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## Why does oxamniquine kill *Schistosoma mansoni* and not *S. haematobium* and *S. japonicum*?

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### ABSTRACT

Human schistosomiasis is a disease which globally affects over 229 million people. Three major species affecting humans are *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*. Previous treatment of *S. mansoni* includes the use of oxamniquine (OXA), a prodrug that is enzymatically activated in *S. mansoni* but is ineffective against *S. haematobium* and *S. japonicum*. The OXA activating enzyme was identified and crystallized, as being a *S. mansoni* sulfotransferase (*SmSULT*). *S. haematobium* and *S. japonicum* possess homologs of *SmSULT* (*ShSULT* and *SjSULT*) begging the question; why does oxamniquine fail to kill *S. haematobium* and *S. japonicum* adult worms? Investigation of the molecular structures of the sulfotransferases indicates that structural differences, specifically in OXA contact residues, do not abrogate OXA binding in the active sites as previously hypothesized. Data presented argue that the ability of SULTs to sulfate and thus activate OXA and its derivatives is linked to the ability of OXA to fit in the binding pocket to allow the transfer of a sulfur group.

### 1. Introduction

Current estimates indicate schistosomiasis affects over 229 million people in 78 countries (Olveda et al., 2016; World Health Organization, 2020), with *S. mansoni* and *S. haematobium* accounting for over 99.5% of human cases (Hotez et al., 2006; Steinmann et al., 2006). Currently, no effective vaccine against human schistosomiasis exists and there is only one method of treatment, a single dose of praziquantel (PZQ), which is effective against all human schistosome species (Vale et al., 2017). PZQ has few adverse side effects and, due to an expired patent, is cost effective. However, PZQ is not effective against immature parasites (Sabah et al., 1986; Pica-Mattoccia and Cioli, 2004). There is concern that emergence of a PZQ resistant strain will be inevitable due to the use of mass preventive chemotherapy of PZQ (Fenwick and Webster, 2006) and the recent efforts to increase mass treatment by 10 fold (Fenwick, 2015) increasing selective pressure.

Previous treatments for *S. mansoni* consisted of a multitude of drugs many of which have fallen out of favor in subsequent years, due to resistance, effectiveness, cost, and side effects (da Rocha Pitta et al., 2013; Siqueira et al., 2017). Oxamniquine (OXA) was one such drug used extensively in Brazil (Katz and Coelho, 2008), where only *S. mansoni* is present, until 1996 when PZQ became the first line drug (Coura and Amaral, 2004). The efficacy of oxamniquine and PZQ is comparable, though in some cases OXA is more effective against *S. mansoni* when PZQ tolerance is observed (Stelma et al., 1997). OXA is only effective against *S. mansoni*. Additionally, resistance is observed in the field (Cioli and Pica-Mattoccia, 1984; Cioli et al., 1989; Gentile and Oliveira, 2008; Chevalier et al., 2016) and was selected for in the laboratory (Rogers and Bueding, 1971). OXA resistant parasites exhibit as high as 500% insensitivity to the drug (Valentim et al., 2013). Previous genetic studies demonstrated that mutations in a single gene of the *S. mansoni* sulfotransferase (*SmSULT-OR*) are responsible for OXA

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resistance both in the field and in lab-derived resistant isolates (Cioli et al., 1992; Pica-Mattoccia et al., 1993; Valentim et al., 2013; Chevalier et al., 2016, 2019).

Sulfotransferases are enzymes that catalyze the transfer of a sulfonyl group ( $\text{SO}_2$ ) from a sulfate donor, such as 3'-phosphoadenosine-5'-phosphosulfate (PAPS), to alcohol (R-OH) or amine (R-NH<sub>2</sub>) acceptors in a variety of molecules to form sulfate (R-O-SO<sub>3</sub><sup>-</sup>) or sulfo-amine (R-NH-SO<sub>3</sub><sup>-</sup>) products, respectively. Sulfotransferases are found in both prokaryotes and eukaryotes and function in many biological processes such as growth, cell signaling, and detoxification (Negishi et al., 2001). Cytosolic sulfotransferases are involved in defense through metabolizing drugs, toxins and carcinogens, by sulfating the substrates to promote their elimination (Negishi et al., 2001; Strott, 2002; Allali-Hassani et al., 2007). Sulfated substrates can result in activation of a prodrug compound into a pharmacologically active form and as a consequence these metabolites can cause pathological repercussions, including cell death (Glatt, 2000), as seen in the case of OXA activation within schistosome parasites (Cioli et al., 1992).

OXA is a prodrug that is enzymatically activated in the schistosome parasite (Cioli et al., 1985; Valentim et al., 2013). OXA binds to a specific *S. mansoni* sulfotransferase, known as *SmSULT*, where it is transiently sulfated. Activated OXA subsequently binds to DNA and other macromolecules, resulting in killing of the worms (Cioli et al., 1985; Valentim et al., 2013; Taylor et al., 2017). Phylogenetic analysis of *SmSULT* in *S. haematobium* and *S. japonicum* identified homologous sulfotransferases (*ShSULT* and *SjSULT*, respectively) (Valentim et al., 2013; Taylor et al., 2017). Each SULT was demonstrated to have sulfotransferase activity. Kinetic analyses allowed direct comparison of *SmSULT*, *ShSULT* and *SjSULT* enzymatic activities and showed *SmSULT* has the highest activity with OXA as substrate based on the  $k_{\text{cat}}/K_M$  values. *SmSULT* indicates no preference for OXA enantiomers while *ShSULT* prefers *S*-OXA (the most potent form) and *SjSULT* prefers *R*-OXA. Furthermore, *ShSULT* is less active than *SmSULT* by a factor of about one-half and *SjSULT* is less active by about an order of magnitude. These lower levels of catalytic efficiency may explain why OXA fails to treat *S. haematobium* and *S. japonicum* infection (Valentim et al., 2013; Taylor et al., 2017). X-ray crystal structures of the three schistosomal SULTs were determined. *SmSULT* and *ShSULT* are 70% identical and have 3 differences in OXA contact residues based on amino acid alignment (Valentim et al., 2013). One of the differences, F39 in *S. mansoni* compared to Y54 *S. haematobium*, was predicted to cause a change in polarity of the amino acid which would negatively impact OXA binding (Valentim et al., 2013). The purpose of the present study is to ascertain if one or any combination of differences in the OXA contact residues is responsible for *S. haematobium* or *S. japonicum* inability to activate OXA.

