (Oppenoorth *et al.*, 1979) and *O*-dearylation. Specific GSTs also catalyse the metabolism of DDT to non-toxic DDE in a dehydrochlorination reaction which does not involve a GSH conjugate intermediate (Clark *et al.*, 1984).

The Epsilon class GST, GSTE2, has been implicated in DDT resistance in both *An. gambiae* and *Ae. aegypti*. The recombinant GSTE2 protein is very efficient at metabolising DDT and expression of this protein is elevated in DDT resistant *An. gambiae* from East Africa and *Ae. aegypti* from Thailand (Ding *et al.*, 2003; Lumjuan *et al.*, 2005; Ortelli *et al.*, 2003). A previous study has implicated an additional GST, referred to as GST-2, in DDT/permethrin resistance in *Ae. aegypti* from South America (Grant *et al.*, 1992) although we found no increased expression of this gene in insecticide resistant *Ae. aegypti* from Thailand (Lumjuan *et al.*, 2005).

The aim of the present study was to characterise the *Ae. aegypti* GST gene family. In doing so we found that the GST-2 described by Grant *et al* (Grant *et al.*, 1992) did not belong to any of the previously recognised classes but had a clear ortholog in *Anopheles* mosquitoes. We expressed and characterised this GST and show that this enzyme is both catalytically active (although not with DDT as a substrate) and is able to bind heme.

2. Materials and methods

2.1. Identification of *Ae. aegypti* GSTs

GST genes from *Ae. aegypti* mosquitoes were identified by searching the sequences in the EST database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=a_aegypti) by keyword (GST) or by using the BLAST algorithm to search with *An. gambiae* GST sequences. ESTs putatively encoding the orthologs of the *An. gambiae* genes were used to identify the *Ae. aegypti* genes by searching against the Whole Genome Shotgun database at the Broad Institute (http://www.broad.mit.edu/annotation/disease_vector/aedes_aegypti/).

2.2. Phylogenetic analysis

Deduced amino acid sequences of GSTs were aligned using ClustalX (Thompson *et al.*, 1997). The alignment was converted to a PHYLIP file using TREECON software (Van de Peer *et al.*, 1993). Phylogenetic trees were generated by TREECON using the distance neighbour-joining method (Saitou *et al.*, 1987).

2.3. cDNA synthesis

Total RNA was extracted from one-day old adults from the PMD-R strain of *Ae. aegypti* using TRI reagent (Sigma) as described previously (Lumjuan *et al.*, 2005). Complementary DNA was synthesised using an oligo $(dT)_{15}$ primer (Promega) and Superscript III reverse transcriptase (Gibco BRL).

2.4. Confirmation of splice variants of *GSTd1* and *GSTs1*

Adult *Ae. aegypti* cDNA was used as a template in PCR reactions to confirm the predicted alternative transcripts derived from two of the GST genes, *GSTd1* and *GSTs1*. For each of these genes we predicted that a common 5' exon could be spliced to alternative 3' exons to generate two (for GSTs1) or three (for GSTd1) distinct transcripts. We therefore designed forward primers within the putative common 5' exon and used these in combination with reverse primers specific to each of the putative alternative 3' exons. Primers used to amplify the *GSTd1* transcripts were GSTd1F forward primer (5'- ATGGATTTCTACTACCTGCCAG-3'), GSTd1-1R reverse primer (5' -TCACTTCTCGAAGTACTTG-3'), GSTd1-2R reverse primer (5' - CTACTGCGCAGGGGCTTTAAC-3'), and GSTd1-3R reverse primer (5'-TTACATTCCGGACAGGAAC-3'). Primers used to amplify the *GSTs1* transcripts were GSTs1F forward primer (5'- ATGCCGGATTACAAG-GTCTAC-3') GSTs1exon4R (5'-TTAGATCTCAGTTTGTGGT-CG-3') and GSTs1exon5R reverse primers (5'-ACTCAGACGGAAATCGTTAATC-3'). PCR products were cloned into the pGEM T easy Vector (Promega) and their sequence was determined using the Beckman Coulter CEQ8000 automatic sequencer.

2.5. Cloning of GSTs

The full-length cDNA of *GSTi1*, *GSTx1* and *GSTx2* genes were amplified using the forward primers *GSTi1F* (5'- ATGAAAATCTATGCCGTATCG -3'), *GSTx1F* (5'-

ATGCCCATGAGTTTGTATTACAG -3') *and GSTx2F* (5'-ATGGCTCCAATTGTGCTGTATC-3') and the reverse primers *GSTi1R* (5'- TCATTTCTTAACTTTTTTGATGGGATG -3'), *GSTx1R* (5'- TACATTCCCTCGGTCACG -3') and *GSTx2R* (5'-TTAGAAAGGTTCCTCCAGCTTG-3'). The initiator methionine and stop codon are underlined in each primer sequence. PCR amplification was performed using ProofStart DNA polymerase (Qiagen). After purification, PCR products were A-tailed by incubation with 0.2 mM dATP and 5 units of *Taq* DNA polymerase at 70^{o}C for 30 minutes, and ligated into pGEM-T easy vector and the plasmids sequenced to confirm the integrity of the product. The GST inserts were then amplified from these plasmids and cloned into the pET SUMO vector (Invitrogen). Positive colonies were screened by PCR using specific forward primers specific to the *GSTs* and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') to determine the correct orientation and plasmids were purified and sequenced.

