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Investigating the Ligandin Activity of *Schistosoma mansoni* Glutathione S-Transferases Toward Testosterone: Implications for Novel Anthelmintic Drug Target Discovery

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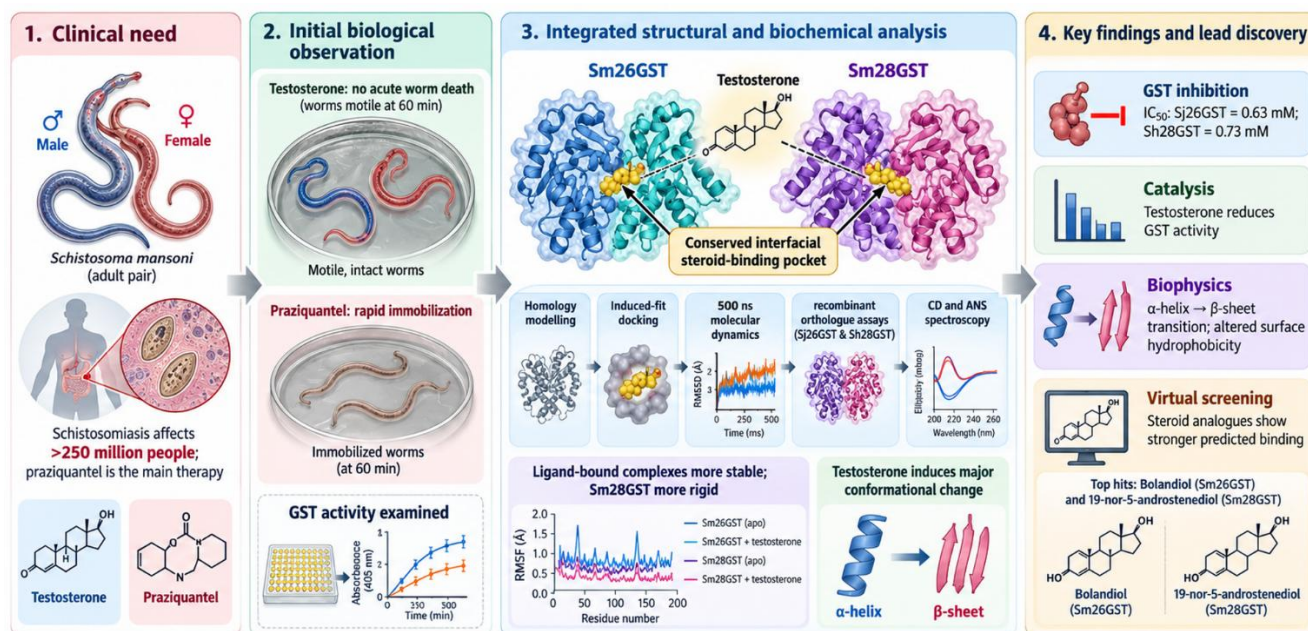
Abstract

Schistosomiasis is one of the most common neglected tropical diseases globally, with more than 250 million people affected, and praziquantel is the only broadly available chemotherapeutic agent. However, concerns regarding decreased susceptibility to the drug and the lack of alternative therapies underscore the need to identify new therapeutic targets. Glutathione *S*-transferases (GSTs) are enzymes with diverse biological functions, including parasite detoxification, antioxidant defense, and ligand transport. This study investigated the interaction of 26- and 28-kDa GST isoforms from *Schistosoma* species with testosterone, which was evaluated as a potential anthelmintic. Testosterone did not exhibit acute vermicide activity, as adult *Schistosoma mansoni* worms remained motile after 60 min, whereas praziquantel caused immediate complete immobilization. Homology models of *S. mansoni* Sm26GST and Sm28GST were validated by Ramachandran analysis and showed conserved catalytic cores with variable substrate-binding loops. Recombinant Sj26GST and Sh28GST were successfully overexpressed and purified. Both enzymes were inhibited by testosterone in a concentration-dependent manner, with IC₅₀ values of 0.63 mM and 0.73 mM, respectively. Far-UV Circular dichroism and extrinsic ANS fluorescence spectroscopy confirmed the conformational changes caused by the ligands. Induced-fit docking showed that the binding pocket is conserved at the GST dimer interface, and the 500-ns molecular dynamics simulations showed that the complexes were more stable than the apo proteins, with Sm28GST being more rigid. Testosterone analogues were identified by high-throughput virtual screening that predicted binding affinities higher than those of testosterone. The results suggest that schistosome GSTs are promising targets for structure-based drug discovery against schistosomes.

Keywords

Schistosoma mansoni; Glutathione *S*-transferase; Ligand activity; Testosterone; Steroid analogues; Molecular dynamics simulation; Induced-fit docking; Antischistosomal drug discovery

Graphical Abstract



Highlights

- Testosterone inhibits schistosome GSTs without causing acute worm death.
- Sj26GST and Sh28GST show IC₅₀ values of 0.63 and 0.73 mM, respectively.
- Testosterone binding induces marked conformational changes in schistosome GSTs.
- Docking and 500-ns MD simulation reveal a conserved interfacial steroid-binding pocket.
- Steroid analogues show stronger predicted GST binding, supporting lead discovery.

1.0 Introduction

Schistosomiasis is a chronic parasitic disease caused by trematode worms of the genus *Schistosoma* and remains one of the world's most important neglected tropical diseases, affecting over 250 million people globally, predominantly in sub-Saharan Africa [1, 2]. It is endemic in more than 70 countries, where it contributes substantially to morbidity, anaemia and impaired childhood development, with correspondingly high social and economic costs [2, 3]. *Schistosoma mansoni*, the agent of intestinal schistosomiasis, accounts for a large share of that burden and, together with *S. haematobium* and *S. japonicum*, is one of the three species responsible for most human infection [3, 4]. Despite decades of control effort, transmission persists wherever sanitation is poor, contact with contaminated freshwater is unavoidable and access to effective interventions is limited [2, 3]. Control therefore still depends on chemotherapy rather than interrupting transmission.

That chemotherapy rests on a single compound. Praziquantel has been the backbone of schistosomiasis control programmes for more than four decades and remains the only broadly available treatment [5-7]. Its limitations are well documented: it is poorly active against juvenile worms, so that recently acquired infections escape treatment; it does not prevent reinfection; and reduced susceptibility has been reported in both laboratory-selected and field isolates [5, 8, 9]. Oxamniquine, the only alternative with established clinical efficacy, is active against *S. mansoni* alone and is no longer widely produced [5]. Recent genetic analysis has identified a transient receptor potential channel as a determinant of praziquantel response, confirming that the drug acts through a defined molecular target that the parasite can alter [10]. This reliance on a single mechanism of action makes identifying new, mechanistically distinct molecular targets a priority [1, 11].

Glutathione transferases (GSTs) are among the most attractive of those targets. They constitute a conserved superfamily of multifunctional enzymes that catalyse the conjugation of reduced glutathione (GSH) to electrophilic centres, the first step in mercapturic acid formation [12, 13], and thereby protect the cell against xenobiotics, oxidative-stress products and reactive endogenous metabolites [13]. In *Schistosoma* spp. they are among the most abundant soluble proteins and are essential for survival in the oxidatively hostile environment of the host bloodstream [14, 15]. Two cytosolic isoforms dominate in *S. mansoni*, the 26 and 28 kDa enzymes (*Sm26GST* and *Sm28GST*), whose fold, catalytic mechanism and inter-subunit architecture have been defined in the schistosome orthologues [16] and which contribute to parasite development, host adaptation and protection from oxidative damage [6, 15, 17]. Their

abundance, surface accessibility and immunogenicity have made them leading vaccine candidates: the recombinant *Sh28GST* vaccine has progressed as far as a phase 3 trial in endemic populations [18, 19], and praziquantel itself has been crystallised in complex with the 26 kDa enzyme [16].

Beyond catalysis, GSTs possess a non-catalytic ligand-binding capacity, termed ligandin activity, that allows them to bind, sequester and transport hydrophobic molecules including fatty acids, haem, bilirubin, drugs and steroid hormones [7, 14, 15, 20]. This second function is far less well understood than the catalytic one, and its contribution to parasite physiology remains largely unresolved [15, 20]. It is of particular interest for steroids, because host steroid hormones, testosterone above all, regulate schistosome growth, reproduction and fecundity while simultaneously shaping the host immune response [21, 22]. Specific, saturable binding of testosterone to the *S. haematobium* 28 kDa GST has been demonstrated directly [22], and a selective ligand-binding site has been mapped to the schistosome GST dimer interface, a region that differs from its human counterpart [23]. What has not been established is whether this recognition extends across the schistosome GST family, what it does to enzyme structure and catalytic output, and whether the site involved is pharmacologically exploitable. These are the questions addressed here.

Advances in recombinant expression, biophysical characterisation and computational modelling now allow such questions to be approached from both directions at once. Combining enzyme kinetics, far-UV circular dichroism, extrinsic fluorescence spectroscopy, induced-fit docking and long-timescale molecular dynamics simulation yields a description of binding affinity, site stability, conformational change and the mechanism by which ligand binding modulates enzyme activity that no single method provides [8, 24, 25]. This study applies that combination to the interaction of *S. mansoni* GSTs with testosterone. Adult worms were exposed *in vitro* to testosterone and to praziquantel and GST activity measured in whole-worm lysates; *Sm26GST* and *Sm28GST* were modelled by homology, and the testosterone-binding site characterised by induced-fit docking, virtual screening of steroid analogues and 500 ns simulations; and recombinant *Sj26GST* and *Sh28GST*, orthologues of high sequence identity to the modelled *S. mansoni* enzymes, whose catalytic and ligand-binding regions were verified by sequence and structural comparison, were expressed, purified and characterised biochemically and biophysically. The aim was to determine whether ligand binding to schistosome GSTs is coupled to a measurable change in enzyme activity and structure, and

thereby to assess the potential of this interaction as a starting point for antischistosomal drug discovery.

Materials and Methods

2.1.1 Materials

Recombinant plasmid constructs *Sj*GST-3_pET-28a(+) and *Sh*GST_pET-11a were synthesized and purchased from GenScript Biotech, USA and the constructs were confirmed by Inqaba Biotec (Pretoria, South Africa). Chemically competent *Escherichia coli* BL21 (DE3) expression cells were purchased from New England Biolabs (Ipswich, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), antibiotics (penicillin, ampicillin sodium, kanamycin sulphate and streptomycin), Tween-20, as well as the protease inhibitor cocktail used during parasite homogenization were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Similarly, the biochemical assays utilized testosterone, praziquantel, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), imidazole, sodium phosphate salts, nickel sulfate (NiSO₄), and isopropyl β-D-1-thiogalactopyranoside (IPTG), were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Likewise, acrylamide, bis-acrylamide, ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, bromophenol blue, and 8-anilino-1-naphthalenesulfonic acid (ANS) were from Sigma-Aldrich. Computational modelling, including homology modelling, induced-fit docking, virtual screening, and molecular dynamics simulations, was performed using the Schrödinger Maestro v13 software suite (Schrödinger LLC, New York, NY, USA). Sequence analyses were performed using UniProt, SWISS-MODEL, PROCHECK, T-Coffee, Clustal Omega. BeStSel web server was used for analyzing protein CD spectra.

2.1.2 Study Design

The study employed a combination of *in vivo*, *in vitro*, and computational methods to investigate the activity of *Sm*GST with testosterone and to assess its therapeutic value. The study had three components. The first part involved maintaining *S. mansoni* in Swiss albino mice and *Biomphalaria pfeifferi* snails, recovering parasites by portal perfusion, exposing adult worms *in vitro* to either testosterone or praziquantel, and biochemically evaluating associated GST activity. The second part used computational and structural biology methods such as sequence analysis, homology modelling, induced-fit molecular docking, virtual screening, and

500 ns molecular dynamics simulations to characterise testosterone interactions with *Sm26GST* and *Sm28GST*. The third component first involved recombinant overexpression of *Sm26GST* and *Sm28GST* proteins in *Escherichia coli* BL21 (DE3) and their purification by immobilized metal affinity chromatography (IMAC). This was followed by their structural and functional analyses using enzyme kinetics, circular dichroism spectroscopy, intrinsic fluorescence spectroscopy, ANS-binding fluorescence spectroscopy, and testosterone inhibition assay to validate the ligandin function of the recombinant GSTs.

2.1.3 Study Area

This study was jointly carried out at three research institutions in Kenya and South Africa. *Schistosoma mansoni* life cycle maintenance, experimental infection of *Biomphalaria pfeifferi* snails and Swiss albino mice, recovery of adult worms by portal perfusion and in vitro parasite culture experiments were conducted at the Centre for Biotechnology Research and Development (CBRD) at the Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya. Laboratory animal husbandry, parasite maintenance and perfusion procedures were carried out according to institutional animal care, biosafety and ethical guidelines. The analyses of sequence retrieval, multiple sequence alignment, homology modelling, validation of homology models, preparation of the ligands, high-throughput virtual screening (HTVS), induced-fit molecular docking (IFD), molecular dynamics simulation setup, and trajectory analyses were performed at the Protein Structure-Function Research Laboratory (PSFRL), located in the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa. These were done using the Schrödinger Maestro software suite. PSFRL provided the high-performance computing resources for HTVS, IFD and molecular dynamics simulations. Recombinant protein overexpression, purification, enzyme kinetics, circular dichroism spectroscopy, and extrinsic fluorescence spectroscopy were also carried out at the PSFRL. General study coordination, data analysis, interpretation of results, and preparation of the manuscript were carried out in the Department of Biochemistry, Faculty of Science and Technology, University of Nairobi, Kenya.

2.1.4 Ethical Approval and Animal Use

All animal experiments were performed following the guidelines of the Care and Use of Laboratory Animals, both at the institution and nationally. The study was approved by the Scientific and Ethics Review Unit (SERU), Kenya Medical Research Institute (KEMRI),

Nairobi, Kenya, with ethics clearance number ACUC/01.10.2025. Swiss albino male mice were used for parasite maintenance and worm recovery.

2.2 *In vivo* Experimental Procedures

2.2.1 Maintenance of the *Schistosoma mansoni* Life Cycle, Snail Infection, and Mouse Infection

Biomphalaria pfeifferi, the intermediate host of *S. mansoni*, were collected from the Rangwena River in Homa Bay County, Kenya. Snails were brought to the laboratory, where they were kept under a 12-hour light/dark cycle, at 25-27 °C, in aerated, dechlorinated water. Fresh lettuce was provided to the snails ad libitum and observed frequently. A laboratory parasite cycle of Swiss albino mice previously infected with *Schistosoma mansoni* was used to obtain miracidia from eggs recovered from the infected mice. To induce miracidial emergence, the eggs were hatched in dechlorinated water in the presence of light. Miracidia were placed on individual snails, which were kept in the laboratory. Cercarial shedding was triggered by light exposure after 4 weeks of infection, and freshly shed cercariae were collected for mouse infection. The experimental infection was carried out in five male Swiss albino mice (*Mus musculus*) between 6 and 8 weeks of age. Mice were kept under standard laboratory conditions and had free access to food and water. About 25-30 cercariae were subcutaneously inoculated in the dorsal region of each mouse. Daily observation and upkeep of animals for eight weeks was made to allow maturation of adult worms. Mice were euthanized via an intraperitoneal overdose of a ketamine and xylazine mixture (10:1 ratio) eight weeks post-infection, in strict accordance with approved institutional animal welfare protocols. Adult *Schistosoma* worms were immediately collected via hepatic portal perfusion using pre-warmed physiological saline. The recovered parasites were washed multiple times in sterile saline to eliminate host blood contaminants and immediately processed for downstream *in vitro* analysis.

2.2.2 The *in vitro* Treatment of Adult *Schistosoma mansoni* Worms

Given the involvement of host-derived hormonal signals and antischistosomal treatment in modulating parasite physiology, adult *S. mansoni* worms were exposed *in vitro* to testosterone and praziquantel to investigate their effects on parasite GST activity. This approach enabled the direct assessment of changes in GST activity following exposure to the hormone and the established antischistosomal drug under controlled experimental conditions. Therefore, adult worms of *S. mansoni* were picked up and washed several times in normal saline to eliminate

host tissue. About 15 worms were transferred into each well of a 6-well culture plate with 5 mL Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin-streptomycin and incubated at 37 °C for 1 hour to acclimatize the worms. Testosterone was dissolved in NaOH-PBS buffer (100 mM stock solution) and considered as a putative GST inhibitor, while praziquantel (PZQ) was dissolved in ethanol and taken as a positive control. Worms were treated with the last dose of 2.5 mM testosterone or PZQ. A number of control groups were supplied with the same volume of the corresponding solvents. After treatment, the parasites were incubated for 5, 10, 15, 30, and 60 minutes, and worm responses, as well as morphological condition, were observed during the incubation period. Worms were then washed in protease inhibitor-containing phosphate-buffered saline (PBS) with 1% (v/v) Tween 20 and kept at -80 °C, followed by homogenization in a tissue homogenizer and stored at -80 °C for further analysis.