## 2. Materials and methods

### 2.1. Parasite life cycle and adult worm harvesting

Life cycles of *Schistosoma mansoni*, *S. haematobium*, *S. japonicum* and HR (an OXA resistant strain of *S. mansoni* (Rogers and Bueding, 1971)) were maintained in the laboratory. The HR mutation was demonstrated to be a glutamate 142 deletion (E142del) in a sulfotransferase enzyme (Valentim et al., 2013). Cercariae collected from previously infected *Biomphalaria glabrata*, *Bulinus truncatus* or *Oncomelania hupensis* were used to infect hamsters by wading (Tucker et al., 2013). Once the schistosome worms developed into adult worms, 30–90 days depending on the species of the parasite, the hamster hosts were euthanized and worms were obtained by perfusion (Duvall and DeWitt, 1967). Collected worms were immediately flash-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Animal infections, perfusions and euthanasia were performed in accordance with the University of Texas Health Science Center at San Antonio IACUC protocol (UTHSCSA IACUC Protocol #08039).

### 2.2. Whole worm extracts

Aliquots of whole frozen male and female adult worms were suspended in Protease Inhibitor Cocktail (PIC) consisting of: 0.1 M HEPES pH 7.4, 0.1 mM leupeptin, 2  $\mu\text{M}$  E-64, 2  $\mu\text{M}$  pepstatin A, 0.1 U of aprotinin. Samples were sonicated (Qsonica) at an amplitude of 50 on ice until a fine homogenous mixture was obtained. The samples were then centrifuged at  $16.1 \times g$ . Supernatant was transferred to ultracentrifuge tubes and centrifuged at  $69522.1 \times g$  for 1 h (Beckman-Coulter Tabletop Ultracentrifuge Optima Max). Supernatant was collected; the whole soluble protein concentration was measured by NanoDrop (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher) and then adjusted to 2 mg/mL using PIC.

### 2.3. *SmSULT*, *ShSULT* and *SjSULT* proteins and mutant proteins

Codon optimization, subcloning, mutagenesis, and expression protocols have been described elsewhere (Valentim et al., 2013; Taylor et al., 2017). The mutant proteins were *SmSULT*: F39Y, L148I, T157S, F39Y/L149I, F39Y/T157S, F39Y/L149I/T157S; *ShSULT*: Y45F, I158L, S166T, Y54/I158L and *SjSULT*: V139G. Inactive *SmSULT*-OR which has a E142del mutation served as a negative control. Recombinant proteins were then prepared for OXA activation assay by adding 1 nM from each recombinant protein to 90  $\mu\text{L}$  of PIC.

### 2.4. Tritiated drug labeling

Tritiated OXA was synthesized by the Center for Innovative Drug Discovery (CIDD) using radiolabeled  $\text{NaBH}_4$  (Moravek Biochemicals, USA) at 100 mCi. For each mole of OXA in the aldehyde form, a single Ci was added and reacted at room temperature to completion and detected by thin layer chromatography. The levels of radioactivity were determined by blotting 10  $\mu\text{L}$  of the final product onto filter paper which were then counted via liquid scintillation counter (Beckman LS 6500 Scintillation Counter, USA) for 10 min for each reaction.

### 2.5. OXA activation assay

The ability of a worm extract or a recombinant protein to activate OXA was tested by quantifying how much tritiated OXA was able to bind DNA. For each reaction, 100  $\mu\text{Ci}$  of [<sup>3</sup>H]OXA was solubilized in 2  $\mu\text{L}$  DMSO and added to 10  $\mu\text{L}$  of a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) mix containing ATP and  $\text{MgCl}_2$  at 50 mM each, and PAPS at 1 mM (Cioli et al., 1989). The radiolabeled OXA and PAPS mix was then added to 90  $\mu\text{L}$  of either whole worm extract or recombinant protein with 10 ng/ $\mu\text{L}$  sheared *S. mansoni* (gDNA) as a final target for activated [<sup>3</sup>H]OXA. The mixture was incubated at  $37^\circ\text{C}$  either for 2.5 h when testing worm extract or for 5 min to 2.5 h when testing recombinant protein. Then the reaction was stopped with 3 vol of 1 mM sodium bicarbonate containing 0.1% SDS (w/v). Afterwards the reaction was extracted 3 times using 2 vol of dichloromethane. A 10  $\mu\text{L}$  aliquot of the aqueous phase was collected onto a small square of filter paper in a scintillation vial and then counted via a liquid scintillation counter (Beckman LS 6500 Scintillation Counter, USA) for 10 min for each reaction.