2.6. *In vitro* expression of GSTs

Plasmids containing the full coding sequence of GSTs were transformed into the expression host *E. coli* BL21 (DE3) pLysS (Novagen). Transformed cells were grown on an LB-agar plate containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml). A single colony was grown overnight in 2 ml LB-medium with antibiotics as above and incubated at 37^{o}C overnight. This culture was used to inoculate 100 ml LB-medium and grown for a further 3 hours. Expression was induced by addition of 1 mM IPTG and, after an additional incubation at 37^{o}C for 3 h, bacterial cells were harvested by centrifugation at 5000g, 4^{o}C for 10 min. Pelleted cells were resuspended in 5 ml of 1X Binding Buffer containing 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, pH 7.9. After snap freezing in liquid nitrogen, the cells were stored at –80^{o}C or used for purification.

2.7. Protein Purification of recombinant GSTs

The cell lysate was incubated with 2 Units/ml of DNase RQI at room temperature for 20 minutes to digest bacterial genomic DNA. After centrifugation to remove the cell debris, the soluble protein was collected for purification. The recombinant protein contained a 6X His tag and SUMO fusion protein (13 kDa) at the N-terminus and this enabled purification using the His•Bind resin (Novagen). The cell lysate was applied to His•Bind resin (Novagen) (1ml bed volume) and, after washing with 10 column volumes of 1X Binding buffer, the column was washed with 10 column volumes of 1 X Wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9), followed by 10 column volumes of Wash buffer containing 80 mM imidazole. The bound proteins were eluted with 6 column volumes of 1X Elute buffer containing 0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole, pH 7.9. The eluted proteins were collected in 1.5 ml/fractions and monitored for protein purity by electrophoresis on 15 % SDS-PAGE. The fractions containing recombinant protein were pooled and loaded onto PD-10 columns to desalt and remove imidazole. Recombinant protein was eluted with 50 mM Tris-HCL pH 8.0 and then concentrated using Microcon YM-30 columns.

The recombinant fusion protein was cleaved by SUMO protease to generate the native protein. Ten units of SUMO protease (Invitrogen) were added to 20 μg of fusion protein in 1X SUMO protease buffer (50 mM Tris-HCl, pH 8.0, 2% Igepal (NP), 10 mM DTT) and incubated overnight at 4^{o}C. The proteins were resolved by SDS-PAGE to verify the efficacy of the cleavage reaction. The SUMO protein and SUMO protease were purified from the recombinant GST using His•Bind resin. Recombinant proteins were collected in the presence of 40% (v/v) glycerol and 15 mM DTT and stored at –20^{o}C. Protein concentration was measured using Bio-Rad Protein Reagent (Bio-Rad) with bovine serum albumin for the standard protein (Bradford, 1976).

2.8. MALDI-TOF Mass Spectrometry Analysis

Matrix Assisted Laser Desorption/Time of Flight (MALDI-TOF) mass spectrometry analysis was performed to confirm the identity of the expressed protein. Briefly, tryptic digestion of purified recombinant protein was carried out using sequencing grade trypsin (Promega). The single band of recombinant GST from SDS-PAGE was cut and washed in dH_2O. The gel slice was dehydrated with 50 % (v/v) acetonitrile and then incubated with 50 mM ammonium bicarbonate for 10 minutes followed by a further dehydration in 50% (v/v) acetonitrile. Digestion was carried out by an overnight incubation in10 ng/μl of trypsin. One microlitre of trypsin-digested sample was mixed with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) on the metal MALDI-TOF target plate. After drying, the sample was then subjected to MALDI-TOF mass spectrometry analysis using a Kratos Analytical mass spectrometer (Shimadzu Group Company). Internal mass calibration was performed using trypsin autodigestion products. A peptide mass map was generated by Kratos PC Axima CFRplus V2.4.1 software (Shimadzu Group Company). Database searching was performed using monoisotopic peptide masses obtained from MALDI-TOF mass spectrophotometry. Mascot search from Matrix Science (http://www.matrixscience.com/search_form_select.html) was used to identify the peptide sequences in the NCBI database.

2.9. Characterisation of GSTX2-2

GST activity against 1-choro-2, 4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) was measured according to the method of Habig (Habig *et al.*, 1974). Glutathione peroxidase activity was determined at 340 nm by coupling the reduction of cumene hydroperoxide (CHP) by GSH to the oxidation of NADPH by oxidized GSSG with glutathione reductase as described previously (Simmons *et al.*, 1989). DDT-dehydrochlorinase activity was determined by conversion of DDT to DDE detected by HPLC as described previously (Prapanthadara *et al.*, 2000).

Hematin binding to GST was monitored by measuring the inhibition of GST activity in the absence or presence of hematin (10-200 µM). The IC_{50} value was determined by plotting sigmoidal dose response of fractional GST activity against log concentration of hematin using GraphPad Prism 4 software. The binding of hematin to GST was also determined by following the quenching of the intrinsic fluorescence of GST in the presence of hematin as described previously (van Rossum *et al.*, 2004). Increasing concentrations of hematin were added to the enzyme solution (1 µM) in 0.02 M potassium phosphate buffer containing 0.1 M NaCl, pH 6.5. After 3 minutes incubation at 25^{o}C, changes in the intrinsic protein fluorescence were monitored using LS50B Luminescence spectrometer (PerkinElmer Life and Analytical Sciences) with the excitation and emission wavelengths of 280 and 363 nm, respectively. The dissociation constant (K_d) value was calculated by double reciprocal plots of the intrinsic fluorescence of GST with the concentration of free hematin. The straight line at the *x*-intercept indicates to 1/ K_d. Finally, the kinetics of hematin inhibition were determined with CDNB varied and GSH held constant at 10 mM. The substrates were incubated with hematin for 3 minutes at 25°C prior to addition of GSTX2-2. This was necessary since the absorption of hematin changed in the presence of GSH and needed to stabilise before the GSH-CDNB conjugation could be observed at 340 nm. The results were analysed by Dixon plots and the data fitted by linear regression analysis at four different concentrations of hematin using GrapPad Prism software.