2.2.3 The preparation of whole-worm lysates and total GST activity assay

To evaluate the effects of the experimental treatments on the glutathione-dependent detoxification capacity of adult *Schistosoma mansoni*, whole-worm lysates were prepared and subsequently used for the determination of total GST activity. The total GST activity assay was carried out to quantify the CDNB-conjugating activity in the worm lysates under the experimental conditions. Concentrated whole-worm homogenates were obtained with centrifugal concentrators with a molecular weight cut-off of 10 kDa. Prior to the enzymatic analysis, protein concentrations were measured with a NanoDrop Microvolume Spectrophotometer (Thermo Scientific, US). Total GST activity in the whole-worm homogenate was determined using the standard CDNB conjugation assay. Concentrated lysates were placed in sodium phosphate buffer with the addition of 5 mM reduced glutathione (GSH), while the reaction was initiated with the addition of 1 mM CDNB. The absorbance at 340 nm of the formation of GSH-CDNB conjugate was measured with a Jasco V-730 spectrophotometer. Using whole-worm lysates meant that total parasite proteins were present in the lysate, allowing relative GST-associated activity to be observed between the testosterone-treated, praziquantel-treated, and solvent-treated control groups.

2.3 Sequence Analysis, Structural Modelling, and Molecular Dynamics Simulations

2.3.1 Sequence Retrieval and Multiple Sequence Analysis

Given the importance of identifying conserved and variable regions that could provide insight into the structural and functional characteristics of the two *Schistosoma* species, and to investigate the sequence characteristics and evolutionary conservation, protein sequences were retrieved from publicly available databases and subjected to multiple sequence analysis. Therefore, the amino acid sequences of the Glutathione *S*-transferase isoforms *Sm26GST* (UniProtKB accession P15964) and *Sm28GST* (UniProtKB accession P09792) of *Schistosoma mansoni* were downloaded from the UniProtKB database in FASTA format. Multiple sequence alignment was performed using Clustal Omega to determine the sequence similarity and conserved and variable regions between the two GST isoforms. GST sequences were also retrieved from *S. haematobium* and *S. japonicum* for comparative analysis. The resulting alignment was analyzed for conservation of residues in the glutathione-binding (G-site) and hydrophobic substrate-binding (H-site) regions, and other residues important for ligand recognition. The conserved and variable residues were then taken into account in the homology modelling and structural analysis of the GST isoforms. GST sequences of *Schistosoma japonicum* and *Schistosoma haematobium* were also obtained for comparison. Multiple sequence alignments were carried out with T-Coffee and Clustal Omega to find conserved catalytic residues, substrate binding regions and sequence similarities between the GST orthologues. The suitability of the recombinant GST constructs as representative models of the native *S. mansoni* GST isoforms was then evaluated by sequence conservation.

2.3.2 Homology Modelling and Structural Validation

Three-dimensional structural models of *Sm26GST* and *Sm28GST* were created by homology modelling in order to provide a consistent structural framework for comparative investigation of testosterone and ligand-induced conformational dynamics. The stereochemical quality and reliability of the predicted models were then evaluated by structural validation prior to molecular docking and molecular dynamics simulations. The SWISS-MODEL homology-modelling server was used to generate three-dimensional structures of *Sm26GST* and *Sm28GST*. The template structures were selected according to sequence similarity, structural coverage and suitability for modelling the respective GST isoforms. The crystal structure of PDB ID 6RWD was chosen as the template for *Sm26GST*, while the crystal structure of PDB ID 1OE7 was chosen as the template for *Sm28GST*. The retrieved template structures and target sequences were then submitted to the SWISS-MODEL modelling pipeline to generate the 3D GST models. The resulting models were then checked for stereochemical quality with PROCHECK. The Ramachandran plot analysis was carried out to assess the distribution of

amino acid residues in the favoured, additionally allowed, generously allowed and disallowed regions. The overall stereochemical quality of the models was evaluated by the percentage of residues in the favoured regions and the calculated G-factor values.

2.3.3 Computational hardware and software used for Molecular Dynamics Simulations

Two high-performance computing units were used for this study. These computing units were equipped with specialized configurations. They are 16 CPU Intel core i7 5960x extreme edition (3.3 GHz, 20 M cache 16× cores), Nvidia GTX 750Ti graphics card, 32 GB DDR4-2133 MHz memory on an MSI X99 motherboard and 264 GB RAM. For the protein-ligand preparation, HTVS, Induced fit docking and also post-dynamics analysis components of the study, an implementable Schrödinger Maestro v13.0 Windows OS desktop was used. Molecular dynamics simulation studies were conducted on a Linux server running the 2019 GPU-enabled version of Schrödinger Desmond.

2.3.4 Protein and Ligand Preparation

To prepare the chemically appropriate and energetically minimized structures of *Sm26GST*, *Sm28GST*, testosterone, and the selected testosterone analogues for docking, protein and ligand preparation was performed. Correct assignment of bond orders, protonation states, hydrogen atoms, and ligand conformations is crucial to ensure accurate predictions of protein-ligand interactions, and hence they were carried out. Testosterone and a library of one hundred testosterone analogues were retrieved from the PubChem database in structure data file (SDF) format and imported into the molecular modelling suite Schrödinger Maestro v13.0 for ligand preparation. The Testosterone and Testosterone analogues structures were subjected to processing in the LigPrep module to obtain energetically favourable three-dimensional structures for molecular docking. LigPrep preparation involved correction of structural representations, generation of protonation and tautomeric states at physiological pH (7.0 ± 2.0) and energy minimization using the OPLS4 force field. The prepared ligands were then converted to Maestro format for induced-fit docking and high-throughput virtual screening. The homology-modelled structures of *Sm26GST* and *Sm28GST* were used as receptor structures for the computational analyses. Both *Sm26GST* and *Sm28GST* were prepared with the Protein Preparation Wizard in Maestro v13.0 before docking. Protein preparation included the assignment of bond orders, the addition of missing hydrogen atoms, the correction of structural and connectivity information, and the optimization of the hydrogen-bonding

network. The protonation states of the ionizable residues were assigned at near-physiological conditions ($\text{pH } 7.0 \pm 0.2$) to ensure a chemically appropriate representation of the proteins during the calculations of ligand binding. The resulting protein structures were then subjected to restrained energy minimization using the OPLS4 force field to eliminate steric clashes and to optimize the local geometry while maintaining the overall experimentally or computationally determined protein fold. Heavy-atom convergence was used in minimization to prevent significant changes to the original protein structures. The stereochemical integrity of the proteins was preserved during the preparation process, and the resulting minimized structures were stored in Maestro format for further receptor-grid generation, high-throughput virtual screening, and induced fit docking.

2.3.5 High-Throughput Virtual Screening

To identify the most promising testosterone analogues with high binding affinity to *Sm26GST* and *Sm28GST*, high-throughput virtual screening (HTVS) was performed using the Virtual Screening Workflow (VSW) implemented in Schrödinger Maestro v13.0. This method allowed for prioritization of a large ligand library prior to more computationally demanding docking and molecular dynamics studies. Testosterone served as the reference ligand, and steroid analogues were chosen as a screening library (of one hundred Testosterone analogues) from the PubChem database. The initial filtration of compounds was done with the help of QikProp to evaluate drug-like properties including molecular weight, LogP, hydrogen bond donors and acceptors, aqueous solubility and Lipinski's Rule of Five. Compounds that passed the filtration step were further subjected to molecular docking analysis. The hierarchical three-step docking process included HTVS, Standard Precision (SP) docking and Extra Precision (XP) docking. In HTVS, the ligand library was screened quickly using the OPLS_2005 force field to identify ligands with high binding affinity. Top-ranked ligands after HTVS were docked again using the SP protocol to increase docking precision. Finally, the best-ranked ligands were docked in the XP stage, where sampling and scoring functions provide the best results in terms of ligands with high binding affinity and geometrical features. Docking score, Glide energy and interactions of compounds with the target protein were used for ranking of ligands. Those steroidal analogues that had the lowest scores and good interactions with residues located at the GST dimer interface were subjected to induced fit docking and molecular dynamic studies.

2.3.6 Induced-Fit Docking

To consider the flexibility of receptors during the ligand docking, Induced-Fit Docking (IFD) was carried out with the IFD protocol in Schrödinger Maestro v13.0. This approach allows simultaneous conformational flexibility of both the ligand and the protein binding site, thus making it possible to represent a more realistic model of the interaction. Docking was performed independently using the full GST homodimer of both *Sm26GST* and *Sm28GST* structure (chains A and B), since structural studies showed that ligand binding often occurs at the hydrophobic interface between the two subunits[19]. The grids were generated in the centre of the putative binding pocket defined by the structural analysis. An OPLS_2005 force-field implicit-solvent model was used during docking. Ligands were first docked in the receptor using Glide (Standard Precision, SP) docking, and then residues around the predicted binding site were optimized in Prime to consider the flexibility of the receptor. Optimized protein-ligand complexes were then re-docked with Glide to yield optimized binding poses. The complexes formed were ranked by IFD score, GlideScore, Emodel, and protein-ligand interaction energies. The most stable testosterone conformation for each GST isoform was determined and then used for the following molecular dynamics simulations. Moreover, the best-scoring testosterone analogues obtained from the virtual screening were run through the same IFD procedure and their top-ranked poses were chosen for subsequent MD simulations: bolandiol for *Sm26GST*, and 19-nor-5-androstenediol for *Sm28GST*.

2.3.7 Molecular Dynamics Simulations

To enhance the reliability of the docking results and to gain a deeper understanding of how Testosterone and its analogues influence the functions of *Sm26GST* and *Sm28GST*, additional computational studies and molecular dynamics simulations (MDS) at 500 ns were performed. This involved the utilization of Maestro v13.0, which incorporates the GPU-enabled Desmond molecular dynamics simulation engine. The use of the System Builder module of the Desmond algorithm was applied in building six different systems namely; *Sm26GST*:Apo, *Sm26GST*:Testo, *Sm26GST*:TestoAnalog, *Sm28GST*:Apo, *Sm28GST*:Testo, *Sm28GST*:TestoAnalog) which were later used in molecular dynamics simulation on the Linux Ubuntu desktop platform for MDS. The complexes were solvated in TIP3P water explicitly in an orthorhombic box with 46 Å buffer around the protein on all sides. Neutralization of the system was achieved through addition of suitable counter ions Na⁺ and Cl⁻, and an ambient salt

concentration of 0.15 M NaCl was added to reproduce physiological conditions. Energy minimization followed by equilibration steps were performed using OPLS_2005 force field. Following equilibration steps, the molecular dynamics simulations were performed for 500 ns in the NPT ensemble at 300 K and 1.01325 bar pressure with the use of a Nose-Hoover thermostat and a Martyna-Tobias-Klein barostat, respectively. Backbone root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and ligand RMSD analyses were performed on the trajectory.

2.4 Recombinant Gene Synthesis, Protein Overexpression, Purification, Enzymatic and Biophysical Characterization of Schistosome Glutathione S-Transferases

2.4.1 Gene Synthesis, Recombinant Overexpression and Purification of *Sj26GST* and *Sh28GST*

In the absence of *Sm26GST* and *Sm28GST* to be used for biochemical and biophysical characterizations, recombinant *Sj26GST* and *Sh28GST* were employed respectively as orthologous representatives for comparative investigations related to *Schistosoma mansoni*. These proteins were included to provide comparative insights into the interactions between testosterone and GSTs within the schistosome GST family. Accordingly, recombinant overexpression and purification of *Sj26GST* and *Sh28GST* were undertaken to obtain sufficient quantities of well-defined proteins for controlled biochemical and biophysical characterizations. Genomic sequences encoding *Schistosoma* GSTs were retrieved from the NCBI database and used for vector design. *Sj26GST*_pET-28a(+) and *Sh28GST*_pET-11a were commercially synthesized by GenScript. Following a heat-shock transformation procedure, chemically competent *Escherichia coli* BL21 (DE3) cells were used for the transformation of *SjGST*_pET-28a(+) and *ShGST*_pET-11a plasmids (GenScript). The transformation mixture was recovered and plated by plating 100 µL of the resuspended cells onto Luria-Bertani (LB) agar plates containing the appropriate selective antibiotic. In particular, LB agar plates with kanamycin (50 µg/mL) were used for the selection of *Sj26GST*_pET-28a(+) transformants and ampicillin (100 µg/mL) was used for the selection of *Sh28GST*_pET-11a transformants. Plates were incubated overnight for 16 h at 37 °C. One single colony from each plate was picked and grown in 2 × yeast tryptone (2YT) medium (1% (v/v) yeast extract, 1.6% (w/v) tryptone, 0.5% (w/v) NaCl) containing the appropriate antibiotic for 16 hours at 37 °C with shaking at 250

rpm. The overnight cultures were then used to inoculate fresh 2YT expression medium at a 1:50 dilution ratio. Cultures were grown until an optical density at 600 nm (OD₆₀₀) of 0.6 was reached, at which point recombinant protein expression was induced with 0.5 mM IPTG. Induction was carried out for 6 h to allow recombinant protein overexpression. Immobilized metal affinity chromatography (IMAC) was used to purify recombinant *Sj26GST* and *Sh28GST* proteins. Purification was carried out on a nickel (Ni²⁺)-charged HisTrap® column (GE Healthcare, Sweden) using an ÄKTA Start Purifier system (Cytiva Life Sciences, USA). To obtain purified *Sj26GST* and *Sh28GST* for downstream analyses, a single-step elution was performed using a buffer containing 100 mM sodium phosphate, 500 mM imidazole, 0.01% (w/v) NaN₃, pH 7.2.

2.4.2 SDS-PAGE Analysis and Protein Quantification

The expression and purification profiles of recombinant *Sj26GST* and *Sh28GST* were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous gel system comprising a 4% stacking gel and a 12.5% resolving gel. Following electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue R-250 and destaining until distinct bands appeared. Gel images were captured under uniform lighting against a white background using a digital camera. Protein concentrations were measured spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) prior to subsequent enzymatic and spectroscopic analyses.

2.4.3 Determination of Recombinant *Sj26GST* and *Sh28GST* Specific Enzymatic Activity

The spectrophotometric determination of the enzymatic specific activity of recombinant overexpressed and purified *Sj26GST* and *Sh28GST* was performed by the standard glutathione *S*-transferase mediated CDNB-GSH conjugation assay. This involves the catalytic activity of *Sj26GST* and *Sh28GST* to conjugate reduced glutathione (GSH) to the electrophilic substrate CDNB [20], [21]. The assay was performed in triplicate in a reaction buffer (100 mM NaH₂PO₄, 0.01% (w/v) NaN₃, pH 7.4) with 5 mM GSH and 1 mM CDNB. While the recombinant *Sj26GST* and *Sh28GST* enzymes were then added at varying concentrations to the reaction mixture, the enzymatic reaction was initiated by adding CDNB. This reaction was similarly carried out in the presence of 1 mM testosterone and PZQ, in order to assess their inhibitory effects. The reaction product, 1-(S-glutathionyl)-2,4-dinitrobenzene (a dinitrophenyl

thioether chromophore), was continuously monitored by measuring the increase in absorbance at 340 nm using a Jasco V-730 UV-Vis spectrophotometer. All measurements were done in triplicate in a 3 mL quartz cuvette at 20 °C over a 60 s interval, using an extinction coefficient (ϵ_{340}) of 9600 M⁻¹ cm⁻¹ for -(S-glutathionyl)-2,4-dinitrobenzene for quantification. The background non-enzymatic rates of CDNB-GSH conjugation were measured under the same conditions in the absence of enzyme and subtracted from the total reaction rates to give corrected enzymatic absorbance.