### 2.6. Structure determination

Mutant sulfotransferase crystals, native and OXA-bound *SmSULT* F39Y and T157S and *ShSULT* Y54F and S166T, were prepared and X-ray crystal structures were determined as previously described (Valentim et al., 2013; Taylor et al., 2017). Data were acquired in the X-ray Crystallography Core Laboratory at the University of Texas Health Science Center at San Antonio, Advanced Photon Source Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C or 24-ID-E and integrated and scaled using XDS (Kabsch, 2010). Models were manually

rebuilt using COOT (Emsley et al., 2010), refined using PHENIX (Adams et al., 2010) and verified using composite omit map analysis (Terwilliger et al., 2008). Data collection and refinement statistics are shown in Supplementary Table S1. Figures were generated using PyMOL (Schrödinger, LLC). Crystal structure coordinates and structure factors were deposited in the Protein Data Bank archive (wwPDB Consortium, 2019) with entry codes 6B4X, 6B4Y, 6B4Z, 6B50, 6B51, 6B52, 6B53 and 6B54.

### 2.7. Amino acid alignment of *SmSULT*, *ShSULT* and *SjSULT*

Sequences sharing similarities with *SmSULT* (Smp\_089320) were identified in the *S. haematobium* and *S. japonicum* genomes (Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012). The crystallographic work performed on *SmSULT* revealed the amino acid side chains that interact with OXA and the depleted form of PAPS, the sulfate-donating cofactor utilized by the vast majority of sulfotransferases (Valentim et al., 2013; Taylor et al., 2015, 2017). All the *SmSULT*, *ShSULT* and *SjSULT* residues that make direct contacts with OXA or PAP at a distance of 4.5 Å were identified using PyMOL.

### 2.8. Statistics

Activation assay data were analyzed using R Studio or Prism (version 8). Student's t-tests were performed to analyze the statistical significance between samples. Wild-type *S. mansoni* serves as the positive control in OXA activation assays using worm extracts or recombinant proteins, while OXA-resistant *SmSULT*-OR (E142del) parasites, or recombinant proteins encoded by *SmSULT*-OR allele, were negative controls.

## 3. Results

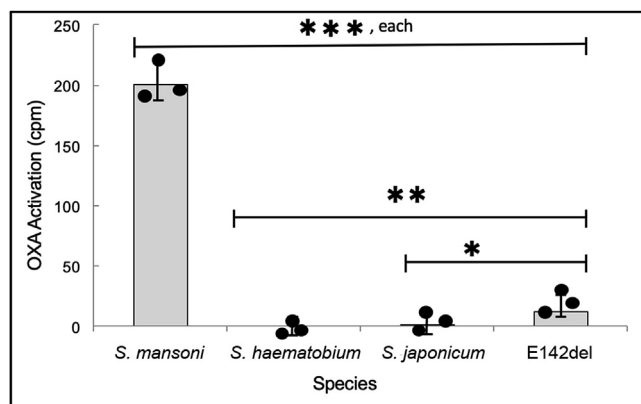
### 3.1. OXA worm activation assay

Previous studies indicated that the whole soluble protein extract from sensitive worms can activate OXA (Pica-Mattoccia et al., 1992). Despite having a sulfotransferase homolog, which has been shown as an active sulfotransferase *in vitro*, *ShSULT* and *SjSULT* are unable to activate OXA to a toxic level in adult worms. To confirm that resistance is present *in vitro*, 2 mg/mL whole worm extract from *S. haematobium* and *S. japonicum* were compared to 2 mg/mL whole worm extracts of OXA sensitive *S. mansoni* (Sm) and OXA resistant *S. mansoni* (E142del). Results showed high levels of OXA activation in the reaction containing sensitive *S. mansoni* extract and residual levels of OXA in the reactions containing resistant *S. mansoni* (E142del), *S. haematobium*, and *S. japonicum* extracts (Fig. 1).

Previous research identified contact residues to OXA and PAPS in *SmSULT* (Smp\_089320) (Valentim et al., 2013). An amino acid alignment between *SmSULT* and *ShSULT* (Sha\_104171) showed 3 differences in OXA contact residues. Adding *SjSULT* (FN317462.1) to the analysis showed that *SjSULT* was 52% identical and 69% similar with 6 differences in OXA contact residues (Fig. 2). The PAPS contact residues were conserved for the three schistosome species (Fig. 2).

### 3.2. X-ray crystal structures

Several mutants of *SmSULT* and *ShSULT* were prepared to examine the impact of exchanging OXA contact residues between the species. Crystal structures for *SmSULT* F39Y and T157S and *ShSULT* Y54F and S166T were determined and all superimposed on their respective wild-type structures (*SmSULT*: PDB entry 4MUA, *ShSULT*: PDB entry 5TIV) with  $\leq 0.32$  Å root mean square deviation indicating that the mutations did not significantly distort the structures. Previous observations revealed that when a racemic mixture of OXA was soaked in *SmSULT* or



**Fig. 1. OXA Activation Assay on Whole Protein Extracts.**

Whole worm soluble extract was obtained and diluted to a concentration of 2 mg/mL [<sup>3</sup>H]OXA was incubated with co-factor PAPS and the extracts. Activated prodrug binds to macromolecules in the extract and remains in the aqueous phase. Unreacted radiolabeled OXA goes to the organic phase. Scintillation counts were determined after a 2.5 h incubation. The Y axis represents scintillation counts per minute (cpm). Sensitive *S. mansoni* extract is known to activate OXA and is a positive control. Resistant E142del extract is known to not activate OXA and is a negative control. Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.