3. Results and discussion

3.1. Classification of *Aedes aegypti* GSTs

We previously reported the identification of nine GSTs in *Ae. aegypti* (Lumjuan *et al.*, 2005). In the present study we searched the *Ae. aegypti* EST database at The Institute for Genomic Research and the partially assembled genome sequence database at the Broad Institute to identify the full complement of cytosolic GSTs in *Ae. aegypti.* The GST genes were categorised into classes according to their amino acid sequence identity and phylogenetic relationship using previously described criteria (Ding *et al.*, 2003). Twenty four distinct GST transcripts derived from 22 GST genes (see below for description of alternatively spliced genes) were identified from the EST database (Table S1). An additional four GST genes were identified from the genome sequence database by querying with the orthologs of these genes from *An. gambiae*. We have not yet detected transcripts for these four *Ae. aegypti* GSTs (*GSTe1*, *GSTe5*, *GSTd5* and *GSTd3*) and one of these, *GSTe1* was also undetectable in microarray experiments using *Ae aegypti* adults and larvae (C. Strode, unpublished data) and may possibly represent a non-transcribed pseudogenes. In addition to these full length GSTs we identified a single partial GST gene in the genome database (within supercontig 1.220) and several sequences with similarity to bacterial GSTs which have subsequently been removed from the genome database.

The six GST classes described in *Anopheles* and *Drosophila* (Ranson *et al.*, 2002) were also found in *Aedes*. The largest classes are the insect-specific Delta and Epsilon classes with eight members in both classes (Figure 1). The Delta GST class in *Ae. aegypti* is reduced in number compared with *Anopheles* (12 Delta GSTs) and *Drosophila* (10). Of the eight *Ae. aegypti* Delta GSTs only one, *GSTd7,* has an ortholog in both *An. gambiae* and *D. melanogaster*

(Figure 2). As discussed below for the Epsilon GSTs, there is little conservation of gene order or orientation between the Delta GSTs from the two mosquito species.

Equal numbers of Epsilon GSTs are found in *Ae. aegypti* and *An. gambiae* (14 Epsilon GSTs are present in *D. melanogaster*) but there are few secure orthologs (Figure 1) and there is local gene shuffling (Figure 2) in this class in the two mosquito species. As with the Delta class, there is a single secure ortholog, *GSTe8*, within the Epsilon GSTs from the mosquitoes and fruitfly. The Epsilon class of GSTs contains the majority of the insect GSTs that have been implicated in insecticide resistance, including the housefly GST, *MdGST6A* (Figure 2).

Single members of the Zeta and Omega GSTs were identified in *Ae. aegypti*, both of which have single orthologs in *An. gambiae*. In contrast, four members of the Theta class, *GSTt1*, *GSTt2*, *GSTt3* and *GSTt4* were identified, representing an expansion of the Theta class in *Aedes* (4 genes) relative to *Anopheles* (2 genes) (Ding *et al.*, 2003).

3.2. Alternative splicing within the GST genes

GSTd1 and *GSTs1* are orthologs of alternatively spliced Delta and Sigma GSTs in *An. gambiae* and the presence of alternative splicing in these *Aedes* genes was investigated (Figure 3 and 4). Two alternatively spliced transcripts of *Ae. aegypti GSTd1* were identified in the EST database and a third, *GSTd1-2*, was predicted from analysis of the genomic sequence. The putative transcripts were amplified from adult cDNA and sequenced to confirm that each of the three splice variants are actively transcribed (data not shown). The three alternative transcripts from *GSTd1* share a common 5' exon but have alternative 3' exons, leading to an increase in the diversity of the GST enzyme family. A similar pattern is also found in the *GSTs1* gene (Figure 4). The full-length of *GSTs1-1* and the partial *GSTs1-2* for the two alternative transcripts were identified in the EST database. These two putative transcripts were used to retrieve the corresponding genomic sequence. The intron and exon structure of these transcripts is shown in Figure 4. These transcripts were amplified using GSTs1F forward primer and GSTs1exon4R and GSTs1exon5R reverse primers to verify the transcription of the alternatively spliced forms. Transcripts of the expected size were detected in adult mosquitoes, indicating that both alternative splice variants are expressed in *Ae. aegypti* (data not shown).

As the carboxy end of the protein contains the majority of the residues constituting the substrate binding site the selection of alternative 3' translated exons is an efficient means of increasing the substrate range of the GSTs. The conservation of splicing sites within the *GSTs1* and *GSTd1* genes in *Aedes* and *Anopheles* suggests that alternative splicing of these genes evolved before the divergence of *Aedes* and *Anopheles*. Four *GSTd1* variants were detected in *Anopheles* (Ranson *et al.*, 1998), but only three *GSTd1* isoforms were identified in *Aedes*, indicating that one variant was lost or gained during its evolution.

3.3. Identification of new classes of mosquito GSTs

The name of 'unclassified' was utilised by Ding *et al.* (Ding *et al.*, 2003) for *An. gambiae* GSTs for which phylogenetic analysis indicates that they do not belong to any of the six classes identified in other species and for which function is unknown. There are three unclassified GSTs in *An. gambiae*, temporarily designated *GSTu1*, *GSTu2* and *GSTu3*. Clear orthologs of each of these three GSTs were found in *Ae. aegypti* (Figure 1). No orthologs of unclassified GSTs were found in *D. melanogaster*, and a search of the database for all organisms did not identify any closely related genes in other species. Thus to date, these three GSTs have been found uniquely in mosquitoes.