2.4.4 Effect of Testosterone and Praziquantel on Recombinant SGST Activity

This experiment aimed to assess the direct effect of testosterone and praziquantel on the catalytic activity of recombinant *Sj26GST* and *Sh28GST*. The effect of testosterone and praziquantel (PZQ) on the catalytic activity of *Sj26GST* and *Sh28GST* was examined by the standard CDNB-GSH conjugation assay, which involves the conjugation of GSH with CDNB by the glutathione *S*-transferase (GST) enzyme. GSH is the nucleophile, and CDNB is the electrophilic substrate, and the formation of the thioether conjugate is followed spectrophotometrically at 340 nm. To ensure that the ligand-protein interaction takes place, the recombinant *Sj26GST* and *Sh28GST* were pre-incubated with either testosterone or praziquantel before the enzymatic reaction was initiated. The pre-incubation was carried out under controlled conditions using concentrations of 1mM testosterone and 1mM praziquantel. This step was designed to test if these compounds could alter the enzymatic activity by binding directly to the enzyme or by changing the conformation of the enzyme, which could alter the catalytic site or nearby structural areas. After pre-incubation, the reaction was initiated by adding 1 mM CDNB or 5 mM GSH to a reaction mixture containing 1 mM GSH in NaH₂PO₄, 0.01% (w/v) NaN₃, pH 7.4. The activity of the enzyme was measured by measuring the absorbance at 340 nm for 60 s at 20 °C in a Jasco V-730 UV/Vis spectrophotometer. Each assay was carried out in triplicate with different concentrations of *Sj26GST* and *Sh28GST* to ensure the reaction progress curves were linear. The initial linear phase of product formation was used for activity calculations, and all data were corrected for background non-enzymatic CDNB-GSH conjugation rates. The molar extinction coefficient ($\epsilon_{340} = 9600 \text{ M}^{-1} \text{ cm}^{-1}$) was used for quantification of product formation. The activity of the enzymes was compared to untreated control reactions, which served as the baseline GST activity under the same experimental conditions. The degree of modulation caused by each ligand was reported as a percentage of the control. This enabled quantitative evaluation of ligand-mediated inhibition or activation of the catalytic function of *Sj26GST* and *Sh28GST*. The activity profiles obtained were then

compared to assess the differential effects of testosterone and praziquantel on the enzymatic efficiency of *Sj26GST* and *Sh28GST* and to infer their potential effects on GST-mediated detoxification processes in *Schistosoma* species.

2.4.5 Far-UV Circular Dichroism (CD) Spectroscopy

The secondary structure of the recombinant *Sj26GST* and *Sh28GST* was determined by Far-UV CD spectroscopy to assess whether binding of testosterone, praziquantel, or glutathione altered the folding of the proteins with respect to their secondary structures. This provided a biophysical evaluation of ligand-induced conformational changes. Their CD spectra were measured using a Jasco J-1500 CD spectropolarimeter over a wavelength range of 180 - 260 nm at 20 °C, and all measurements were performed in triplicate. A 6µM each of *Sj26GST* and *Sh28GST* were measured in their apo state and also after incubation with 1mM GSH, 1mM testosterone or 1mM praziquantel. All samples were prepared in 100 mM NaH₂PO₄, pH 7.4, to determine the changes in protein secondary structure upon substrate or ligand binding. The CD data were reported as mean residue molar ellipticity [θ] using Equation 1

$$[\theta] = \frac{100 \times \theta}{C \times n \times l} \quad (1)$$

where θ is the observed ellipticity in degrees, l is the optical path length in cm, C is the protein concentration in mg.mL⁻¹, M is the molecular mass of the protein, and n is the number of amino acid residues. The mean residue molar ellipticity is reported in deg cm² dmol⁻¹. This normalization allows direct comparison of CD data from different sample preparations and spectropolarimetric systems, ensuring consistency and reproducibility in structural analyses. The secondary structure content was then estimated using the BeStSel web server.

2.4.6 Extrinsic (8-anilino-1-naphthalenesulfonic acid) ANS Fluorescence Spectroscopy

To investigate changes in the solvent-accessible hydrophobic regions of the GSTs after ligand binding, ANS fluorescence spectroscopy was used. ANS fluorescence spectroscopy was employed. Changes in the ANS fluorescence spectrum were used to monitor changes in hydrophobic pocket accessibility and to assess ligand-induced conformational changes. The tertiary structures of *Sj26GST* and *Sh28GST* apo proteins and their bound forms were evaluated using a JASCO FP-6300 spectrofluorometer with Spectra Manager software v1.5.00 (Jasco Inc., Tokyo, Japan). The structural conformation and interactions of *Sj26GST* and *Sh28GST* in

the presence and absence of testosterone and the anthelmintic drug PZQ were assessed using 8-anilino-1-naphthalenesulfonic acid (ANS) as an extrinsic fluorescent probe that binds to protein hydrophobic pockets. The apo *Sj26GST* and *Sh28GST* samples, 6 μ M each, in 100 mM NaH_2PO_4 , pH 7.4, with 200 μ M ANS, were spectroscopically analyzed using a 10 mm pathlength quartz cuvette at 20 °C. Fluorescence emission spectra were recorded at an excitation of 395 nm and between 400 and 650 nm. A 1 cm path length, an excitation bandwidth of 5 nm, a 2.5 nm emission bandwidth with a data pitch of 1 nm, and a scanning speed of 200 nm/min were used, and measurements were recorded in triplicate. The readings were buffer-corrected before averaging. This procedure was repeated in the presence of reduced glutathione (GSH), a GST substrate. testosterone and PZQ and their binding effects on the hydrophobic cleft of both *Sj26GST* and *Sh28GST* were also assessed. Before spectral measurements, 1 mM Testo and 1 mM PZQ were separately incubated with 6 μ M *Sj26GST* and 6 μ M *Sh28GST* in the presence and absence of GSH for 30 min. During this monitored spectroscopic analysis, spectra from three accumulations were averaged and recorded. Recorded spectra were buffer-corrected, and fluorescence intensities in the presence of Testo and PZQ were also corrected for the inner filter effect to account for absorption of excitation (primary) and emission (secondary) light.

2.4.7 Determination of Testosterone IC₅₀

The testosterone IC₅₀ assay was carried out to quantify the amount of testosterone needed to inhibit the catalytic activity of the recombinant *Sj26GST* and *Sh28GST* by 50%. The half-maximal inhibitory concentration (IC₅₀) is a quantitative measure of a ligand's or drug's potency: the concentration needed to inhibit a particular enzyme activity or biological response by 50% [19]. The half-maximal inhibitory concentration (IC₅₀) of testosterone against recombinant *Sj26GST* and *Sh28GST* was spectrophotometrically determined under identical experimental conditions for both enzymes, using the CDNB-GSH conjugation assay. The activity of the enzymes was measured at 340 nm by measuring the time course for 60 s on a Jasco V-630 UV/Vis spectrophotometer. The recombinant *Sj26GST* and *Sh28GST* were pre-incubated with increasing concentrations of testosterone before initiating the reaction with CDNB. The reaction mixture contained 12.5 nM of the respective recombinant *Sj26GST* or *Sh28GST*, 5 mM GSH, 1 mM CDNB, and 100 mM NaH_2PO_4 , pH 7.4. In each case, an enzyme control (without testosterone) was included to represent 100% *Sj26GST* or *Sh28GST* activity. The percentage residual activity was calculated relative to the corresponding untreated control and plotted against the logarithm of testosterone concentration. Nonlinear regression analysis

was performed in GraphPad Prism to fit dose-response data, and characteristic S-shaped (sigmoid) inhibition curves were generated using a four-parameter variable-slope sigmoidal (logistic) curve. The IC₅₀ was determined as the concentration of testosterone needed to inhibit enzymatic activity by 50% along the fitted sigmoidal dose-response curve for each SGST isoform. The resulting IC₅₀ values were then compared to the inhibitory potency of testosterone against *Sj26GST* and *Sh28GST*.

2.5 Statistical Analysis

GraphPad Prism software was used for all the analysis of experimental data. Non-linear regression models were used to fit dose-response curves, and $p < 0.05$ was considered to be statistically significant. Since these datasets did not satisfy the assumptions of normality, comparisons among multiple simulation systems were performed using the non-parametric Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests for post hoc comparisons. The Mann-Whitney U test was used to analyze the difference between two groups, namely ligand RMSD (root mean square deviation) and ligand solvent-accessible surface area (Lig-SASA). A P value < 0.05 was considered to be statistically significant. The dimeric *Sm26GST* and *Sm28GST* molecular dynamics trajectories were analyzed to extract residue-wise Ca RMSF values for each individual monomeric subunit (subunit 1 and subunit 2). RMSF data were exported for statistical analysis and comparison of residue fluctuations across the N-terminal and C-terminal domains of each GST monomer in the different simulation systems. To identify the most prevalent collective motions and conformational changes of the GST proteins during the simulations, principal component analysis (PCA) was carried out on the MD trajectories using Python. The resulting principal components were then compared between the apo, testosterone-bound, and testosterone-analogue-bound systems.

3.0 Results

3.1.1 Effects of Testosterone and Praziquantel on Adult Worms

This assay was performed to compare the acute activity of testosterone and praziquantel against adult *S. mansoni* viability and motility, as a biological control for subsequent activity against schistosome GST activity. The antischistosomal drug, praziquantel, was used as the positive control, and testosterone was evaluated to determine whether its interaction with GSTs had a direct schistosomicidal effect. Adult *Schistosoma mansoni* worms exposed to testosterone remained viable throughout the 60-minute observation period, exhibiting motility comparable

to that of the solvent control groups (NaOH-PBS and ethanol). In contrast, worms treated with praziquantel showed complete loss of viability from the first observation at 5 minutes and remained non-viable for the duration of the experiment. Assessment of adult *S. mansoni* viability following exposure to testosterone and praziquantel, relative to the corresponding solvent controls, is summarized in Table 1. Similarly, Figure 1 shows the morphological appearance of adult male and female *S. mansoni* worms after exposure to PZQ and testosterone, as observed under a dissecting microscope.

Table 1. Viability assessment of adult *Schistosoma mansoni* worms following exposure to testosterone, praziquantel, and solvent controls.

Treatment	5 min	10 min	15 min	30 min	60 min
Testosterone	M	M	M	M	M
Praziquantel	NM	NM	NM	NM	NM
Ethanol (solvent control)	M	M	M	M	M
NaOH-PBS (solvent control)	M	M	M	M	M

where M = Motile (motile and morphologically intact); NM = non-motile (absence of motility and loss of viability).

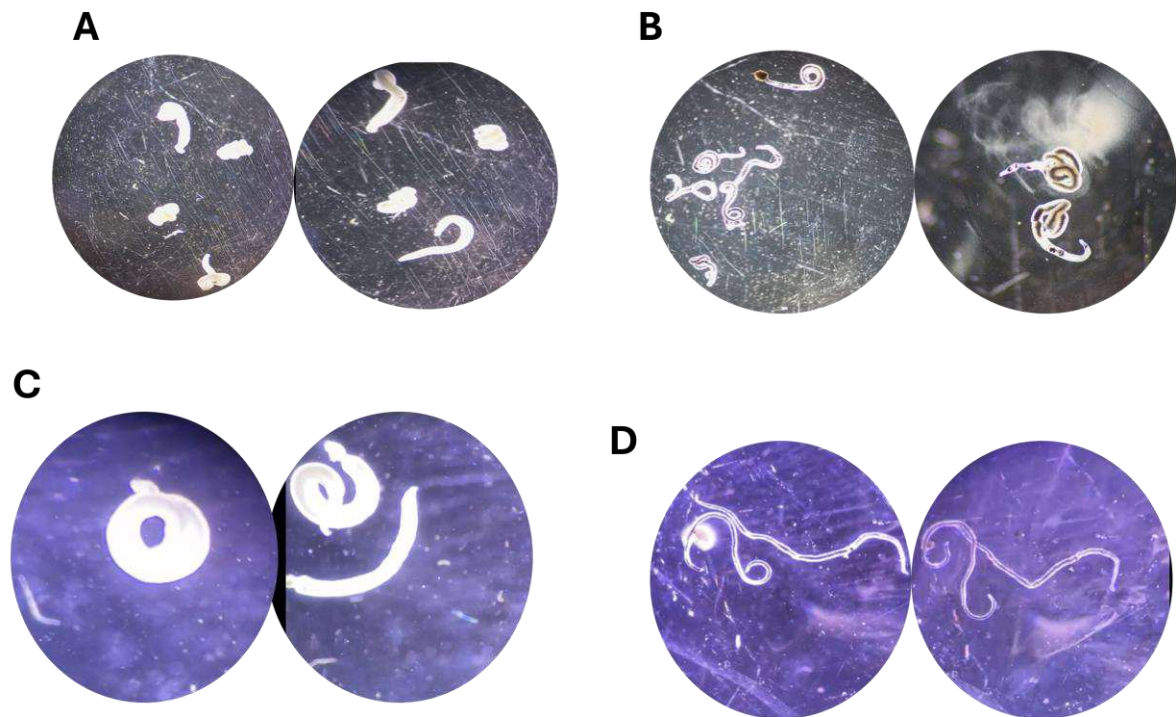


Figure 1. The morphological appearance of adult male and female *S. mansoni* worms after exposure to (A) Male worms exposed to PZQ, (B) Female worms exposed to PZQ, (C) Male worms exposed to testosterone (Testo), and (D) Male worms exposed to testosterone (Testo), as observed under a dissecting microscope.

3.1.2 GST-Associated Total Enzyme Activity Assay for the Treated Whole-Worm Lysate

To determine whether the experimental treatments altered the glutathione-dependent enzymatic activity of adult *S. mansoni*, GST-associated total activity was assessed in the prepared whole-worm lysates. The measured CDNB-conjugating activity was subsequently compared among the respective treatment groups to determine treatment-associated changes in total GST activity. GST-associated enzymatic activity in whole-worm lysates was measured after *in vitro* treatment of adult *Schistosoma mansoni*. GST-associated activity was assessed using the CDNB-GSH conjugation assay by monitoring absorbance at 340 nm over a 1-minute reaction period, as shown in Figure 2.

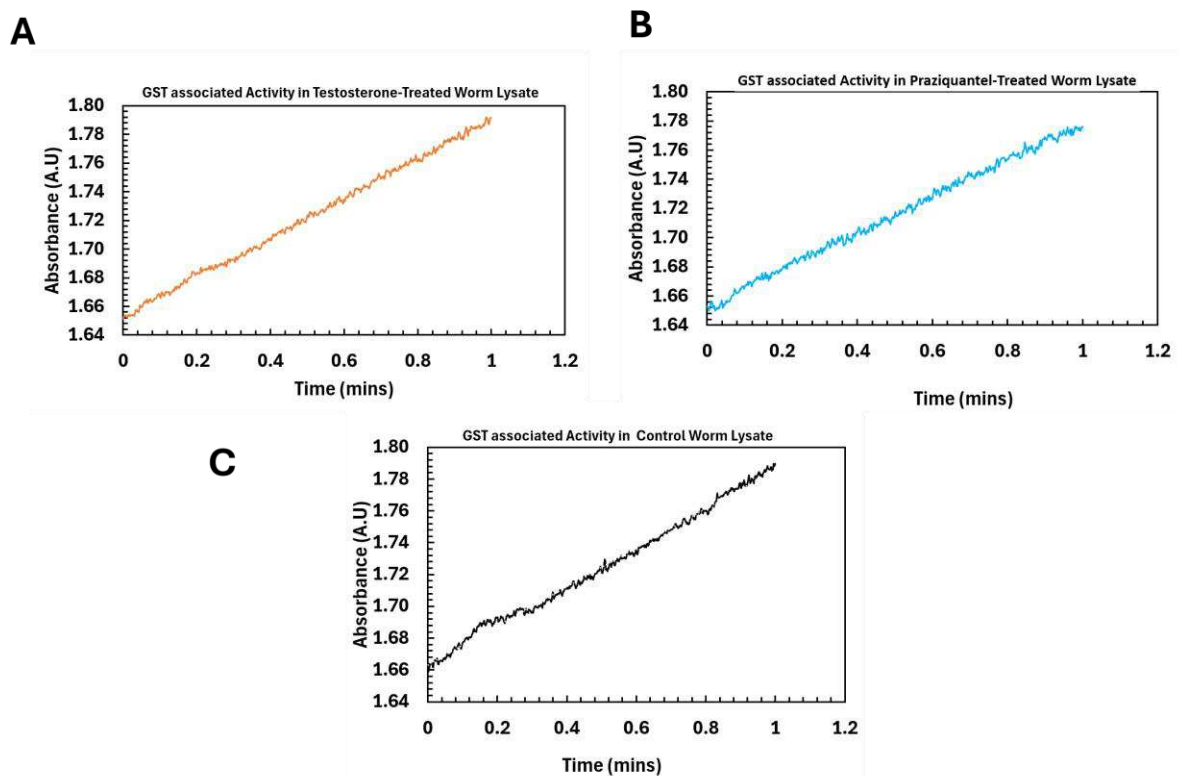


Figure 2. GST-associated activity in (A) testosterone-treated, (B) praziquantel-treated, and (C) control worm lysates, measured by CDNB-GSH conjugation over a 1-minute reaction period. A similar progressive increase in absorbance was observed in testosterone-treated, praziquantel-treated, and control worm lysates, demonstrating detectable CDNB-conjugating activity in the respective protein extracts. The assay confirmed the presence of total GST-associated enzymatic activity in the recovered worm lysates following *in vitro* treatment. Because the protein concentrations of the lysates were not equivalent, the observed reaction profiles were reported descriptively and were not used for direct comparison of GST activity between treatment groups.