*ShSULT*, the S enantiomer binds preferentially (Taylor et al., 2015, 2017). Likewise, all crystallized mutants bound S-OXA from soaks with a racemic mixture (Supplementary Fig. S1). Furthermore, the *ShSULT* tyrosine substitution for phenylalanine in *SmSULT* and its position in the active site was found to allow more than one binding mode for OXA in *ShSULT* (Taylor et al., 2017). This observation was duplicated in the F↔Y mutants where OXA bound in *SmSULT* F39Y shifted into the “haematobium-like” binding position and in *ShSULT* Y54F, OXA shifted into the “mansoni-like” binding position (Fig. 3A). Clearly, exchanging tyrosine for phenylalanine at this position in *ShSULT* (and possibly *SjSULT*) does not prevent OXA binding, but it does have an impact by allowing multiple OXA positions in the active site unlike the fixed OXA position in *SmSULT*. In *SmSULT* T157S and *ShSULT* S166T structures, OXA bound in the positions observed for their wild-type OXA complex structures (Fig. 3B and C).

### 3.3. OXA recombinant protein activation assay

We repeated activation assays using recombinant proteins, rather than whole worm extracts. We observed that recombinant proteins from *S. mansoni* and *S. haematobium* (r*SmSULT* and r*ShSULT*) both showed strong OXA activation compared to the r*SmSULT*-OR (E142del). Consistent with this, we found that introducing *S. haematobium* residues into *S. mansoni* recombinant proteins, or *S. haematobium* residues into *S. mansoni*, had no impact on activation activity. The mutations to interconvert *SmSULT* and *ShSULT* are *SmSULT* F39Y, L149I, T157S and *ShSULT* Y54F, I158L, S166T (Fig. 4). Single, double and triple mutant proteins to interconvert r*SmSULT* and r*ShSULT* showed no effect on the ability of the recombinant sulfotransferase to activate OXA (Figs. 4–6). Therefore, our working hypothesis predicting the differences in contact residues between *SmSULT* and *ShSULT* are responsible for the OXA resistance exhibited by *S. haematobium* does not explain the natural resistance of the *S. haematobium* worms to OXA.

In the case of *S. japonicum*, r*SjSULT* failed to activate OXA. Our previous studies suggested that a glycine to valine change at residue 139 might account for the inability of *SjSULT* to activate OXA (Fig. 3D) (Taylor et al., 2017). We made the mutation and tested the ability of the Val139Gly mutation to activate OXA in a recombinant protein activation assay. The mutation results in an approximate 10-fold increase in



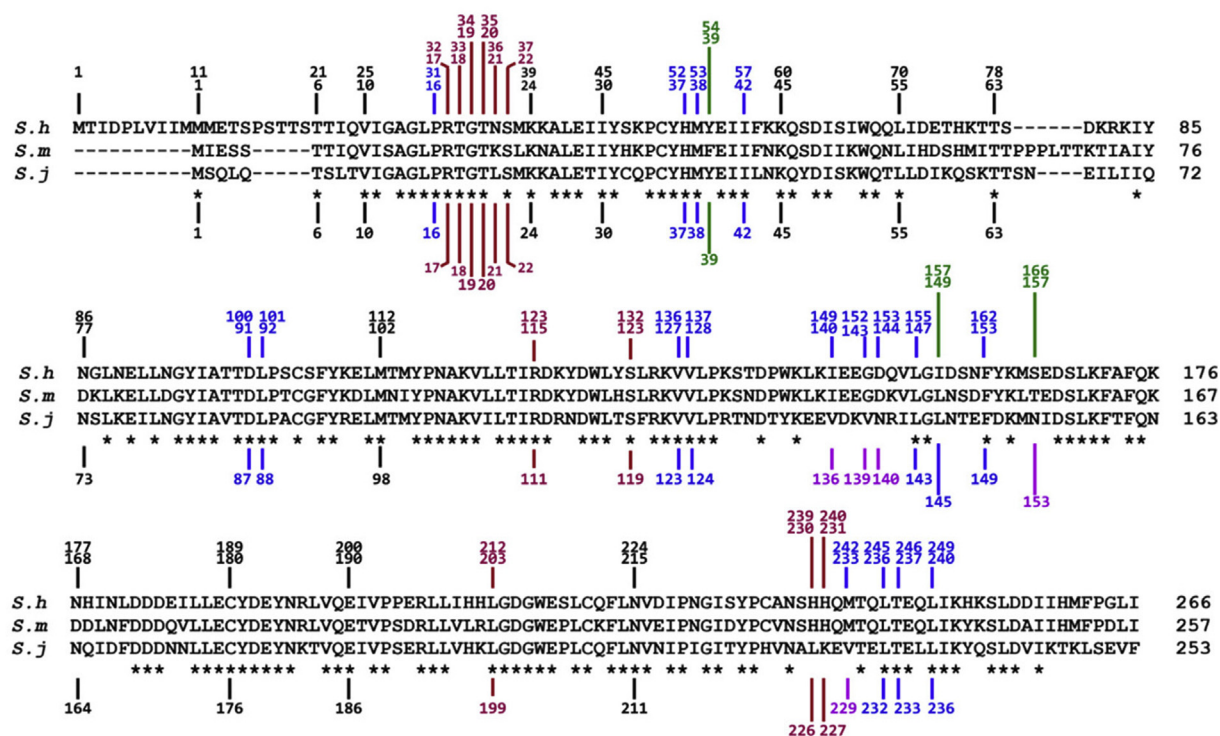


Fig. 2. Amino acid alignment of *S. mansoni* SULT, *S. haematobium* SULT and *S. japonicum* SULT.