The deduced amino acids of the three 'unclassified' *Ae. aegypti* and *An. gambiae* GSTs are aligned in Figure 5. *GSTu2* and *GSTu3* consist of 218 amino acids, whereas *GSTu1* contains

231 amino acids. The percentage identities between the *Aedes* and *Anopheles* orthologs range from 58 to 75.9 at the amino acid level (Table 1). The maximum degree of sequence identity between these GST proteins and those from the Delta and Epsilon classes is 44 %, although the majority of pairwise comparisons are well below this level of identity. Furthermore the phylogenetic analysis resolved *GSTu2* and *GSTu3* from both mosquito species as a single clade (89% bootstrap support) (Figure 1). We propose that these two GSTs belong to a new class of GSTs which we have named Xi or X. *GSTu1* shows low levels of identity to any of the other members of the GST enzyme family (<40% identity) and had been renamed as class Iota (*GSTi1*).

3.4. Expression of GSTX2-2

The three genes belonging to the new GST classes were cloned into expression vectors and expressed in *E. coli*. Despite repeated attempts at optimisation, two of the recombinant proteins, GSTI1 and GSTX1, were constantly retained in the insoluble fraction and could not be recovered as active proteins. However, sufficient GSTX2 was retained in the soluble fraction to enable purification by His-tag chromatography. The purified recombinant protein has a Mwt of approx. 25 kDa on SDS-PAGE and its identity was confirmed by MALDI-TOF mass spectrometry analysis. Mascot search result confirmed that the tryptic peptides matched to GSTX2 from *Ae. aegypti* with a score of 99%.

The substrate specificities of recombinant GSTX2-2 are shown in Table 2. GSTX2-2 has high activity with CDNB (152 ± 5 μmol/min/mg) and DCNB (5.83 ± 0.72 μmol/min/mg) and detectable, but very low, activity against cumene hydroperoxide. These studies were performed with recombinant GSTX2-2 alone, but it is noteworthy that the CDNB-GSH conjugation rate was not significantly different for the SUMO-GSTX2-2 fusion protein (data not shown).

The DDT dehydrochlorinase activity of recombinant GSTX2-2 was measured as the conversion of DDT to DDE but the enzyme had no detectable activity with this substrate. This result confirms our earlier prediction that GSTX2 is not responsible for DDT resistance in *Ae. aegypti* from Northern Thailand (Lumjuan *et al.*, 2005) but also questions the role of this enzyme in conferring DDT resistance in South America. The studies implicating this enzyme in DDT resistance were conducted 15 years ago before the extent of the GST enzyme family in insects was realised. Two classes of GSTs were recognised and elevated levels of the group named class 2 were reported based on northern and western blots. It is possible that the elevated levels of GST-2 (now renamed GSTX2) reported in this earlier study could have been attributed to increased expression of an alternative, closely related enzyme. Of course it is not unreasonable to imagine that different GST enzymes confer DDT resistance in different geographical regions, but the absence of any DDTase activity in GSTX2 does not support a direct role for this enzyme in DDT resistance.

3.5.Hematin binding to GSTX2-2

The affinity of GSTX2-2 for hematin was initially measured by varying the concentration of hematin in the presence of fixed concentrations of GSH and CDNB. The resulting IC_{50} value, 37.5 ± 0.1 μM, was approximately 200 fold higher than that reported for blood feeding nematodes and similar to that observed for the GST CE07055 from the free living nematode *Caenorhabditis elegans* (van Rossum *et al.*, 2004). As in nematodes, the binding of hematin is non- competitive with respect to CDNB (Figure 6A). The K_i (hematin/CDNB) for GSTX2-2 is estimated as 13± 2 μM but this measurement is complicated by the reduction in V_{max} caused by the interaction between hematin and GSH. From the relationship between the apparent V_{max} and hematin concentration (shown in Figure 6B) the V_{max} in the absence of hematin is 98 ± 4 µmol/min/mg (Figure 6B). However, by direct measurement we determined the V_{max} for GSTX2-2 (in the absence of hematin) to be 152 ± 5 µmol/min/mg. This discrepancy appears to be due to a non-enzymatic interaction between GSH and hematin. The binding of hematin to recombinant GSTX2-2 was also analysed by monitoring the quenching of intrinsic protein fluorescence of aromatic amino acids, mostly tryptophan. The dissociation constant (K_d) value for hematin is 2.58 ± 0.46 μM (Figure 6C), similar to that of *H. contortus* GST, 1.72 ± 0.1 uM (van Rossum *et al.*, 2004).

The differences observed in GST affinity for hematin in competitive enzymatic based assays and in assays measuring changes in intrinsic fluorescence in the current study and for other, non-insect GSTs (van Rossum *et al.*, 2004; Vander Jagt *et al.*, 1985) suggest the heme-binding site is separated from the active site. This is the first report of a mosquito GST with an affinity for heme. Although the affinity for heme is lower than that found in blood feeding nematodes, it is interesting to note that this property is found in an enzyme belonging to a class that has so far only been identified in blood feeding insects.

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4. Conclusions

The GST supergene family is surprisingly conserved between *Ae. aegypti* and *An. gambiae.* Many clear orthologs can be identified between the two species and in both *Aedes* and *Anopheles* (but not in *Drosophila*) two GST genes are alternatively spliced to create additional diversity in this enzyme family. The major difference is in the representation of the GST classes, with more Theta class GSTs and fewer Delta GSTs in *Ae. aegypti* compared to *An. gambiae*. Comparison with *D. melanogaster* suggests that GST genes have been differentially lost from these two classes in mosquitoes but as so few insect GSTs have been functionally characterised the significance of this for the mosquito's ability to adapt to different niches, if any, is unclear.

Two classes of GST have so far been identified only in mosquitoes. Here we report that an enzyme from one of these classes, GSTX2-2 binds hematin and it is possible that this GST may play a protective role in the insect midgut by reducing heme toxicity after a blood meal.