3.2 Sequence Conservation and Structural Modelling of *Sm*GSTs

3.2.1 Multiple Sequence Alignment Reveals High Conservation Among Schistosome GSTs

Multiple Sequence Alignment was done with GSTs of various species of *Schistosoma* to test the sequence conservation of different species. The sequence alignment of the 26 kDa GST isozyms from *S. mansoni* and *S. japonicum* (Figure 3A) and the 28 kDa GST isozyms from *S. haematobium* and *S. mansoni* (Figure 3B) indicated that there was a high degree of conservation between each of the two classes, reflecting a high degree of evolutionary conservation between the orthologous schistosome GSTs. The *Sm26GST* and *Sm28GST* sequences were aligned, and comparatively low levels of conservation were detected (Figure 3C), suggesting a higher sequence divergence between the two classes of GST.



Figure 3. T-Coffee multiple sequence alignments of *Schistosoma* GST isozyms. (A) GST26 sequences from *S. mansoni* (P15964) and *S. japonicum* (P08515). (B) GST28 sequences from *S. haematobium* (P30113) and *S. mansoni* (P09792). (C) Alignment of *S. mansoni* (*Sm26GST* vs. *Sm28GST*), showing conservation between isozyms classes. Colors indicate alignment confidence (BAD/AVG/GOOD); in the "cons" row, * = identical, : = strongly similar, . = weakly similar residues.

3.2.2 Structures from Homology Modelling and Structural Validation by Ramachandran plots

Homology models of *Sm26GST* and *Sm28GST* were then generated to provide three-dimensional structures for downstream docking and molecular dynamics studies. Both the two models predicted had the characteristic glutathione *S*-transferase (GST) fold, consisting of an N-terminal thioredoxin-like domain followed by an α -helical C-terminal domain, with the greatest structural variation occurring in the loop regions between the two domains that

distinguish the two isoforms, as illustrated in Figure 4. The superimposition of the two homology models also revealed that the overall structure of *Sm26GST* and *Sm28GST* was highly conserved, with only minor differences in the flexible loop and terminal regions (Figure 5)

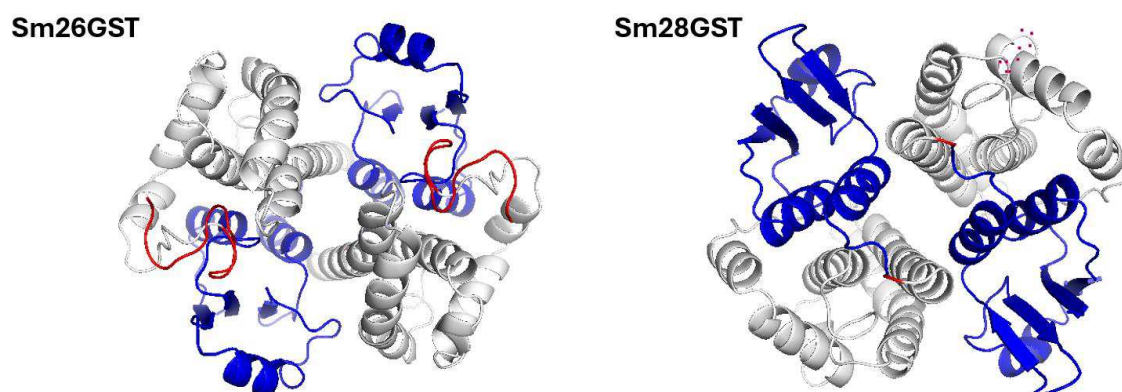


Figure 4. Predicted three-dimensional structures of *Sm26GST* and *Sm28GST* generated by homology modelling. The tertiary structures of *Sm26GST* (left) and *Sm28GST* (right) are shown in ribbon representation. The N-terminal domain is coloured **blue**, while the C-terminal domain is coloured **grey**. Regions highlighted in **red** represent loop segments exhibiting structural variability between the two GST isoforms.

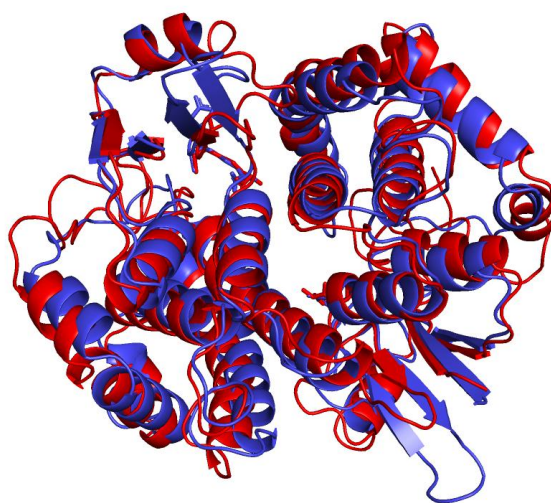


Figure 5. Structural superimposition of *Sm26GST* and *Sm28GST* homology models. Ribbon superimposition of the predicted three-dimensional structures of *Sm26GST* (**red**) and *Sm28GST* (**blue**) showing the high degree of structural conservation between the two GST isoforms. Extensive overlap of the α -helices and β -strands demonstrates conservation of the overall protein architecture, while minor structural deviations are observed primarily within flexible loop regions and terminal segments.

To assess the stereochemical and conformational quality of the predicted GST structures, both models were structurally validated using Ramachandran plot analysis with PROCHECK. It was found that 95.8% of non-glycine/proline residues in the *Sm26GST* model and 92.9% in the *Sm28GST* model in the most favoured regions, which is above the 90% threshold set from the

analysis of 118 high-resolution structures (≤ 2.0 Å resolution, R-factor ≤ 20.0), as shown in Table 2. The G-factors of 0.02 for Sm26GST and -0.00 for Sm28GST also indicated good stereochemical quality for both models. The only significant difference between the two models was in the distribution of omega dihedral angles (*Sm26GST*: -0.79 ; *Sm28GST*: -0.64), which were flagged as unusual but not highly unusual and are typical of minor deviations observed in homology-modelled structures. These results collectively indicate that both homology models were suitable for further structural and docking studies.

Table 2 PROCHECK Ramachandran plot and G-factor statistics for the *Sm26GST* and *Sm28GST* homology models.

Structure	<i>Sm26GST</i>	<i>Sm28GST</i>
Template (PDB)	6rwd	1oe7
Total residues	434	418
Non-Gly/Pro residues	380	364
Most favoured regions	364 (95.8%)	338 (92.9%)
Additional allowed regions	14 (3.7%)	22 (6.0%)
Generously allowed regions	2 (0.5%)	2 (0.5%)
Disallowed regions	0 (0.0%)	2 (0.5%)

3.3.1 High-Throughput Virtual Screening of Testosterone Analogues

High-throughput virtual screening (HTVS) was carried out to identify testosterone analogues with good predicted binding to *Sm26GST* and *Sm28GST*. The screened compounds were ranked by docking score, with bolandiol being the top hit for *Sm26GST* with a docking score of -6.54589 kcal/mol (Glide Gscore -6.54589 , Glide Emodel -26.8338 kcal/mol, Glide energy -25.5171 kcal/mol), followed by 19-nor-5-androstenedione and 19-nor-5-androstenediol (Table 3). The best hit for *Sm28GST* was 19-nor-5-androstenediol with a docking score of -5.65495 kcal/mol (Glide Gscore -5.65495 , Glide Emodel -55.009 kcal/mol, Glide energy -39.5089 kcal/mol), followed by norethynodrel and 17-methyandrost-4-ene-3 β ,17 β -diol (Table 4).

Table 3. High-throughput virtual screening (HTVS) results for *Sm26GST*.

Rank	Compound_CID	Compound_name	docking_score	glide_gscore (kcal/mol)	glide_emodel	glide_energy (kcal/mol)
1	9835303	Bolandioli	-6.54589	-6.54589	-26.8338	-25.5171
2	18633059	19-Nor-5-androstenedione	-6.43214	-6.43214	-24.5003	-21.8064
3	9900246	19-Nor-5-androstenediol	-6.33061	-6.33061	-30.2084	-23.0415
4	10634	Androstenediol	-5.86905	-5.86905	-15.0248	-14.0871
5	69259387	17-Methylandro-4-ene-3 β ,17 β -diol	-5.63583	-5.63583	-20.0834	-1.44718
6	136297	4-Androstenediol	-5.40401	-5.40401	-20.453	-13.1862

Table 4. High-throughput virtual screening (HTVS) results for *Sm28GST*.

Rank	Compound_CID	Compound_name	Docking_score	Glide_Gscore (kcal/mol)	Glide_Emodel	Glide_energy (kcal/mol)
1	9900246	19-Nor-5-androstenediol	-5.65495	-5.65495	-55.009	-39.5089
2	6231	Norethynodrel	-5.59739	-5.59739	-55.4856	-41.2677
3	69259387	17-Methylandro-4-ene-3 β ,17 β -diol	-5.22558	-5.22558	-48.5702	-36.1343
4	10634	Androstenediol	-5.02813	-5.02813	-50.5764	-37.5552
5	9835303	Bolandioli	-4.94353	-4.94353	-48.8835	-36.9504
6	160531	Androst-5-ene-3,17-dione	-4.8643	-4.8643	-48.3712	-36.1777

3.3.2 Induced Fit Docking Analysis

Following HTVS and the identification of testosterone analogues with good predicted binding, induced-fit docking (IFD) was performed using the selected testosterone analogues against *Sm26GST* and *Sm28GST* to further characterize their predicted binding modes. The resulting 2D interaction maps showed that the ligands occupied an interfacial binding region involving residues from both subunits of the GST homodimers. Several residues, particularly Leu, Glu, and Arg, were recurrently observed among the predicted ligand-protein interactions (Figure 6). Three-dimensional inspection of the docked complexes showed that this interfacial site lies between the two symmetrical subunits, with the ligand interacting with residues from both chains and thus bridging the dimer interface, which may help stabilize the dimeric assembly (Figure 7).

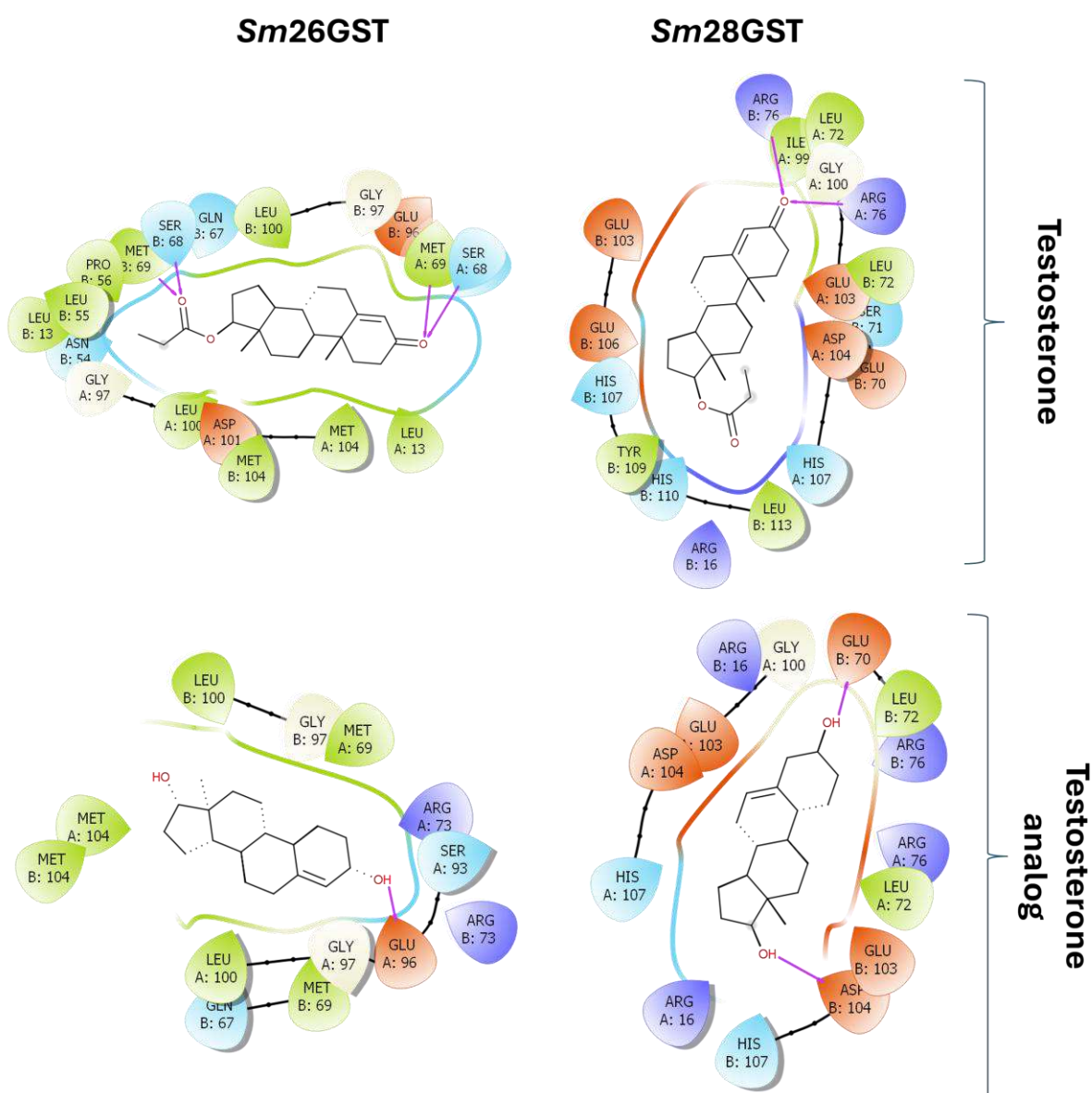


Figure 6. Induced fit docking 2D Ligand Interaction Diagram for both *Sm26GST* and *Sm28GST* with Ligands (testosterone and a representative testosterone analog). These 2D interaction maps detail how the ligand anchors itself within the interfacial site through a network of hydrophobic contacts (green/lime bubbles) and hydrogen bonds (purple arrows) with residues from both subunits. The consistent involvement of residues like Leu, Glu, and Arg in both chains across all four panels highlights a stable, symmetrical binding environment where the ligand acts as a central bridge between the dimeric halves.