Red lines indicate residues that contact co-factor (PAPS), blue lines indicate residues that contact OXA. The green lines indicate amino acids of special interest that differ between *SmSULT* and *ShSULT*. The purple lines indicate amino acids that differ between *SmSULT* and *SjSULT*. All contacts are  $\leq 4.5 \text{ \AA}$ . *S. haematobium* SULT vs *S. mansoni* SULT: 70.6% Identity; 80.9% Similarity; *S. japonicum* SULT vs *S. mansoni* SULT: 52% Identity; 69% Similarity. Modified from Valentim et al. (2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activation of OXA (Fig. 7), indicating that *SjSULT* Val139 can account in part for the lack of *SjSULT*'s activation of OXA. These results are consistent with the hypothesis that amino acid changes are, in part, responsible for the lack of OXA activation in *S. japonicum*.

Since the OXA activation assay determines an endpoint in an *in vitro* setting, it is possible that the results are an artifact of the long incubation and high protein concentrations. If efficiency of the sulfotransferase in *ShSULT* is significantly delayed compared to *SmSULT* it may account for OXA resistance. To determine if sulfotransferase efficiency is the reason for *S. haematobium* resistance to OXA, we measured activation at 15 min intervals over the 2.5 h incubation. There was little difference at most of the time points except for the first 30 min (Fig. 8A). To better define the difference, we repeated this experiment using 5 min increments over 1 h. Results again showed marginal statistically significant OXA activation at between 25 min and 45 min ( $P = 0.018$ ), however, the reactions all plateau by the 1 h mark (Fig. 8B).

An alternative hypothesis could be the presence of a component in the *S. haematobium* whole worm extract that inhibits *ShSULT*. To determine if *SmSULT* could complement OXA activation in *S. haematobium* extracts, each of the whole worm extracts – sensitive *S. mansoni*, resistant *S. mansoni* (E142del) and *S. haematobium* – were supplemented with varying amounts of r*SmSULT* from 1  $\mu\text{M}$  to 100 fM. At high concentrations – from 100 nM to 1  $\mu\text{M}$  of r*SmSULT* – all of the extracts activated OXA equally. However, when 10 nM of r*SmSULT* was added, the E142del extract showed the same levels of OXA activation as *S. mansoni* extract but the *S. haematobium* extract showed background levels (Fig. 9). At 1 nM, *ShSULT* is no longer complemented by *SmSULT* and at 1 pM E142del is still complemented by *SmSULT*.

#### 4. Discussion

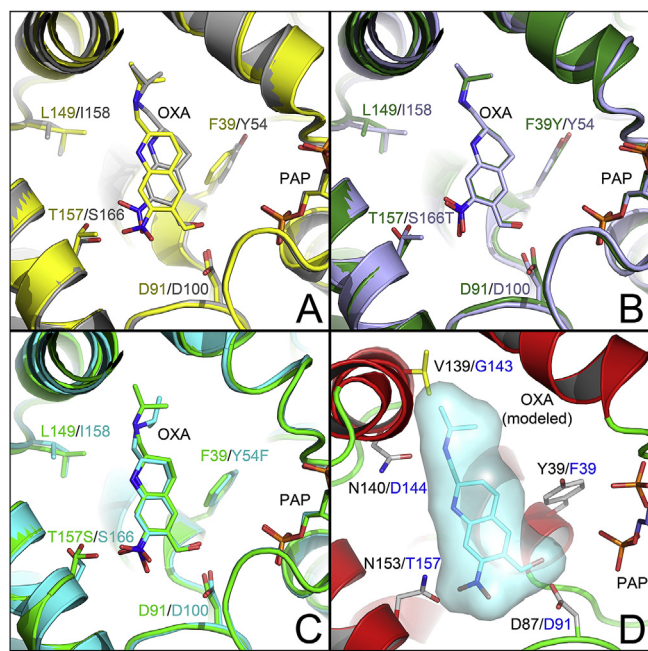
Oxamniquine kills *S. mansoni*, but not *S. haematobium* and *S.*

*japonicum*. We previously hypothesized that amino acid and resulting structural differences between *SmSULT* and *ShSULT* or *SjSULT* would explain the specificity of the drug towards *S. mansoni* (Valentim et al., 2013). These experiments were designed to test this hypothesis.

##### 4.1. Do amino acid changes in schistosome sulfotransferase explain species specific drug action?

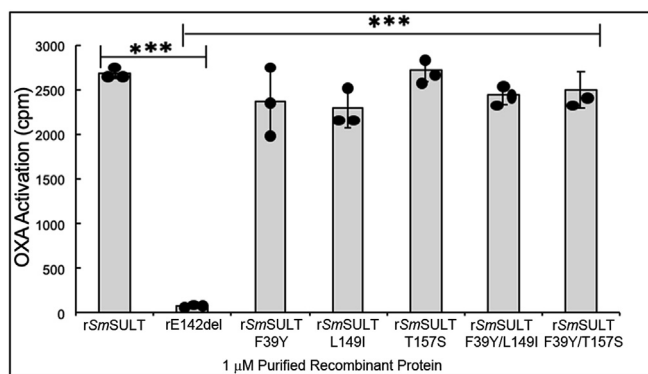
Initially, we used OXA activation assay using the soluble fraction from whole worm extracts incubated with [ $^3\text{H}$ ]OXA to quantify OXA activation after 2.5 h. This end point assay used conditions of saturation for all elements except for the endogenous sulfotransferase protein present in the extracts. Previous studies showed OXA sensitive *S. mansoni* extract caused high levels of OXA activation based on scintillation counts of the aqueous phase. These results closely parallel killing activity against adult worms. We observed that extracts from OXA-sensitive *S. mansoni* showed strong OXA activation activity, while extracts from OXA resistant *S. mansoni*, *S. haematobium* and *S. japonicum* extracts showed low to background levels of OXA activation based on scintillation counts in the aqueous phase (Fig. 1).