Abbreviations

GST: glutathione S-transferase, GSH: glutathione; CDNB: 1-chloro-2, 4-dinitrobenzene; DCNB: 1,2-dichloro-4-nitrobenzene; CHP: cumene hydroperoxide; EST: expressed sequence tag, IPTG: isopropyl β-D-thiogalactoside; DDT: 1,1,1-trichloro-2, 2-bis-(*p*-chlorophenyl)ethane; DDE: 1,1-dichloro-2,2-bis--(*p*-chlorophenyl)ethane; HPLC: high performance liquid chromatography; NADPH: nicotinamide adenine dinucleotide phosphate (reduced form); DTT: Dithiothreitol.

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Figures

Figure 1 - Phylogenetic relationship between GST proteins from *Aedes aegypti*, *Anopheles gambiae* and *Drosophila melanogaster*. Amino acid sequences were aligned using ClustalX (Thompson *et al.*, 1997) and a distance neighbor-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >70 % are shown. Right square braces indicate to the novel classes of mosquito GSTs. Aa (pink) = *Ae. aegypti* Dm (yellow) = *D. melanogaster*. Ag (blue) = *An. gambiae*. Md (green) = *Musca domestica*.

Figure 2 - Genomic organisation of the Epsilon GST class in *Aedes aegypti* and *Anopheles gambiae*. Secure orthologs are linked with a blue line. Not to scale.

Figure 3 - Alternative splicing in GSTd1. **(A)** Gene organisation showing location of exons and introns of *Ae. aegypti GSTd1*. The grey rectangles indicate exons and the black lines represent introns. The upper and lower numbers indicate intron and exon sizes (bp), respectively. **(B)** Schematic representation the three alternatively spliced products. **(C)** Nucleotide sequences at the exon/intron boundary in the GSTd1 gene.

Figure 4 - *Ae. aegypti* GSTs1 gene organisation and alternative splicing. **(A)** Exon-intron structure of *GSTs1*. Light grey areas indicate exons, whereas horizontal lines represent intron positions. Upper and lower numbers correspond to intron and exon size (bp), respectively. **(B)** Exon usages in the splice variants *GSTs1-1* and *GSTs1-2*. Light grey boxes indicate utilized exons. White boxes represent skipped exons. Arrows represent the position of primers used to confirm the expression cDNA of *GSTs1-1* and *GSTs1-2*. The small letter a, b and c

indicates GSTs1 forward primer and GSTs1exon4R and GSTs1exon5R reverse primers, respectively.

Figure 5 - Multiple alignment of deduced amino acid sequences of unclassified GSTs from *Ae. aegypti* and *An. gambiae*. *Aedes* GST sequences shown are from PMD-R strain. The amino acid sequences were aligned using ClustalW (Thompson *et al.*, 1994). Gray shade represents amino acids conserved in all six genes. Dashes are used to denote gaps introduced for maximum alignment. Ag = *An. gambiae*.

Figure 6. Inhibition studies on *Ae. aeypti* GSTX2-2**.** GSTX2-2 activity was measured in 10 mM GSH, at pH 6.5 and 25°C, with hematin and CDNB concentrations varied. Data shown are three replicate experiments with standard deviations**.** (A) Dixon plot showing non-competitive inhibition. (B) Effect of haematin on Vmax. The apparent Vmax was calculated by non-linear regression. The regression line ($r^2 = 0.985$) was calculated using the observations in the presence of hematin only. (C) Double reciprocal plot of the intrinsic fluorescence intensity of GSTX2-2 against the concentration of free hematin. Changes in the intrinsic fluorescence of GSTX2-2 were monitored in the presence of increasing concentrations of hematin up to1.25 µM in a fixed enzyme concentration (1 µM). Dissociation constant (K_d) = 2.58 ± 0.46 µM.

Table 1

Amino acid identities between *Aedes* and *Anopheles* GSTs.

	Percent identity					
	GSTi1	***GSTx1***	***GSTx2***	***AgGSTu1***	***AgGSTu2***	***AgGSTu3***
GSTi1	-	26.5	27.9	75.9	29.1	26.0
GSTx1		-	37.4	25.1	58.0	32.4
GSTx2			-	26.5	39.7	62.1
AgGSTu1				-	29.1	26.9
AgGSTu2						38.8
AgGSTu3						-

Table 2

Substrate specificities for recombinant GSTX2-2 from *Ae. aegypti*.

Substrate	Specific activity of GSTX2-2
CDNB (µmol/min/mg)	152 ± 5
DCNB (µmol/min/mg)	5.83 ± 0.72
CHP (µmol/min/mg)	0.093 ± 0.054
DDTase activity (nmol DDE/µg)	ND