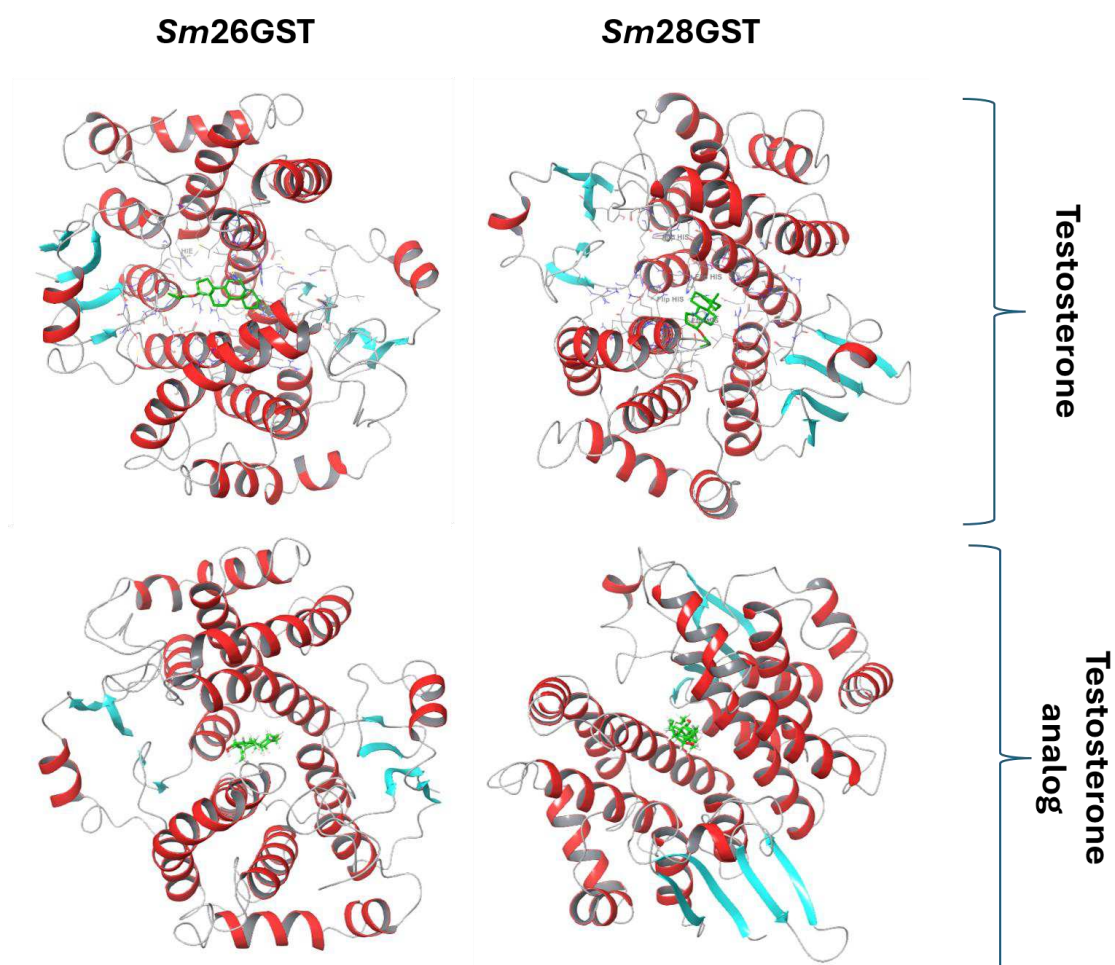


Figure 7. Three-dimensional images for Induced Fit Docking for *Sm26GST* and *Sm28GST*. The active site is strategically nestled between the two symmetrical subunits. In this orientation, the ligand makes contact with residues from both chains, effectively bridging the interface and potentially stabilizing the dimeric assembly.

Table 5. IFD analysis for *Sm26GST* with the selected testosterone analogues

Entry ID	Docking score	glide gscore (kcal/mol)	Glide Emodel	Glide energy (kcal/mol)
1	-8.7425	-8.7425	-68.3490	-45.1650
2	-7.9494	-7.9494	-66.8893	-45.4310
3	-8.4347	-8.4347	-65.4686	-44.0740
4	-8.4038	-8.4038	-63.0755	-43.1390
5	-7.4357	-7.4357	-60.7089	-42.4170
6	-8.1562	-8.1562	-63.1237	-43.1400

Table 6 IFD analysis for *Sm28GST* with the selected testosterone analogues

Entry ID	Docking score	Glide Gscore (kcal/mol)	Glide Emodel	Glide energy (kcal/mol)
1	-6.1609	-6.1609	-55.3758	-39.1880
2	-6.1095	-6.1095	-50.8968	-35.4390
3	-5.9385	-5.9385	-51.0908	-35.5240
4	-6.1509	-6.1509	-53.4871	-38.6570
5	-5.6972	-5.6972	-50.7890	-37.0370
6	-6.2495	-6.2495	-54.0358	-38.5770

3.3.3 Molecular Dynamics Simulation Analysis

To evaluate the impact of ligand binding on protein stability and dynamics, 500 ns molecular dynamics simulations (MDS) were carried out for the apo *Sm26GST* and *Sm28GST*, and their testosterone-bound as well as testosterone-analogue-bound forms. The C α -RMSD trajectories of *Sm26GST*, *Sm26GST*:Testo, *Sm26GST*:Testo-Analog, *Sm28GST*, *Sm28GST*:Testo, and *Sm28GST*:Testo-Analog were analyzed to assess structural deviations relative to their respective initial structures. Molecular dynamics simulations were performed for 500 ns under isothermal-isobaric (NPT) conditions at 300 K and approximately 1 bar, using the OPLS_2005 force field and TIP3P water model. The C α -RMSD values were plotted as a function of simulation time to evaluate the structural stability and conformational behaviour of the apo and ligand-bound GST systems. The C α -RMSD profiles revealed that the apo forms of both isoforms were more flexible than their respective complexes with the ligands, suggesting that the binding of the ligands stabilizes both *Sm26GST* and *Sm28GST*, with the testosterone-analogue complex showing the most consistent profile for *Sm26GST*, and *Sm28GST* showing lower RMSD values and less fluctuation than *Sm26GST*, indicating greater intrinsic structural stability (Figure 8A and B).

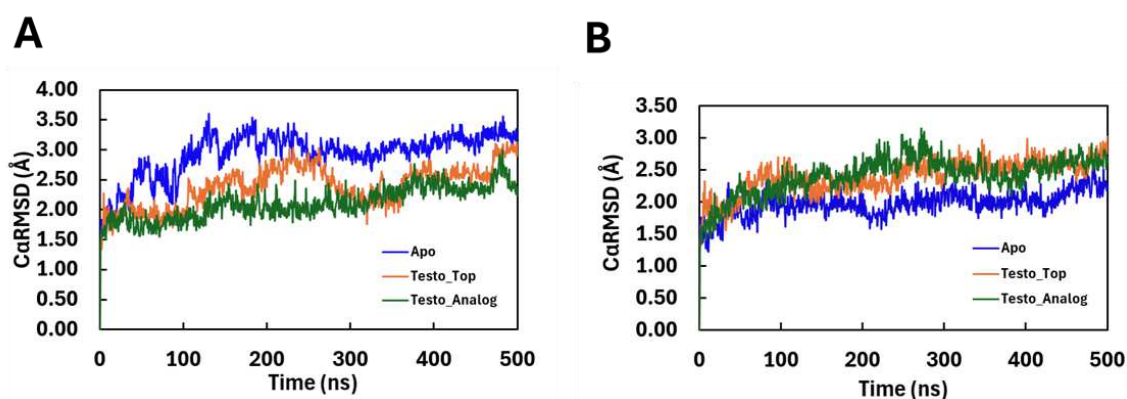


Figure 8. Molecular dynamics simulations of (A) Ca RMSD profile of *Sm26GST* and (B) *Sm28GST* for 500 ns. The apo form (blue), testosterone-bound complex (orange), and testosterone-analogue-bound complex (dark green) are shown.

Similarly, to assess residue-level flexibility and ligand-induced changes in protein dynamics, the side-chain root-mean-square fluctuation (RMSF) profiles of *Sm26GST*, *Sm26GST:Testo*, *Sm26GST:Testo-Analog*, *Sm28GST*, *Sm28GST:Testo*, and *Sm28GST:Testo-Analog* were calculated from the 500 ns MD trajectories and plotted against residue number. RMSF provides a residue-wise measure of atomic positional fluctuations throughout the simulation and was therefore used to compare local flexibility among the apo and ligand-bound systems. Ca-RMSF analysis showed that the backbone fluctuations were lower in the bound state than in the apo state for *Sm26GST*, while *Sm28GST* had lower fluctuations overall, regardless of the presence of the ligand, and that the bound state was further stabilized by increased rigidity in the protein (Figure 9A and B).

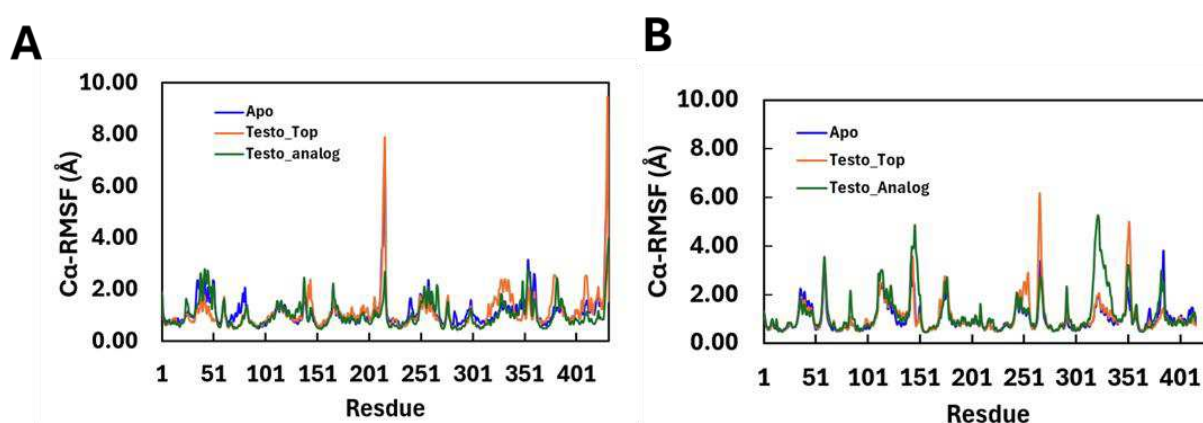


Figure 9. A Residue-wise Ca-RMSF profile of *Sm26GST* and B. Residue-wise Ca-RMSF profile of *Sm28GST* for the apo form (blue), testosterone top-ranked complex (orange), and testosterone analogue complex (dark green).

To evaluate changes in ligand compactness and conformational stability during the simulations, ligand radius of gyration (RoG) was analyzed. Analysis of ligand RoG over the simulation trajectory showed that, for *Sm26GST*, the apo protein maintained the lowest RoG values, while the top-ranked testosterone pose and the testosterone analogue maintained slightly higher but stable RoG profiles, indicating compact, conformationally stable ligand geometries. The RoG values for all the ligand-bound systems in *Sm28GST* were found to be very stable with very small fluctuations, indicating that there is little change in the shape of the ligand and the two ligands are of similar compactness, as seen in Figure 10 A and B.

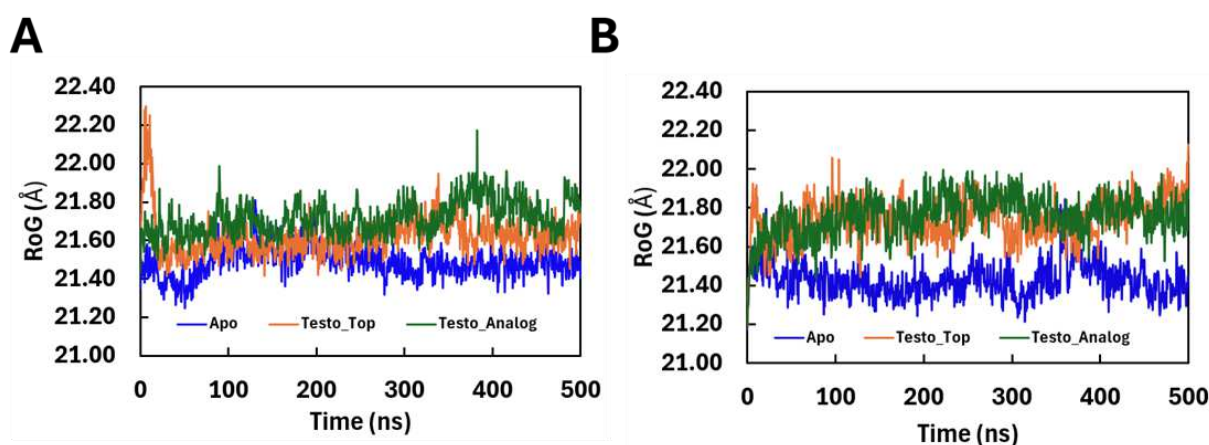


Figure 10. (A) Time evolution of ligand radius of gyration (RoG) for *Sm26GST* systems over 500 ns and (B) Time evolution of ligand RoG for *Sm28GST* systems for the apo form (**blue**), testosterone top-ranked complex (**orange**), and testosterone analogue complex (**dark green**).

Furthermore, the ligand-RMSD and solvent-accessible surface area (SASA) of the *Sm26GST*, *Sm26GST*:Testo, *Sm26GST*:Testo-Analog, *Sm28GST*, *Sm28GST*:Testo, and *Sm28GST*:Testo-Analog systems were analyzed throughout the 500 ns MD trajectories. Ligand-RMSD was used to monitor the conformational stability and positional deviation of testosterone and its analogue within the respective GST binding environments, whereas SASA was evaluated to assess changes in solvent exposure and overall structural compactness of the protein-ligand systems. The resulting ligand-RMSD and SASA profiles were plotted against simulation time to compare the dynamic behaviour of the apo and ligand-bound systems. Testosterone had higher ligand RMSD and more fluctuations than the analogue in *Sm26GST* (Figures 11A and B), suggesting that it is more mobile in the binding site, while the analogue is more stable. Likewise, the analogue had lower and more consistent SASA values, indicative of deeper or more persistent pocket burial, while testosterone had higher and more variable SASA values, indicative of greater solvent exposure. The Ligand-RMSD and SASA profiles of both ligands were relatively stable in *Sm28GST*, with the analogue having a slightly higher SASA profile,

suggesting differences in binding orientation and pocket shielding (Figures 12A and B). In general, the *Sm28GST* complexes were more stable towards the ligand than the *Sm26GST* complexes.

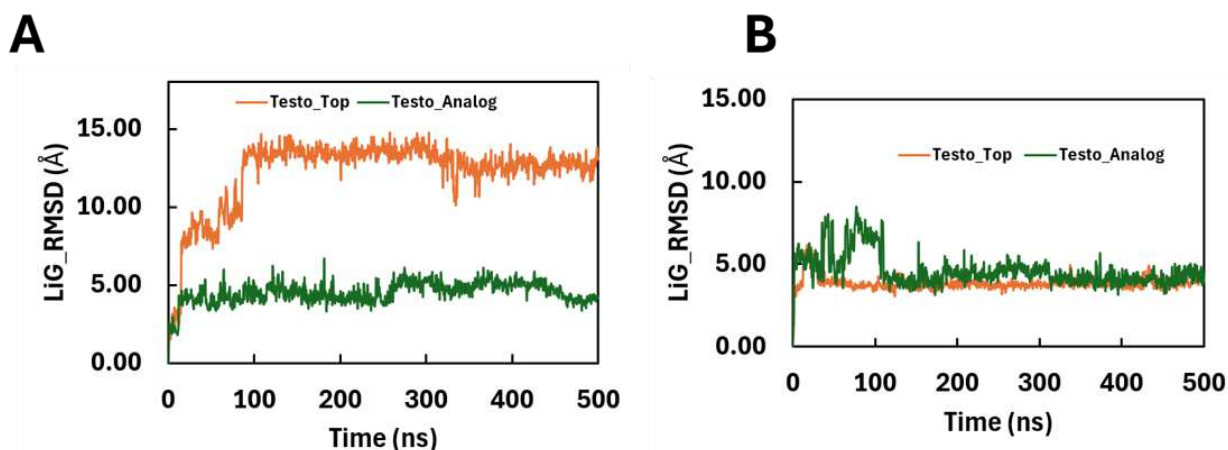


Figure 11 (A) Time evolution of ligand RMSD for *Sm26GST* complexes during the 500 ns molecular dynamics simulation and (B) for ligand RMSD for *Sm28GST*.

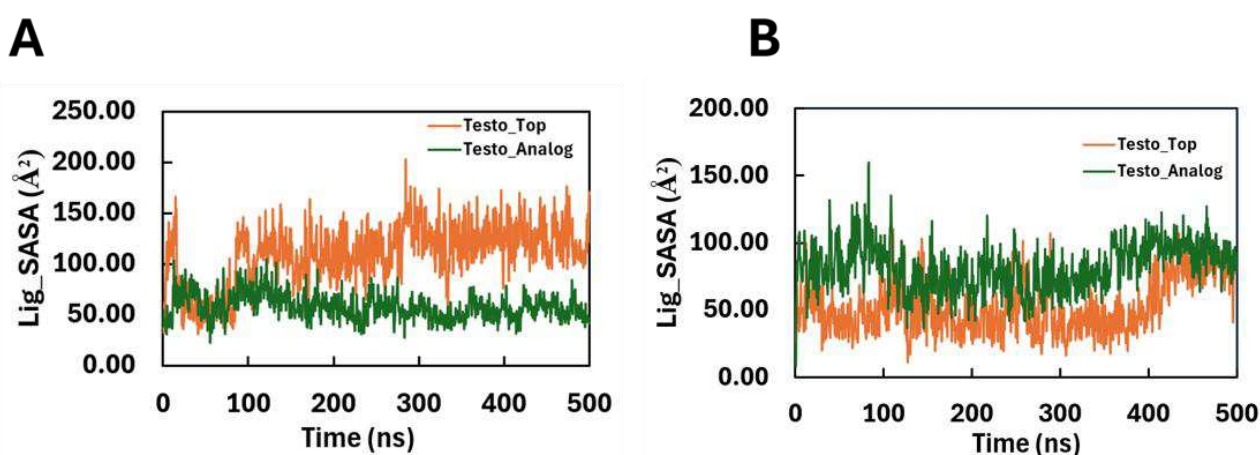


Figure 12. (A) Time evolution of ligand SASA for *Sm26GST* complexes over 500 ns and (B) Time evolution of ligand SASA for *Sm28GST* for testosterone top-ranked complex (orange), and testosterone analog complex (dark green).