To investigate sulfotransferases in a controlled environment, a recombinant protein OXA activation assay was developed. The recombinant protein OXA activation assay eliminates the use of whole soluble worm extract and replaces it with a known concentration of recombinant sulfotransferase, radiolabeled substrate, co-factor PAPS, ATP, and  $\text{MgCl}_2$ , suspended in a protease inhibitor cocktail. Importantly, the use of a recombinant protein OXA activation assay demonstrates the differences in OXA activation between enzymes without any effects from endogenous inhibitors or enhancers. OXA-activation using the recombinant proteins r*SmSULT*, r*ShSULT* and r*SjSULT* revealed very different results from those using crude worm extracts. We found that proteins from both *S. mansoni* and *S. haematobium* showed comparable levels of activity, while recombinant

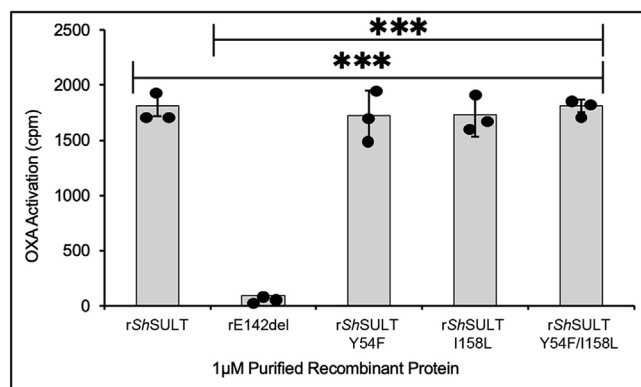


**Fig. 3.** Structural depiction of amino acid differences between *SmSULT*, *ShSULT* and *SjSULT*.

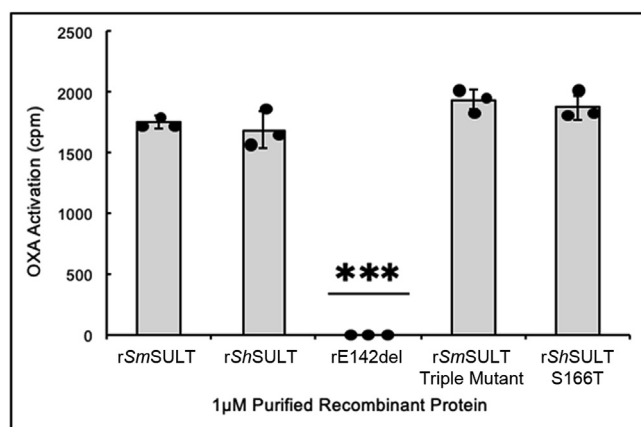
A) OXA-bound *SmSULT* (yellow) superimposed on OXA-bound *ShSULT* (gray). Three critical OXA contact residues from *SmSULT* (L149, F39, T157) are shown as sticks along with *ShSULT* counterparts (I158, Y54, S166). OXA, PAP and catalytic D91 (*SmSULT*) and D100 (*ShSULT*) are also shown in stick representation. Note the slightly different positions of OXA when bound in *SmSULT* (yellow) and *ShSULT* (gray). B) OXA bound in *ShSULT* S166T (light blue) and *SmSULT* Y54F (dark green) adopts the position of OXA-bound wild-type *ShSULT* shown in panel A. C) OXA bound in *ShSULT* Y54F (cyan) and *SmSULT* T157S (green) adopts the position of OXA-bound wild-type *SmSULT* shown in panel A. D) Crystal structure of *SjSULT* (PDB entry 5TIZ) with OXA (cyan) superimposed on the active site. *SjSULT* amino acid labels are shown in black and equivalent *SmSULT* amino acids are shown in blue. The V139 side chain of *SjSULT* creates a steric clash with OXA (at top) if it were to bind in the same position and conformation observed for OXA-bound *SmSULT* or *ShSULT*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** *SmSULT* Mutants in Recombinant Protein OXA Activation Assay. Purified recombinant proteins were incubated with [<sup>3</sup>H]OXA and the co-factor PAPS. Activated prodrug binds to sheared gDNA added to the mix and remains in the aqueous phase. Unreacted radiolabeled OXA goes to the organic phase. Scintillation counts were determined after a 2.5 h incubation. The Y axis represents scintillation counts per minute (cpm). *rSmSULT* is known to activate OXA and is a positive control. E142del is known to not activate OXA and is a negative control. Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.



**Fig. 5.** *ShSULT* Mutants in Recombinant Protein OXA Activation Assay. [<sup>3</sup>H]OXA was incubated with co-factor PAPS and the recombinant proteins. Activated prodrug binds to sheared gDNA added to the mix and remains in the aqueous phase. Unreacted radiolabeled OXA goes to the organic phase. Scintillation counts were determined after a 2.5 h incubation. The Y axis represents scintillation counts per minute (cpm). Higher OXA activation is indicated by higher cpm. *rSmSULT* is known to activate OXA and is a positive control. E142del is known to not activate OXA and is a negative control. Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.