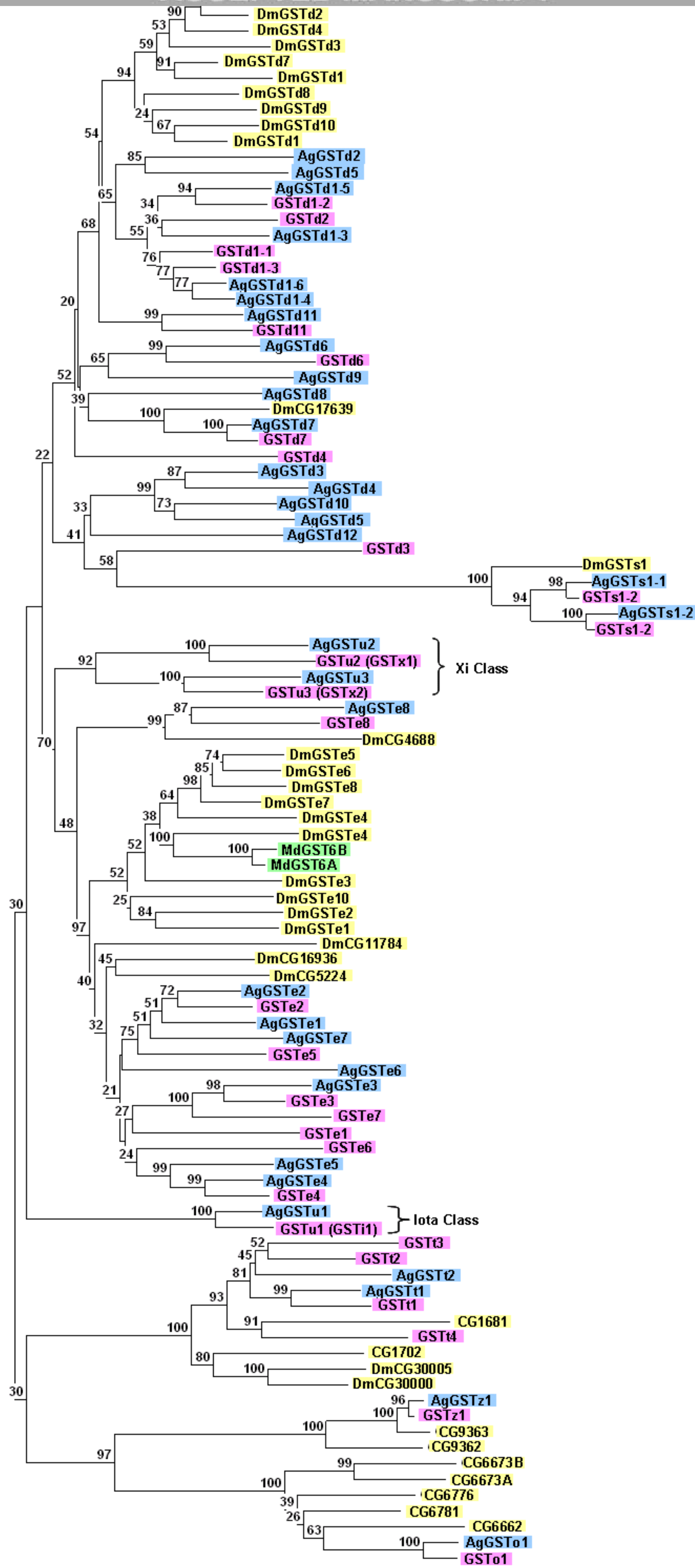
Three independent assays were performed. Results show mean ± SD. ND indicates not detectable.

Table S1

***Aedes aegypti* GST transcripts identified from EST sequence database and WGS database.** The TC number refers to tentative consensus sequences, created by TIGR (tigr.org) by assembling ESTs into virtual transcripts. The final column represents the Vectorbase ID (vectorbase.org).

GST Classes	Gene name	No. of Transcripts	Protein Size	Gene size (bp)	TCs number	Vectorbase ID
Delta	*GSTd1*	3	209, 211 and 219	2771	TC52522, TC51556	AAEL001061 AAEL001078
	GSTd2	1	209	930	TC67297	AAEL001078
	GSTd3	1	215	701	ND	AAEL001059
	GSTd4	1	218	720	TC53163	AAEL001054
	GSTd5	1	209	627	ND	AAEL001071
	GSTd6	1	249	917	TC57303	AAEL010591
	GSTd7	1	218	3438	TC55645	AAEL001090
	GSTd11	1	222	739	TC67189	AAEL010582
Epsilon	*GSTe1*	1	209	824	ND	AAEL007954
	GSTe2	1	222	1233	TC54011	AAEL007951
	GSTe3	1	222	725	TC54476	AAEL007947
	GSTe4	1	224	812	TC65731	AAEL007962
	GSTe5	1	221	795	ND	AAEL007964
	GSTe6	1	219	820	TC64617	AAEL007946
	GSTe7	1	220	724	TC67175	AAEL007948
	GSTe8	1	220	858	TC56599	AAEL007955
Theta	*GSTt1*	1	229	805	TC58005	AAEL009017
	GSTt2	1	227	1151	TC65938	AAEL009016
	GSTt3	1	232	764	TC56273	AAEL009020
	GSTt4	1	227	4951	TC62912	AAEL004229
Sigma	*GSTs1*	2	203 and 203	7687	TC67856, TC63301	AAEL011741
Omega	*GSTo1*	1	248	3790	TC62645	None

Zeta	*GSTz1*	1	233	9994	TC67856	None
Iota	*GSTi1*	1	231	770	TC61046	AAEL011752
Xi	*GSTx1*	1	218	784	TC57857	AAEL000092
	GSTx2	1	218	3430	TC54577	AAEL010500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GSTd3
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AgGSTs1-1
GSTs1-2
AgGSTs1-2
GSTs1-2
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GSTu2 (GSTx1)
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GSTu3 (GSTx2)
Xi Class
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MdGST6B
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GSTe7
GSTe1
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GSTe4
AgGSTu1
GSTu1 (GSTi1)
Iota Class
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Figure 2