3.4.1 Recombinant Expression and Purification of SGSTs

To enable comparative biochemical and biophysical characterization of schistosome GSTs, recombinant *Sj26GST* and *Sh28GST* were selected as orthologous representatives for the *S. mansoni* GSTs investigated in biological and computational studies. This is given the unavailability of *Sm26GST* and *Sm28GST* constructs. Recombinant *Sh28GST* and *Sj26GST* were expressed in *E. coli* BL21(DE3) and purified for further downstream applications. The bacterial cells containing the *Sj26GST*_pET-28a(+) and *Sh28GST*_pET-11a constructs were grown on LB agar plates supplemented with kanamycin and ampicillin, respectively, and

positive colonies were expanded in overnight LB cultures at 37 °C. Protein expression was induced by adding 0.5 mM IPTG to the cultures at 30 °C, and the cells were harvested by centrifugation. The recombinant proteins were purified using Ni²⁺-affinity chromatography, and the purified fractions were subjected to 12.5% SDS-PAGE analysis. The purified proteins ran at the expected molecular weights of ~28 kDa for *Sh28GST* and ~26 kDa for *Sj26GST*, indicating successful overexpression and purification of both enzymes for further functional and structural studies. The workflow of the procedure is as summarized in Figure 13.

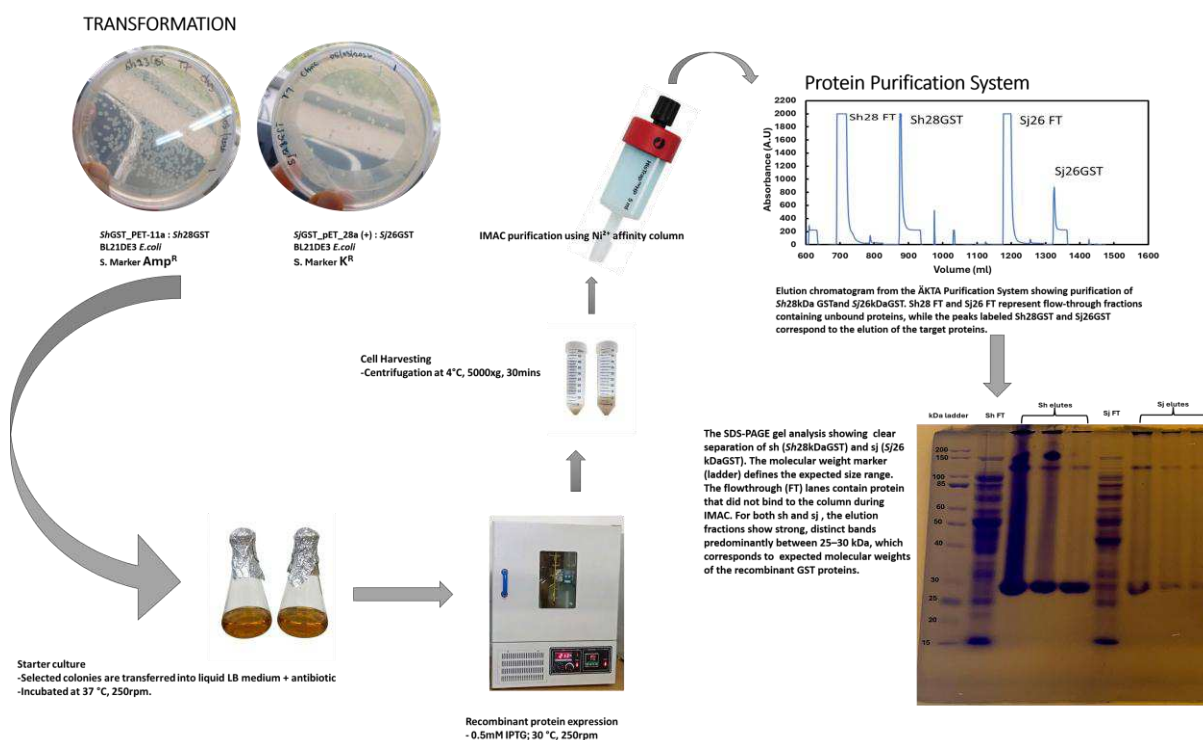


Figure 13. Schematic workflow for recombinant *Sh28GST* and *Sj26GST* expression, purification, and analysis.

3.4.2 Specific Enzymatic Activity of Recombinant *Sj26GST* and *Sh28GST*

The purified recombinant enzymes were subsequently evaluated for catalytic activity in the absence and presence of praziquantel and testosterone (using the GSH-CDNB assay) to determine whether either compound altered their enzymatic function. Specific enzyme activity was calculated from the slope of the linear regression generated by plotting GST activity (μmol/min) against enzyme concentration (mg) (Figure 14). Praziquantel did not significantly alter the activity of *Sj26GST* relative to the untreated enzyme, whereas testosterone significantly reduced its specific activity by 47.36%. In contrast, praziquantel increased the specific activity of *Sh28GST* relative to baseline, while testosterone produced a decrease in activity, consistent with the response observed for *Sj26GST*. A direct comparison of the

untreated enzymes also revealed that *Sj26GST* had a significantly higher intrinsic specific activity (slope = 134.5) than *Sh28GST* (slope = 58.28), suggesting that the two schistosome GST isoforms have significantly different intrinsic catalytic efficiencies, despite their differential sensitivity to testosterone and praziquantel.

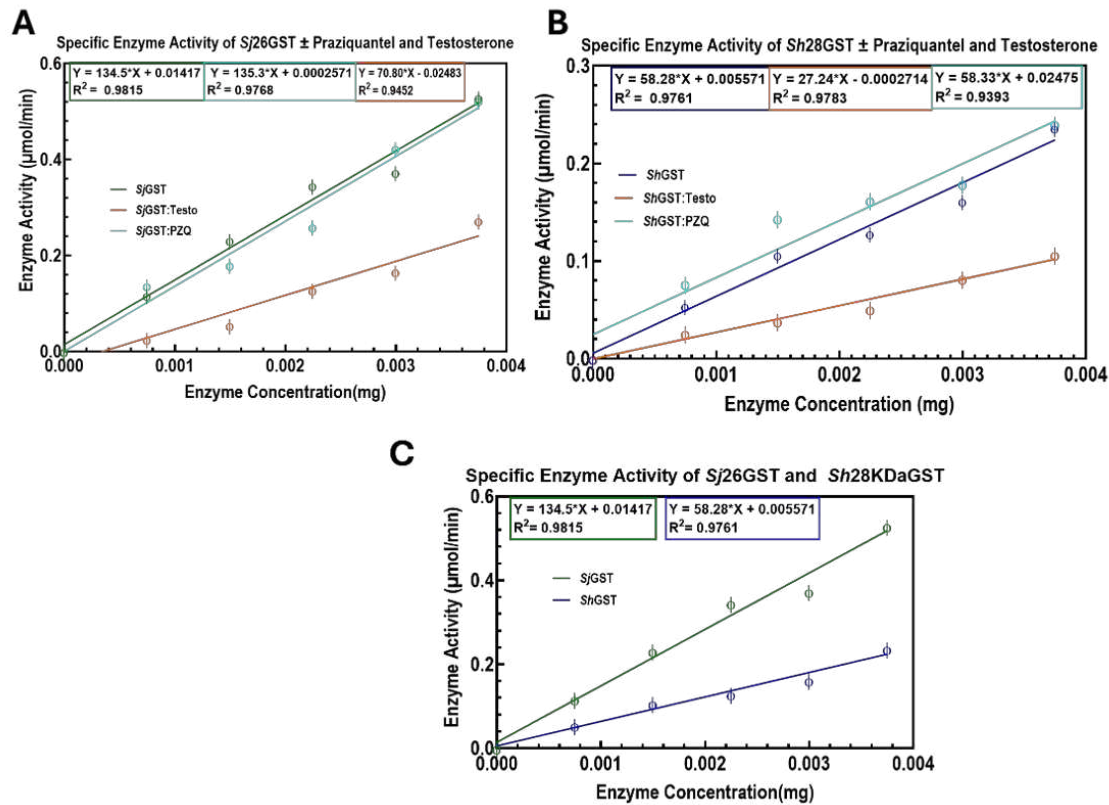


Figure 14. Specific enzyme activity of *Sj26GST* and *Sh28GST* is modulated by praziquantel and testosterone. Specific enzyme activity ($\mu\text{mol}/\text{min}$) was measured as a function of enzyme (*Sj26GST* and *Sh28GST*) concentration (mg). This was also repeated in the presence of praziquantel (PZQ) and testosterone (Testo). Data points represent mean $\pm$ SEM, and solid lines show linear regression fits; the slope of each line reflects the specific enzyme activity, with corresponding equations and R^2 values shown in the inset boxes (colour-coded to match each condition). (A) *Sj26GST* activity assayed alone (green), with testosterone (orange), or with PZQ (cyan). PZQ had little effect on *Sj26GST* activity relative to the untreated enzyme, whereas testosterone markedly reduced its specific activity. (B) *Sh28GST* activity assayed alone (blue), with testosterone (orange), or with PZQ (cyan). PZQ increased *Sh28GST* specific activity above baseline, while testosterone reduced it, similar to the pattern observed for *Sj26GST*. (C) Direct comparison of baseline (untreated) specific enzyme activity between *Sj26GST* (green) and *Sh28GST* (blue), showing that *Sj26GST* exhibits a higher intrinsic specific activity (slope = 134.5) than *Sh28GST* (slope = 58.28).

3.4.3 Structural Characterization by Far-UV CD Spectroscopy

Far-UV circular dichroism (CD) spectroscopy was used to investigate the effect of ligand binding on the secondary structure of the recombinant enzymes over 190-250 nm wavelength. Both apo *Sj26GST* and *Sh28GST* showed CD spectra typical of significant α -helical content, with only minor changes upon binding of the natural substrate, glutathione (GSH), suggesting that GSH alone does not cause significant secondary structural changes in either isoform.

(Figures 15A and B). Both enzymes, however, showed a significant conformational change in the presence of testosterone, with or without GSH (Figures 15C and D), whereas praziquantel showed relatively small spectral changes with respect to testosterone in both proteins (Figures 15E and F).

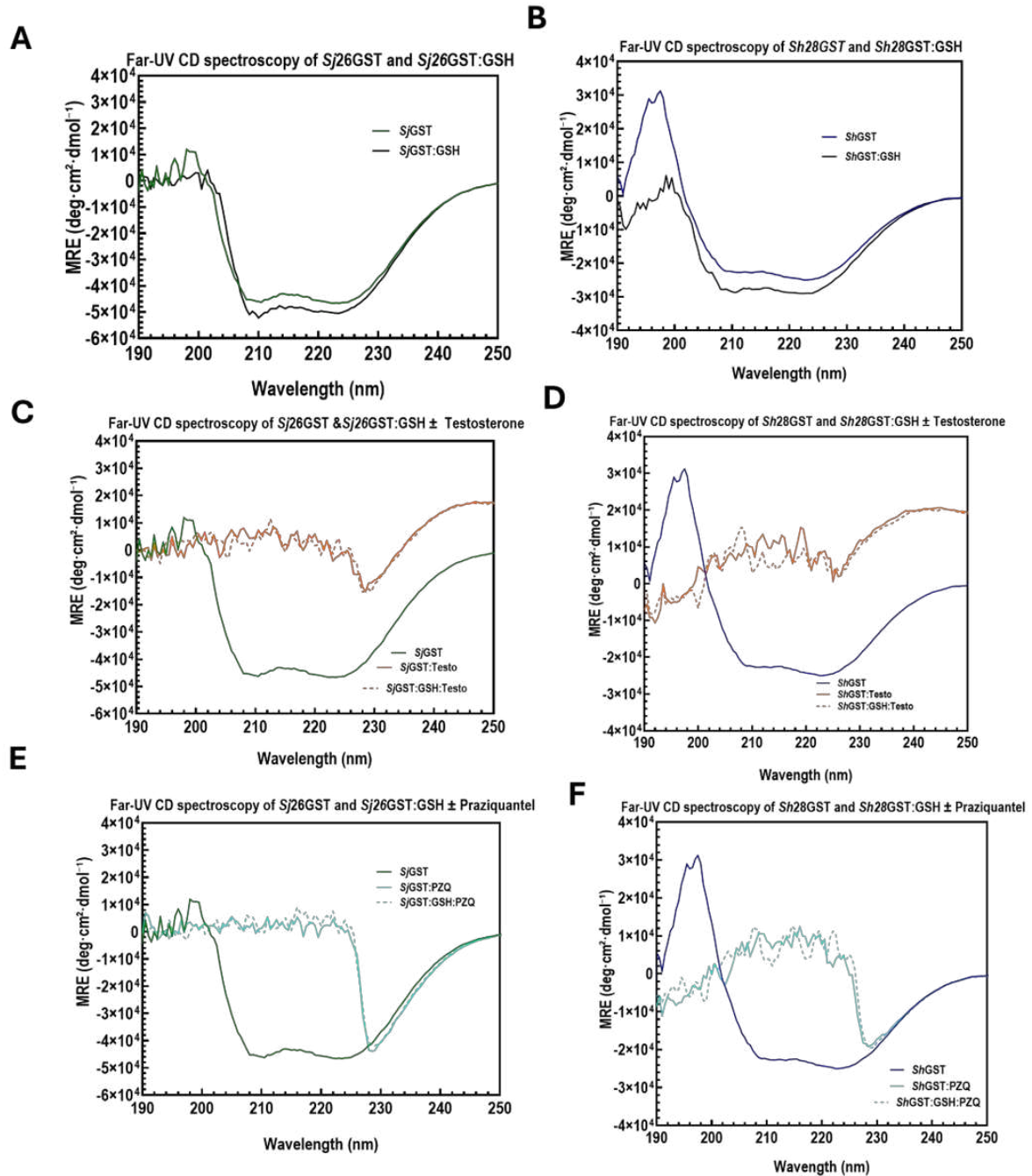


Figure 15. Secondary structure changes in *Sj26GST* and *Sh28GST* upon GSH binding and in the presence of testosterone and praziquantel are detected by Far-UV CD spectroscopy. The spectra are shown as mean residue ellipticity (MRE, $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) against wavelength (nm). Apo protein and GSH complex (A,B). Free enzyme:GSH complex (*Sj26GST*, *Sh28GST*). (C,D) Effect of testosterone, with and without GSH, for *Sj26GST* and *Sh28GST*, respectively. (E,F) Effect of praziquantel, with and without GSH, for *Sj26GST* and *Sh28GST*, respectively. The minima in the spectrum at 208-222 nm is typical of α -helical secondary structure, and is present in all panels.

These changes in ellipticity are most pronounced in the 208 - 222 nm region, which is diagnostic of α -helical structure, and the results are compared with the no-and substantial inhibition in the specific enzymatic activity of *Sj26GST* and *Sh28GST* with PZQ and testo, respectively. These qualitative observations were confirmed by quantitative deconvolution of the CD spectra performed with the BeStSel web server. The apo enzyme of *Sj26GST* was mostly α -helical (58.2%) with a small β -sheet component, and this α -helical content was mostly preserved after GSH binding (52.4%) as seen in Table 7. Testosterone binding, however, was accompanied by a near-complete loss of α -helical signal (0.0%) and the appearance of a strong antiparallel β -sheet component (63.4%) even in the presence of GSH (65.1% antiparallel β -sheet, 0.0% α -helical). PZQ caused a relatively small decrease in α -helical content (2.9 - 4.2%) and did not induce an antiparallel β -sheet component but rather increased the amount of turns and random-coil content. The same trend was seen for *Sh28GST* (Table 8): the apo form and the GSH-bound form had significant α -helical content (55.4% and 52.4%, respectively), whereas binding to testosterone completely lost the α -helical signal (0.0%) and gave rise to a strong antiparallel β -sheet component (82.7-84.9%), and praziquantel again gave a more modest reduction in α -helical content (4.8-5.8%). These results collectively suggest that testosterone, but not GSH or praziquantel, induces a significant α -helix to β -sheet transition in both GST isoforms.