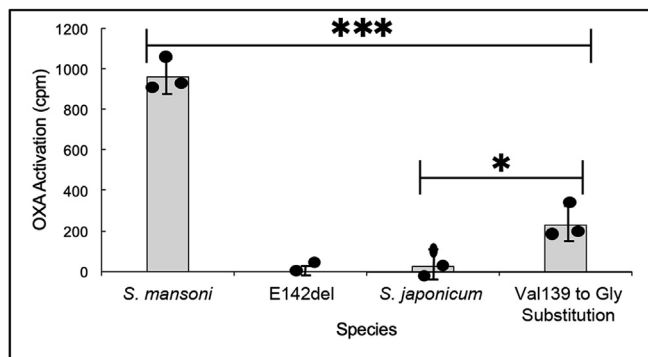
**Fig. 6.** *SmSULT* and *ShSULT* Mutants in Recombinant Protein OXA Activation Assay.

[<sup>3</sup>H]OXA was incubated with co-factor PAPS and the recombinant proteins. Activated prodrug binds to sheared gDNA added to the mix and remains in the aqueous phase. Unreacted radiolabeled OXA will go to the organic phase. Scintillation counts were determined after a 2.5 h incubation. The Y axis represents scintillation counts per minute (cpm). Higher OXA activation is indicated by higher cpm. *rSmSULT* is known to activate OXA and is a positive control. E142del is known to not activate OXA and is a negative control. Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.

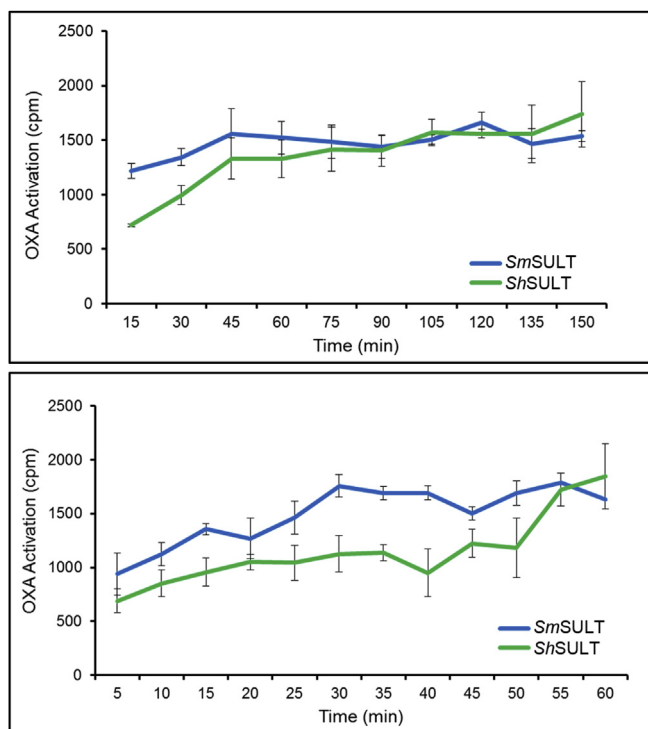
proteins from resistant *S. mansoni* or *S. japonicum* showed minimal activity (Figs. 4–7). These recombinant activation assays suggest that amino acid and structural changes between *SmSULT* and *ShSULT* do not explain why OXA kills *S. mansoni* but not *S. haematobium*. However, the results do suggest that structural changes may contribute to the inability of *SjSULT* to activate OXA and in part for the inability of this drug to kill *S. japonicum*.

These results are broadly consistent with kinetic analyses of sulfo-transferase activity in the three species. These experiments demonstrated a 2-fold greater  $k_{cat}/K_m$  value for *rSmSULT* relative to *rShSULT*, but 240-fold greater  $k_{cat}/K_m$  value for *rSmSULT* relative to *rSjSULT* (Taylor et al., 2017).

To further examine the role of amino acid differences between these three enzymes, we conducted experiments using chimeric recombinant



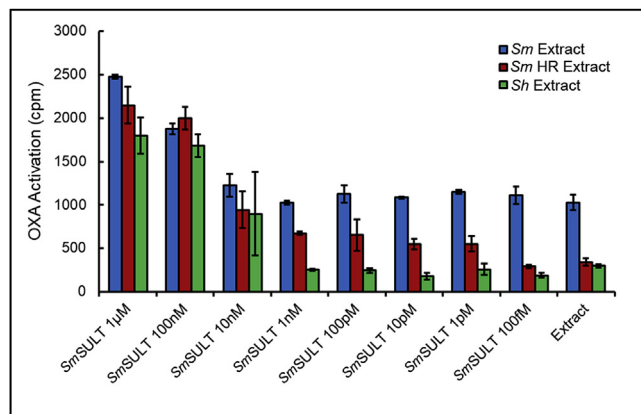
**Fig. 7. SjsULT Mutant in Recombinant Protein OXA Activation Assay.**  $^{[3H]}$ OXA was incubated with co-factor PAPS and the recombinant protein. Activated prodrug binds to sheared gDNA added to the mix and remains in the aqueous phase. Unreacted radiolabeled OXA goes to the organic phase. Scintillation counts were determined after stopping the reaction in 2.5 hr. The Y axis represents scintillation counts per minute (cpm). Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.