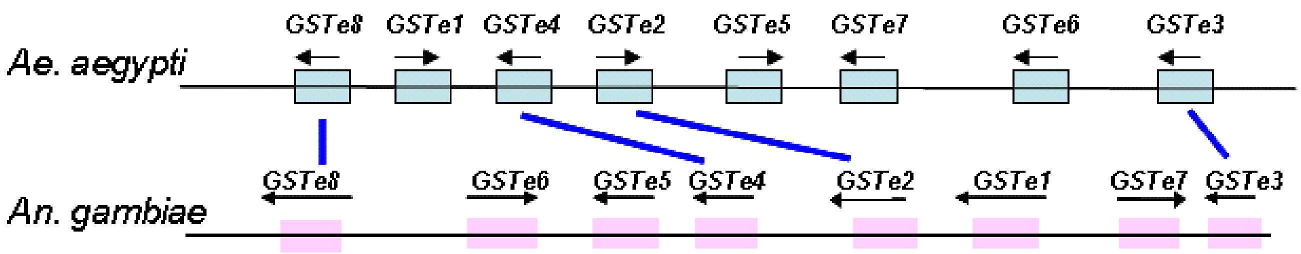
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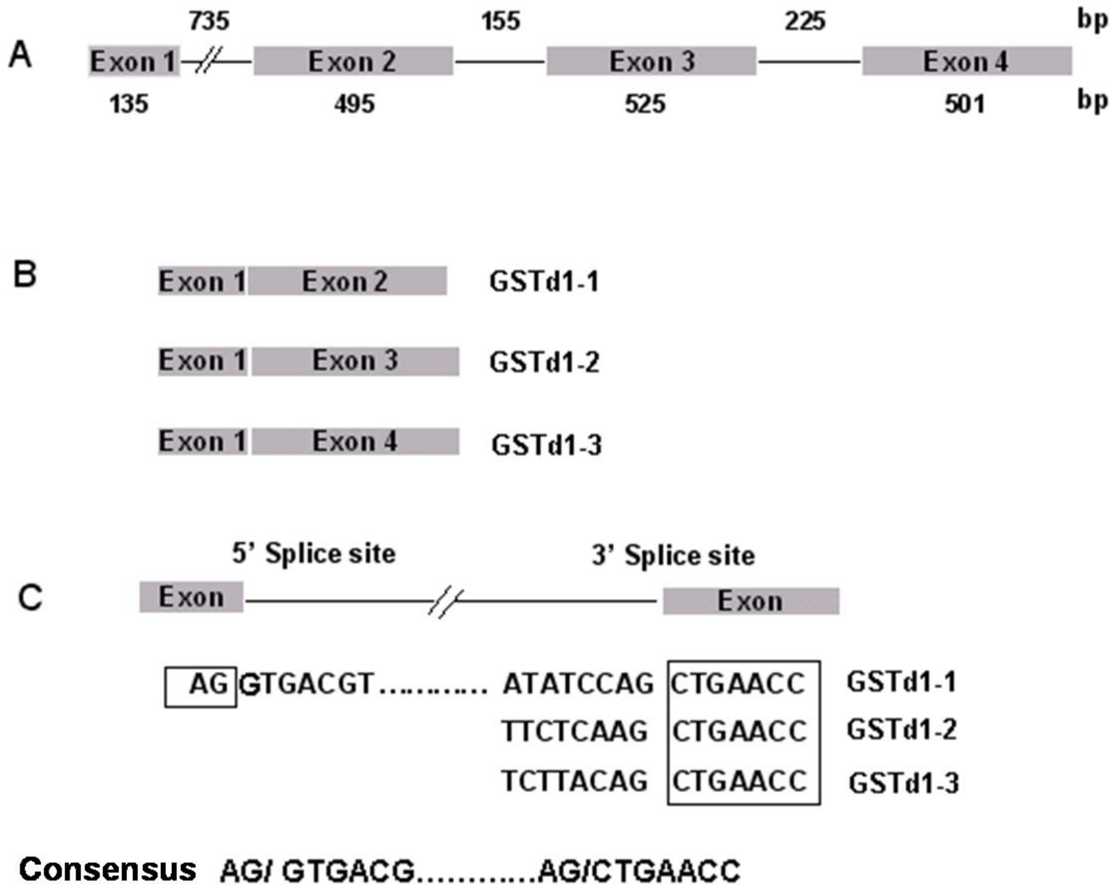
Figure 3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Exon 1
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Exon 1
Exon 4
GSTd1-3
C
5' Splice site
3' Splice site
Exon
Exon
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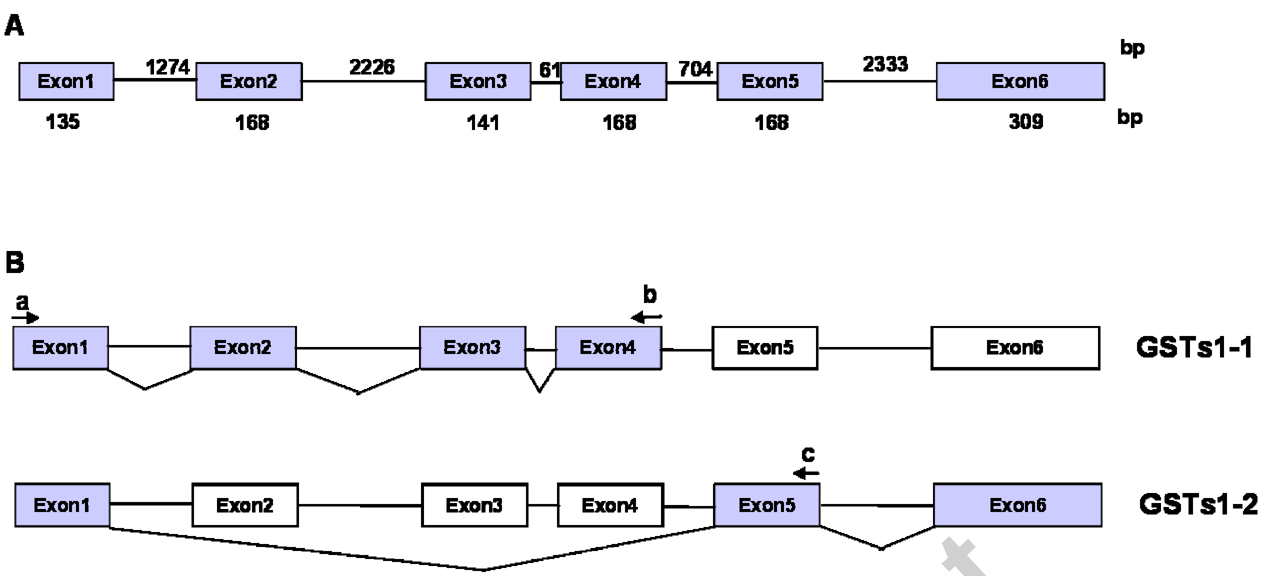
Figure 4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168
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bp
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a
b
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
GSTs1-1
c
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
GSTs1-2