Table 7. Comparison of the secondary structure composition of recombinant *Sj26GST* under different ligand-binding conditions as estimated by the BeStSel web server from far-UV CD spectra.

Secondary structure (%)	<i>Sj26GST</i>	<i>Sj26GST</i> : GSH	<i>Sj26GST</i> : Testo	<i>Sj26GST</i> : PZQ	<i>Sj26GST</i> :GSH: Testo	<i>Sj26GST</i> :GS H: PZQ
α -Helix	58.2	52.4	0.0	2.9	0.0	4.2
β -Sheet (Antiparallel)	0.0	0.0	63.4	0.0	65.1	0.0
β -Sheet (Parallel)	13.8	22.9	0.0	19.0	0.0	16.6
Turns	0.0	0.0	16.5	33.9	14.7	34.0
Random coil/loops	28.0	24.7	20.2	44.2	20.3	45.2
RMSD	2.8751	3.1240	2.2362	2.4865	2.1931	2.4745
NRMSD	0.1634	0.1935	0.2350	0.1677	0.2223	0.1625

Table 8. Comparison of secondary structure composition of recombinant *Sh28GST* under different ligand-binding conditions as estimated by the BeStSel web server from Far-UV CD spectra.

Secondary Structure (%)	<i>Sh28GST</i>	<i>Sh28GST</i> :GSH	<i>Sh28GST</i> :Testo	<i>Sh28GST</i> :PZQ	<i>Sh28GST</i> :GSH:Testo	<i>Sh28GST</i> :GSH:PZQ
α -Helix	55.4	52.4	0.0	5.8	0.0	4.8
β -Sheet (Antiparallel)	0.0	0.0	82.7	0.0	84.9	0.0
β -Sheet (Parallel)	4.3	3.9	0.0	11.1	0.0	30.2
Turns	0.0	0.0	8.4	35.1	7.6	26.4
Random coil/loops	40.3	43.7	8.9	48.0	7.5	38.7
RMSD	1.4565	1.5022	2.7868	0.9472	2.6615	1.1176
NRMSD	0.0719	0.1223	0.3998	0.1010	0.3890	0.1125

3.4.4 ANS Fluorescence Analysis

To further assess whether ligand-induced conformational changes were accompanied by alterations in protein surface hydrophobicity, extrinsic fluorescence spectroscopy using 8-anilidonaphthalene-1-sulfonic acid (ANS) was performed on both enzymes under the corresponding ligand-bound conditions. This is as ANS fluorescence provides information on changes in the microenvironment and accessibility of hydrophobic regions on proteins, as changes in fluorescence intensity and emission maximum can reflect alterations in protein conformation and the exposure of hydrophobic sites. The ANS fluorescence spectra of both *Sj26GST* and *Sh28GST* showed only minor changes following binding of the substrate (GSH) compared with their respective apo forms (Figures 16A and B). This suggests that substrate binding alone produced limited alterations in the accessibility of hydrophobic regions on the protein surfaces. In contrast, testosterone binding resulted in a noticeable decrease in ANS fluorescence intensity for both *Sj26GST* and *Sh28GST*, indicating reduced accessibility of ANS to hydrophobic microenvironments, which may reflect ligand-induced conformational compaction or shielding of previously accessible hydrophobic regions (Figures 16C and D). This observation is consistent with the conformational changes detected by Far-UV CD spectroscopy, particularly the transition from α -helical to β -sheet-associated structural features, suggesting that testosterone binding induces a substantial rearrangement of the protein conformation. Praziquantel produced little or no appreciable change in ANS fluorescence intensity for *Sj26GST*, indicating that its binding has limited effects on ANS-accessible hydrophobic regions in this isoform (Figure 16E). In contrast, praziquantel increased the ANS fluorescence intensity of *Sh28GST*, suggesting increased accessibility of hydrophobic regions and/or the formation of a more favourable ANS-binding environment upon ligand binding (Figure 16F). This differential response indicates that praziquantel induces distinct

conformational effects in the two GST isoforms, with *Sh28GST* exhibiting greater exposure or accessibility of hydrophobic regions following ligand binding.

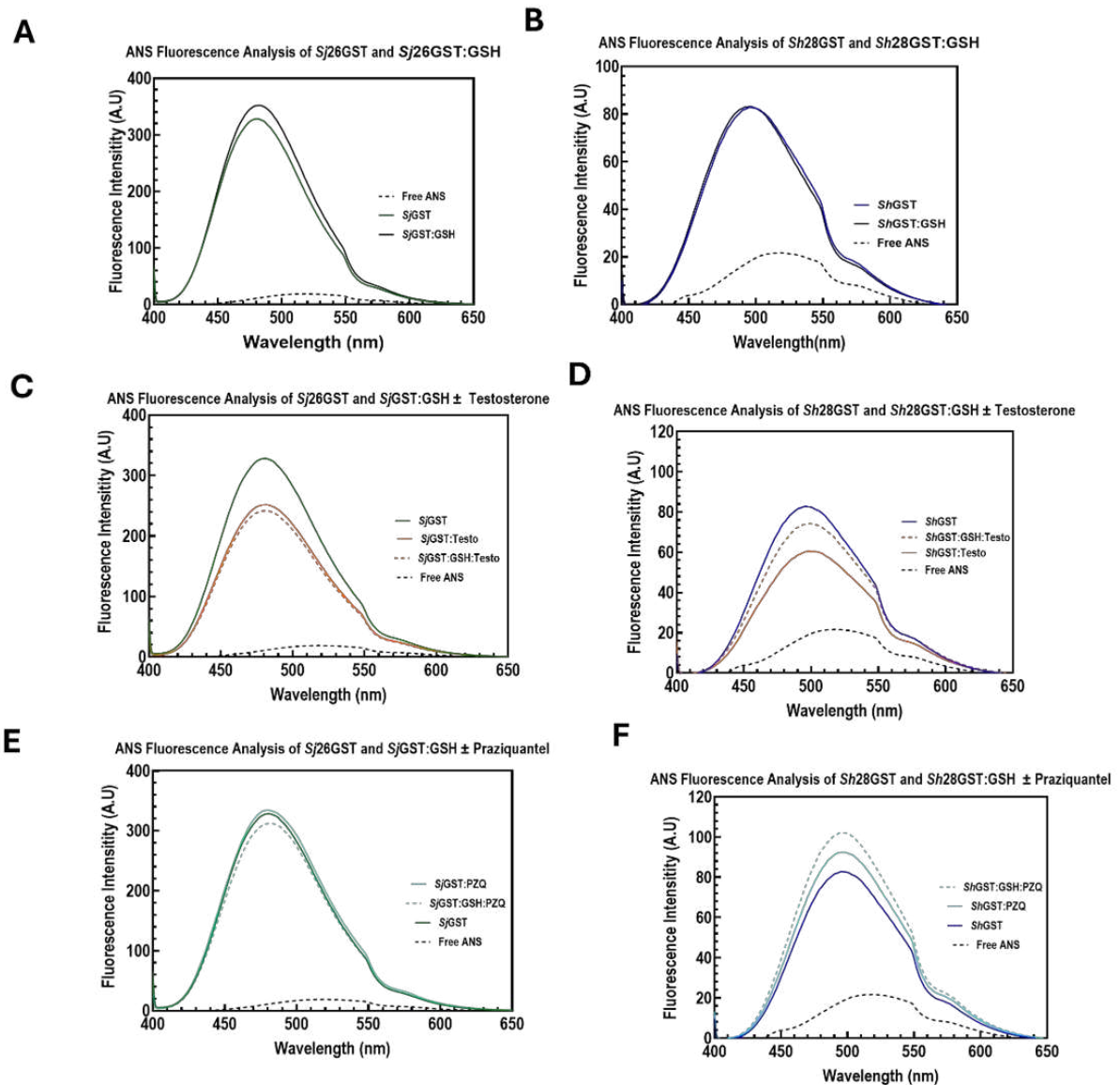


Figure 16. ANS fluorescence analysis reveals surface hydrophobicity changes in *Sj26GST* and *Sh28GST* upon GSH binding and in the presence of testosterone or praziquantel. All panels include a baseline control of free ANS dye (dashed black line). (A,B) Apo enzyme/Enzyme:GSH complex for *Sj26GST* and *Sh28GST*, respectively. (C,D) Effect of testosterone, with and without GSH, for *Sj26GST* and *Sh28GST*, respectively. (E,F) Effect of praziquantel, with and without GSH, for *Sj26GST* and *Sh28GST*, respectively.

3.4.5 Testosterone Inhibition and IC_{50} Determination

IC_{50} (half-maximal inhibitory concentration) is the concentration of an inhibitor required to reduce the measured activity of an enzyme to 50% of its uninhibited activity under the specific experimental conditions used. Testosterone was selected for IC_{50} determination because it demonstrated both functional inhibition of GST activity and interaction with the recombinant GST proteins. In contrast, PZQ did not inhibit specific GST enzymatic activity or show

detectable binding to *Sj26GST* and *Sh28GST* under the experimental conditions used. As shown in Figure 17, increasing testosterone concentrations produced a progressive, sigmoidal decrease in the relative enzymatic activity of both *Sj26GST* (Figure 17A) and *Sh28GST* (Figure 17B). The fitted curves therefore demonstrate a concentration-dependent inhibitory effect of testosterone on both GST isoforms. The transition from the upper plateau, representing relatively uninhibited enzyme activity, to the lower plateau reflects increasing inhibition as the testosterone concentration increases.

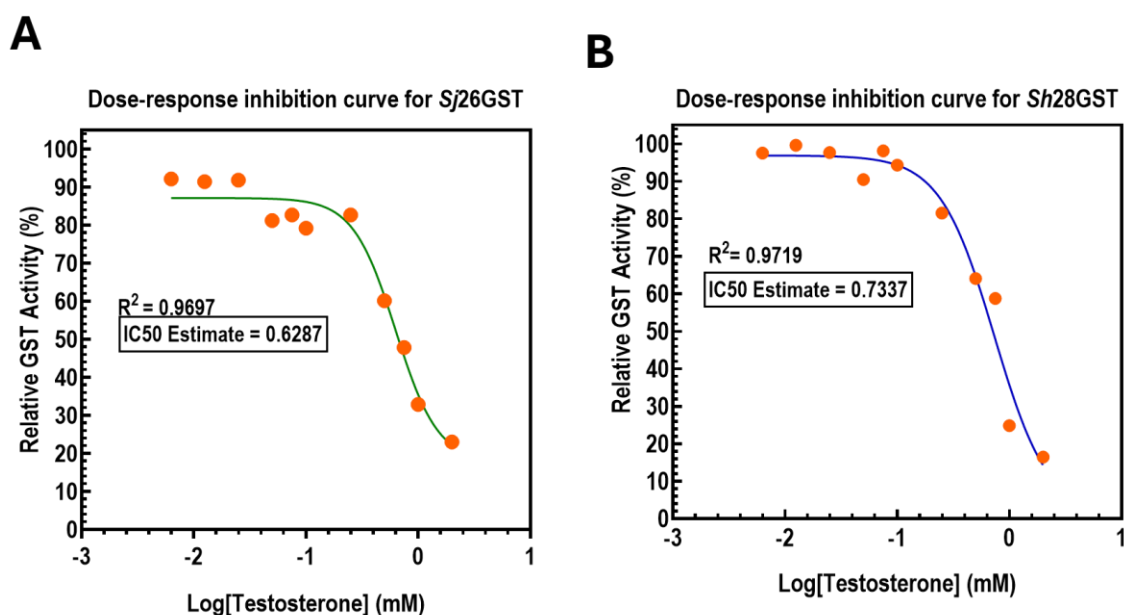


Figure 17. Dose-dependent inhibition of (A) *Sj26GST* (green fit line) and (B) blue fit line *Sh28GST* enzymatic activity by testosterone. Relative GST activity (%) was plotted against log[Testosterone] (mM). Orange circles are individual experimental data points, and the solid line is the non-linear regression fit to calculate IC50.

3.5 Statistical Analysis of

To statistically compare the dynamic behaviour of the two GST isoforms and their ligand complexes, the median values of the trajectory-derived structural parameters were compared. Comparative analysis of C α -RMSD, C α -RMSF and RoG was carried out for the apo and ligand-bound systems to determine the differences in intrinsic flexibility and ligand-induced structural stabilization between the two GST isoforms using one-way ANOVA with Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests for post hoc comparisons as illustrated in Table 7. The ligand-RMSD and ligand-SASA were compared between the testosterone-bound and the testosterone analogue-bound systems to evaluate the stability of the ligand, the compactness of the ligand, and the amount of ligand buried in the GST binding pockets using the Mann-Whitney U test as shown in Table 8.

Table 7. Comparative analysis of structural dynamics of *Sm26GST* and *Sm28GST*

Parameter	GST	Apo median	Testo median	Analog median	Overall p-value	Apo vs Testo	Apo vs Analog	Testo vs Analog
Cα RMSD (Å)	<i>Sm28GST</i>	1.980	2.430	2.460	<0.0001 ****	<0.0001 ****	<0.0001 ****	0.027 *
	<i>Sm26GST</i>	3.025	2.454	2.089	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****
Radius of gyration (Å)	<i>Sm28GST</i>	21.42	21.74	21.76	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****
	<i>Sm26GST</i>	21.49	21.61	21.70	<0.0001 ****	<0.0001 ****	<0.0001 ****	<0.0001 ****
N-terminal RMSF (Å)	<i>Sm28GST</i>	0.677	0.740	0.809	ND	0.442 ns	0.031 *	0.653 ns
	<i>Sm26GST</i>	0.952	0.910	0.806	ND	0.308 ns	<0.0001 ****	0.014 *
C-terminal RMSF (Å)	<i>Sm28GST</i>	0.808	0.907	0.977	ND	0.268 ns	0.017 *	0.548 ns
	<i>Sm26GST</i>	0.886	1.147	1.294	ND	<0.0001 ****	<0.0001 ****	0.699 ns

C α -RMSD and radius of gyration were evaluated using the Kruskal-Wallis test followed by two-tailed Mann-Whitney U post-hoc comparisons. N- and C-terminal RMSF values were compared using pairwise Mann-Whitney U tests. $p < 0.05$; $\mathbf{p} < 0.01$; $\mathbf{p} < 0.001$; $\mathbf{p} < 0.0001$; ns, not significant; ND, not determined.

Table 8 Comparative analysis of ligand conformational behaviour and solvent exposure

Parameter	GST	Testo median	Testo analog median	p-value
Ligand RMSD (Å)	<i>Sm28GST</i>	3.774	4.357	<0.0001 ****
	<i>Sm26GST</i>	13.00	4.526	<0.0001 ****
Ligand SASA (Å²)	<i>Sm28GST</i>	48.58	81.86	<0.0001 ****
	<i>Sm26GST</i>	114.0	56.59	<0.0001 ****

Ligand-RMSD and solvent-accessible surface area (SASA) were compared between the testosterone and testosterone-analogue systems using the two-tailed Mann-Whitney U test. $\mathbf{p} < 0.0001$.

To visualize the overall conformational landscape sampled by each isoform, principal component analysis (PCA) of the MD trajectories was performed (Figure 18). For *Sm26GST*, PC1 explained 52.34% of the total variance and PC2 explained 18.93%, whereas for *Sm28GST*,

PC1 explained 50.93% and PC2 explained 18.19%. For *Sm26GST*, the apo and testosterone-bound systems overlapped significantly, suggesting that testosterone binding did not substantially alter the major collective motions, while the testosterone-analogue system formed a well-defined, distinct cluster along PC1 for both isoforms. The separation of the analogue-bound trajectories from the apo-bound states suggests that the analogue causes a different global conformational response in both *Sm26GST* and *Sm28GST*, supporting the RMSD and RMSF results, and indicating that the analogue has a stronger conformational effect than testosterone.