**Fig. 8. Recombinant Protein OXA Activation Assay at 15 (A) and 5 min (B) Time Points.**

$^{[3H]}$ OXA was incubated with co-factor PAPS and the recombinant proteins. Activated prodrug binds to sheared gDNA added to the mix and remains in the aqueous phase. Unreacted radiolabeled OXA goes to the organic phase. Scintillation counts were determined after stopping the reaction in 15 (A) or 5 min (B) increments. The Y axis represents scintillation counts per minute (cpm). Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.

proteins, in which residues were swapped between SmSULT and ShSULT or SjsULT. We predicted that chimeric proteins between SmSULT and ShSULT would not impact OXA activation, because the proteins from each of these species show strong activation activity. This prediction was clearly upheld: the recombinant protein OXA activation assay showed no significant change in the ability of the mutated *S. mansoni* sulfotransferase to activate OXA (Figs. 4–6). These results



**Fig. 9. rSmSULT Titration Assay.**

Fresh worm extracts of *S. mansoni*, *S. mansoni* E142del and *S. haematobium* were made (2 mg/mL) and incubated with varying amounts of rSmSULT. Scintillation counts were determined after a 2.5 h incubation. The Y axis represents scintillation counts per minute (cpm). Unsupplemented extracts acted as internal controls. Background levels of radiation were subtracted from all reactions prior to graphing. Experiments were run in triplicate.

challenge the hypothesis that ShSULT and SjsULT bind OXA poorly compared to SmSULT put forward by Valentim et al. (2013) and indicate that changes in the amino acid sequence are not fully responsible for OXA resistance in *S. haematobium* or *S. japonicum* (see below). In fact, a recent study shows similar binding affinities ( $K_D$ ) for OXA to SmSULT (66 μM) and SjsULT (Kd 59 μM) (Guzman et al., 2020). However, for SjsULT it seems that Val139 contributes to the inability of SjsULT to activate OXA. Structural analyses suggested that this mutation would impact enzyme function (Taylor et al., 2017). Consistent with this hypothesis, Val139Gly substitution in the rSjsULT protein partially restores OXA activation. Hence, for *S. japonicum* amino acid and associated structural changes do impact OXA activation, consistent with Valentim et al. (2013) prediction. Furthermore, SjsULT possesses additional active site amino acids Asn140 and Asn153 that substitute for aspartate and threonine/serine, respectively (Fig. 3D). While not predicted to abrogate OXA binding in the crystal structure of ShSULT gives some insight on this possibility. Multiple binding modes for OXA could impact ShSULT catalytic efficiency and its Tyr54 substitution for SmSULT active site Phe39 plays a role in the binding position (Taylor et al., 2017) Notably, SjsULT also has a Tyr39 at the SmSULT Phe39 position and may therefore experience a similar impact to OXA catalysis as a result.

#### 4.2. What other factors explain low OXA activation in *S. Haematobium* and *S. japonicum*

Recombinant rShSULT activates OXA effectively, so it is unclear why OXA treatment does not kill adult *S. haematobium*. We proposed two hypotheses to explain the poor killing activity: (i) substances within homogenates of *S. haematobium* might inhibit or detoxify OXA and (ii) amounts of ShSULT in *S. haematobium* homogenates may be too low to effectively sulfate OXA.

To test the first hypothesis we conducted a titration experiment in which whole worm extract from sensitive *S. mansoni*, HR, E142del and *S. haematobium* were supplemented with rSmSULT at varying concentrations. At high concentrations – from 1 μM to 100 nM of rSmSULT – all of the extracts activated OXA about equally. However, when less than 10 nM of rSmSULT was added, the E142del extract showed roughly half the levels of OXA activation as *S. mansoni* extract but the *S.*



*haematobium* extract measured at background (Fig. 9). These results suggest that an inhibitory component in *S. haematobium* extract might exist to prevent enough OXA sulfation or prevent the sulfated OXA product from exerting its toxic reactivity. We can speculate for example, that the inhibitory component, if it exists, could be a part of a *S. haematobium* or *S. japonicum* detoxification process that converts OXA to a product (as in oxidation) that the sulfotransferase will not use as a substrate. Another possibility is that the sulfated OXA product is detoxified in *S. haematobium* or *S. japonicum* before it kills the worms. If *S. mansoni* has a similar inhibitory component, the *S*mSULT may simply overcome such a process with a sufficient catalytic efficiency to produce a large enough pool of sulfated OXA to kill the worm unlike the less efficient *S*hSULT (Fig. 9). Enzyme efficiency cannot be ruled out as a factor for OXA resistance in *S. haematobium*. This experiment provides some suggestion that inhibition may contribute to the poor killing of *S. haematobium* by OXA.

## 5. Conclusions

Our results rule out the possibility that active site sequence variation between schistosome species abrogates OXA binding for *S. haematobium*, but this remains a contributory factor for *S. japonicum*. However, differences in sulfotransferase enzyme efficiency, variation in detoxification processes between species, and differences in sulfotransferase concentration remain possible explanations for species-specific resistance and may be interdependent in establishing OXA toxicity. Therefore, one answer to the question is that OXA kills *S. mansoni* but not *S. haematobium* or *S. japonicum* because it does not fit into the SULT binding pocket productively and does not get activated to a sufficiently toxic level.

Schistosomiasis is a neglected tropical disease which disproportionately affects poor communities in developing nations. While the current drug, PZQ, is effective and extraordinarily economical there is no second line drug to turn to when mass chemotherapy causes enough selective pressure for resistance to sweep through the pathogen parasite population. The recently elucidated mechanism of action for an older drug, OXA, has created an exploitable opening for directed drug development (Guzman et al., 2020; Hess et al., 2017; Taylor et al., 2017). A new generation of drugs are being developed using a rational approach to drug design (Neves et al., 2015; Hess et al., 2017; Rugel et al., 2018; Bibo-Verdugo et al., 2019).

## Declaration of competing interest

The authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2020.04.001>.

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