Figure 5

```
GSTu2 (GSTx1)   -MPMSLYYSKMSPPARSVLLLIQELGLTGIQLKEVDVQGGGTRTEEFLKMNPEHTIPTLD 59
GSTu3 (GSTx2)   MAPIVLYHFPMSPPSRSALLVARNLGLD-VEVKILNLMAGEHMQEEFVKINPQHTVPTVV 59
GSTu1 (GSTi1)   ---MKIYAVSDGPPSLAVRMALKALDIA-HEHVPVDYGKGEHMTEDYAKMNPQKEIPVLD 56

GSTu2 (GSTx1)   DNGFYLWESRAILTYLVDAYRPGHDLYPNIPREKAQINRVLHHELSAFHPKTLGQMGAIY 119
GSTu3 (GSTx2)   DDDYVLWESKAIATYLVEQHQPDSTLYPADPKQRGIINQRLYFDSTVLFARAYAAVAPLM 119
GSTu1 (GSTi1)   DDGFFLSESNAILQYLCDKYAPDSPLYPKDPKERALVNHRLCFNLSFLYPQISAYVMAPI 116

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GSTu3 (GSTx2)   RQGATSIPQDKKDAILEALGTLNGYLD--GQDWVAGENTTVADLCLLATVSSLEKLGVDL 177
GSTu1 (GSTi1)   FFDYERTPMGLK-KLHIALAAFETYMSRLGSKFAAGDHLTIADFPLVTSVMCLEGINFNI 175

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GSTu3 (GSTx2)   SDLPNITAWLERCK---------------SLPGFEENEEGASMFGNGLKSKLEEPF 218
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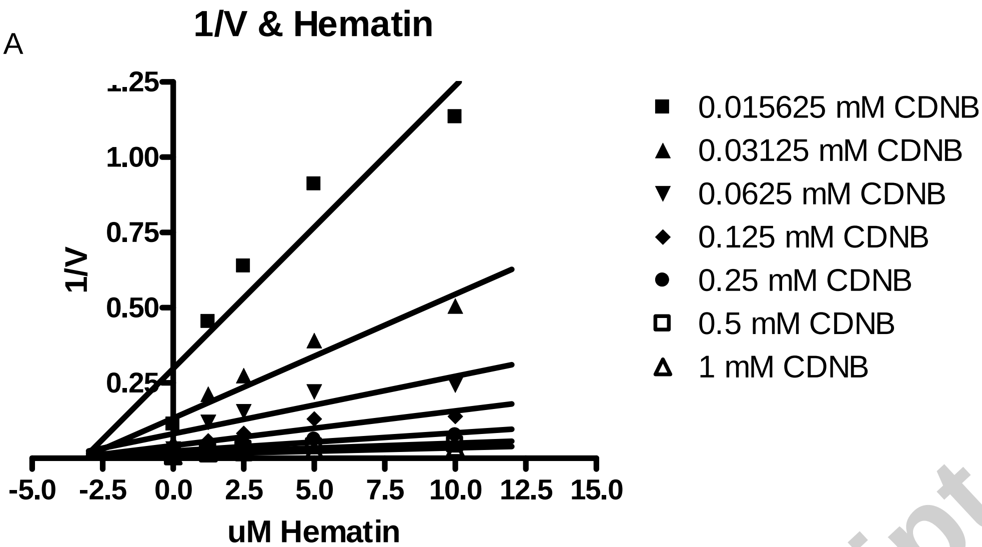
Figure 6

A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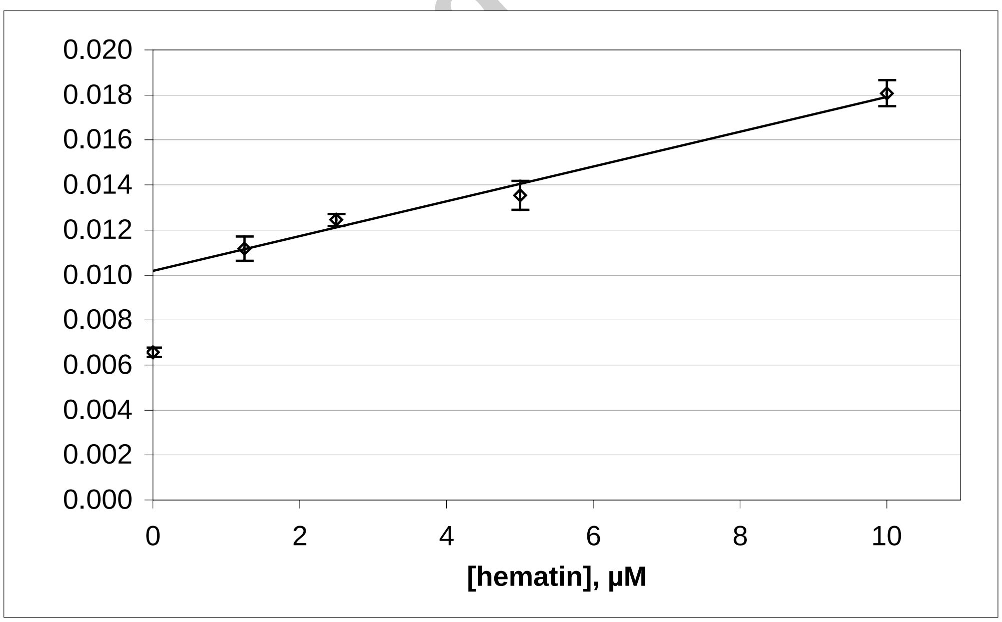
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