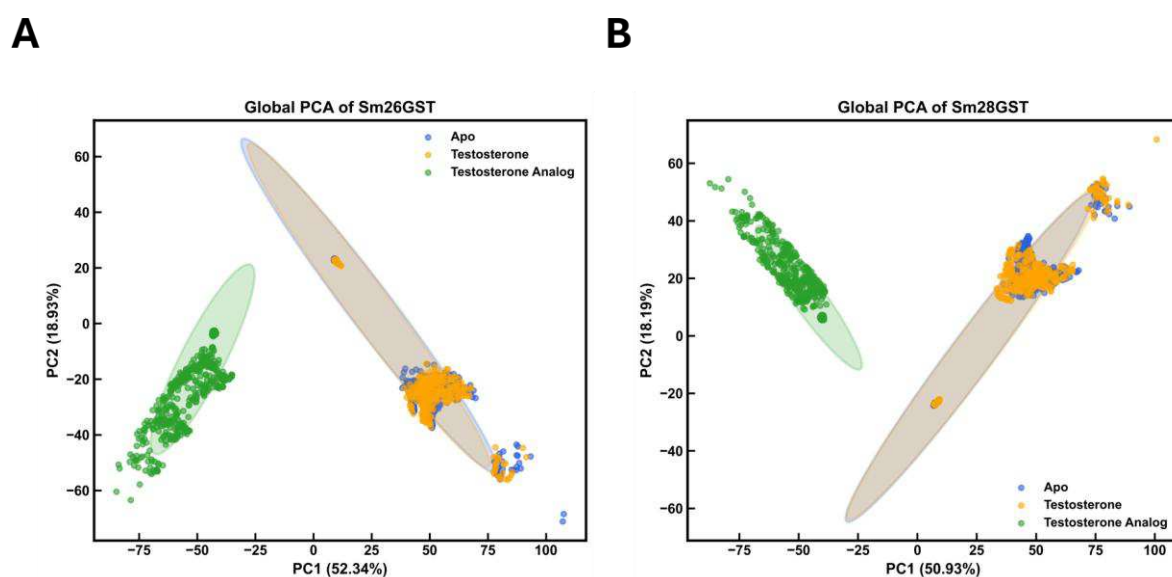


Figure 17. Principal component analysis (PCA) of *Sm26GST* and *Sm28GST* molecular dynamics trajectories. PCA plots showing the conformational distribution of apo (blue), testosterone-bound (orange), and testosterone-analog-bound (green) systems for *Sm26GST* (left) and *Sm28GST* (right). PC1 and PC2 account for 52.34% and 18.93% of the total variance for *Sm26GST*, respectively, and 50.93% and 18.19% for *Sm28GST*. Shaded ellipses represent the conformational distribution of the respective trajectories.

The PCA profiles show two different conformational states for each of the two GST isoforms, depending on the presence of the ligand. The testosterone and apo systems exhibit significant overlap for *Sm26GST*, while the testosterone-analog system is a well-defined cluster along PC1, suggesting a different conformational ensemble. Likewise, in *Sm28GST*, the system for the testosterone analog is distinctly different from the apo and testosterone-bound systems. The separation of the analogue-bound trajectories from the other states indicates that the analog causes a unique global conformational response in both GSTs. The similar positioning of the apo and testosterone trajectories suggests that the dominant collective motions are relatively unaffected by the binding of testosterone. In general, the PCA confirms the results of the RMSD/RMSF and indicates that the testosterone analogue causes more significant changes in the global conformational landscape of both *Sm26GST* and *Sm28GST* than does testosterone.

4.0 Discussion

The central question addressed in this study is whether testosterone acts on *Schistosoma* GSTs as a lethal agent, as a classical active-site inhibitor, or as a conformational modulator of detoxification capacity. The evidence is considered below along four converging lines: the response of intact worms, the sequence and structural basis of isoform divergence, the computational description of where and how testosterone binds, and the biochemical and biophysical behaviour of the purified orthologues.

4.1 Effects of Testosterone and Praziquantel on Adult Worm GST Activity

GSTs of *Schistosoma* spp. have long been pursued as vaccine and drug targets because they combine electrophile detoxification with a ligandin capacity for lipophilic host molecules, including steroid hormones [22, 24]. The whole-worm cultures separated these two functions clearly. Praziquantel immobilised worms immediately and completely, consistent with its established action on Ca^{2+} homeostasis, tegumental integrity and subsequent worm death [26], whereas testosterone-treated worms remained motile for the full 60 min while their lysates showed reduced GST activity. Testosterone is therefore not vermucidal at the concentrations used; it modulates a detoxification pathway in an otherwise viable parasite. This is the behaviour expected of a host steroid that influences schistosome development, reproduction and fecundity rather than survival [6, 22], and it places the testosterone-GST interaction in the domain of long-term physiological regulation. Importantly, CDNB-conjugating activity remained measurable in all three treatments [27], so the effect is a graded reduction in detoxification capacity rather than ablation of the enzyme. What that reduction means mechanistically depends on which isoforms are present and how far they differ from one another, which is considered next.

4.2 Sequence Conservation and Homology Modelling of *Sm26GST* and *Sm28GST*

Two cytosolic GST isoforms dominate the *S. mansoni* complement, and the whole-worm activity measured above is their combined output. Multiple sequence alignment showed that *Sm26GST* and *Sj26GST*, and *Sm28GST* and *Sh28GST*, are near-identical within each class, whereas the 26 kDa and 28 kDa classes diverge substantially from one another while retaining the canonical cytosolic GST architecture: a conserved thioredoxin-like N-terminal G-site and a variable α -helical C-terminal domain that shapes the H-site [28]. This is the pattern seen in the solved schistosome GST structures, in which the catalytic machinery is essentially invariant

while the substrate-binding loops carry the class-specific differences [11, 23, 29]. Two consequences follow. First, the within-class conservation justifies treating recombinant *Sj26GST* and *Sh28GST* as experimental proxies for the computationally modelled *S. mansoni* enzymes, an approach already validated for engineered schistosome GST constructs [30]. Second, the between-class divergence concentrated in the H-site region generates a testable expectation that the two isoforms will differ in ligand recognition and in their response to the same compound, a prediction examined directly in the kinetic and spectroscopic experiments below. The homology models were of adequate quality to support this analysis, with well over 90% of residues in the favoured Ramachandran regions and no outliers in the catalytic core.

4.3 High-Throughput Virtual Screening of Testosterone Analogues

If schistosome GSTs are to be exploited as drug targets rather than merely described, the steroid scaffold must be improved upon. We therefore used high-throughput virtual screening to identify analogues with predicted binding affinities equal to or greater than those of testosterone itself. The top-ranked hits were dominated by nor-androstane derivatives, bolandiol, 19-nor-5-androstenedione and 19-nor-5-androstenediol for *Sm26GST*, and 19-nor-5-androstenediol, norethynodrel and 17-methylandrost-4-ene-3 β ,17 β -diol for *Sm28GST*, indicating a pocket that is sterically permissive and preferentially accommodates the steroid nucleus with reduced C-19 substitution. A comparable enrichment of steroid and flavonoid scaffolds was reported for the structurally related *S. bovis* 28 kDa GST, where a single high-affinity ligand class recurred across related binding pockets [18], and structure-based screening is now applied routinely to schistosome targets [1, 3, 7, 21]. The pharmacological rationale is the continued dependence on praziquantel as the only broadly available treatment and the demonstration that praziquantel response in schistosome populations is heritable and genetically tractable [10, 31]. These analogues are therefore defensible starting points for lead development, provided the site they occupy is genuine, which induced-fit docking was used to define.

4.4 Induced-Fit Docking Reveals an Interfacial Testosterone Binding Site

Testosterone was docked onto both modelled isoforms using a Glide/Prime induced-fit protocol benchmarked to sub-2.5 Å pose accuracy across a large set of protein-ligand complexes [32]. Testosterone and each of the top-ranked analogues docked reproducibly not into the canonical glutathione (G-) site but into a cavity at the interface between the two subunits of the *Sm26GST* and *Sm28GST* homodimers, engaging conserved Leu, Glu and Arg residues contributed by

both protomers. An interfacial ligand site of this kind has precedent in this family: the schistosome GST dimer interface binds bromosulfophthalein selectively relative to its human counterparts [25], and praziquantel itself has been located crystallographically at a non-catalytic site on the 26 kDa enzyme [23]. Glide GScore, Emodel and Glide energy values were consistently more favourable for *Sm26GST* than for *Sm28GST* across all top-ranked poses, predicting a tighter binding mode for the 26 kDa isoform, a prediction matched by the greater conformational and kinetic sensitivity of *Sj26GST* reported in Sections 4.7 and 4.8. Because cytosolic GSTs are obligate homodimers whose active sites are completed by inter-subunit contacts [11, 28], occupancy of this cavity provides a direct route by which testosterone could depress catalytic activity without competing with glutathione. Whether such a pose is stable, rather than merely favourable at a single docked geometry, required simulation.

4.5 Molecular Dynamics Simulations

Molecular dynamics over 500 ns supported the docked model and refined it. The ligand-bound complexes behaved consistently differently from their apo counterparts, confirming that testosterone occupancy alters the conformational space sampled by both isoforms. *Sm28GST* showed lower C α -RMSD values, a smaller radius of gyration and reduced residue fluctuation than *Sm26GST*, identifying it as the more rigid of the two. The two computational measures did not agree straightforwardly: although testosterone scored better in docking against *Sm26GST*, it was the more positionally mobile ligand within that pocket during simulation, whereas the analogue remained essentially fixed throughout. Docking score is therefore a poor proxy for residence stability, and the two approaches are complementary rather than interchangeable [16]. The residues responsible for the additional flexibility lie in the C-terminal α -helical domain, the region repeatedly implicated in substrate recognition and ligand specificity in cytosolic GSTs [11, 28], which links the sequence divergence described in Section 4.2 directly to the dynamic behaviour observed here.

Principal component analysis, which resolves the dominant collective motions and conformational substates sampled along a trajectory [33] showed that the analogue drives both isoforms into a distinct, well-separated conformational state, whereas the sampling of the testosterone-bound complexes overlaps largely with the apo ensemble, consistent with a modest, modulatory perturbation rather than a large allosteric switch. The opposing ligand RMSD and SASA trends, with greater burial of the analogue in *Sm26GST* and greater solvent exposure in *Sm28GST*, indicate that a conserved pocket within an almost identical fold can

nevertheless present the same ligand class in different geometries. Testing these predictions required the purified proteins.

4.6 Recombinant Overexpression and Purification

Recombinant *Sj26GST* and *Sh28GST* were obtained in a form suitable for that testing. IMAC elution profiles showed peaks clearly resolved from the flow-through fractions, NanoDrop quantification confirmed adequate yields, and SDS-PAGE gave dominant bands migrating between 25 and 30 kDa, consistent with the expected subunit masses of the two isoforms. Complete saturation of the affinity column was not achieved for either protein, a limitation also reported for an engineered pseudo-26-kDa *S. bovis/haematobium* GST expressed in *Escherichia coli* and attributed there to incomplete tag exposure or partial misfolding of a fraction of the expressed material. Because all downstream analyses were performed on the eluted, homogeneous fraction, this constrains yield rather than the interpretation of the kinetic and spectroscopic data that follow.

4.7 Differential Enzymatic Modulation of SGSTs

Under identical CDNB-conjugation conditions [27], *Sj26GST* was approximately 2.3-fold more active than *Sh28GST*, confirming that schistosome GSTs are catalytically non-equivalent [25]. The most economical explanation is the H-site and substrate-binding-loop divergence identified in Section 4.2, which governs the orientation and turnover of the electrophilic substrate while leaving the G-site intact, the same basis proposed for the activity differences between engineered and native schistosome GSTs.

The two ligands then separated the isoforms pharmacologically and catalytically. Testosterone reduced the specific activity of both *Sj26GST* and *Sh28GST* by about half, whereas praziquantel left *Sj26GST* essentially unchanged and marginally increased *Sh28GST* activity. Praziquantel therefore does not compete with testosterone for the inhibition site under the conditions tested, consistent with both its crystallographically defined, non-catalytic binding mode on the 26-kDa enzyme [21] and earlier ligandin analyses of engineered schistosome GSTs [30]. The testosterone effect, by contrast, extends the specific binding previously demonstrated for the *S. haematobium* 28 kDa enzyme [22] to a 26-kDa isoform, indicating that steroid recognition is a property of the schistosome GST family rather than of a single class. This provides direct biochemical support for the long-standing proposal that binding of host androgen to parasite GST underlies the antifecundity effects attributed to these hormones [22, 24]. The divergence between the two ligands, and between the two isoforms, in their response

to praziquantel argues against a single shared inhibitory mechanism and for distinct modes of interaction, a distinction the spectroscopic data resolve.

4.8 Structural and Conformational Effects of Testosterone and Praziquantel on SGSTs

Far-UV CD and extrinsic ANS fluorescence define the structural consequence of testosterone binding. Native spectra for both recombinant enzymes were typical of the α -helix-rich cytosolic GST fold [28]. Testosterone induced a near-complete conversion of α -helical to antiparallel β -sheet content in both *Sj26GST* and *Sh28GST*, deconvoluted using BeStSel [34-36], which resolves the spectral diversity of β -sheet geometries more reliably than earlier algorithms. The change was considerably larger than that produced by either glutathione or praziquantel, occurred independently of glutathione, and was not reversed by subsequent glutathione addition. Simple competition at the G-site can therefore be excluded, and the loss of catalytic activity reported in Section 4.7 is better explained as the functional consequence of this conformational transition, precisely the behaviour expected of the interfacial, non-competitive site identified by docking and simulation. Ligand-driven secondary-structure rearrangement of this magnitude, altering both stability and function, is documented for other globular proteins binding small amphipathic molecules [37]. The accompanying decrease in ANS fluorescence intensity indicates that the hydrophobic surface becomes more compact and less solvent-exposed upon testosterone binding, since ANS enhancement is an established reporter of exposed hydrophobic surface on conformationally altered proteins [37]. Together, CD and ANS describe testosterone as a conformational modulator acting on both secondary and tertiary structure, mechanistically distinct from the smaller, isoform-conservative changes caused by praziquantel. The remaining question is the concentration at which this occurs.

4.9 Testosterone Inhibition and IC₅₀ Determination

IC₅₀ values of 0.63 mM for *Sj26GST* and 0.73 mM for *Sh28GST* place testosterone in the moderate-affinity range characteristic of a physiological modulator rather than of an optimised pharmacological inhibitor. The slightly lower value for *Sj26GST* is consistent with the more favourable docking scores, greater ligand mobility, and higher kinetic and conformational sensitivity reported for this isoform throughout. These values are compatible with a ligandin interaction of the kind established by direct binding measurements on the *S. haematobium* 28 kDa enzyme [12] and with a GST-mediated contribution to the hormonal and reproductive disturbances associated with schistosome infection [24], although the millimolar range implies

that any such contribution reflects local rather than circulating steroid concentrations. It also defines the task for medicinal chemistry: the analogues prioritised in Section 4.3 must improve on this baseline by orders of magnitude to be therapeutically useful, and the isoform-specific binding geometries revealed by simulation provide the structural handles for doing so.

5. Conclusion

This study integrates and extends previous studies linking testosterone to schistosome GSTs activity by combining structural and dynamic effects of the ligand on enzyme activity. The findings show that while testosterone binding to schistosome GSTs does not cause immediate parasite death, the enzyme contracts its binding sites, increases surface hydrophobicity, and decreases catalytic activity. The subtle structural and flexibility differences in the binding sites of Sm26GST and Sm28GST, though relatively minor, suggest they may be targeted in the design of selective inhibitors for the respective enzymes. These results highlight schistosome GSTs as promising therapeutic targets and offer a structural basis for developing steroid-inspired antischistosomal compounds with better specificity and efficacy.

Data Availability

The data that support the findings of this study are openly available in Zenodo at [<https://10.5281/zenodo.22901621>, with reference number v1.

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Contributions

IA conceptualised the overall study; MM and VM co-conceptualised and affirmed the study; IA, MM, VM and IM designed the methodology of the study. CN and TN carried out the study; CN wrote the initial draft; OO and IA supervised the review and manuscript production; OO supervised the biophysicochemical aspect of the study; IA and VM supervised the *in silico* aspect of the study; MM and IM supervised the parasitological aspect of the study; IA, MM and VM supervised CN; IA provided the funding, software and hardware for the study; IA, MM and VM administered the project.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The Scientific and Ethics Review Unit (SERU), Kenya Medical Research Institute (KEMRI), Nairobi, Kenya, approved this study under ethics clearance number ACUC/01.10.2025.

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