Molecular characterization of zebrafish Gstr1, the only member of teleost-specific glutathione S-transferase class

Authors:

Branka Bašica[a], Ivan Mihaljević[b], Nikola Maraković[c], Radmila Kovačević[a], Tvrtko Smital[b,*]

Affiliations:

[a] University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovica 2, 21000 Novi Sad, Serbia
[b] Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenicka 54, 10000 Zagreb, Croatia
[c] Instiute for Medical Research and Occupational Medicine, Ksaverska cesta 2, 10000 Zagreb, Croatia
These authors contributed equally to this work

*Corresponding author
Tvrtko Smital, PhD
Laboratory for Molecular Ecotoxicology
Division for Marine and Environmental Research
Ruđer Bošković Institute
Bijenicka 54
10 000 Zagreb, CROATIA
Tel.: **385 1 45 61 039
E-mail: smital@irb.hr

Highlights

- First functional characterization of the teleost-specific Gstr1 in zebrafish (*Danio rerio*);
- Recombinant zebrafish Gstr1 protein used for screening inhibition assay;
- New insight into structural characteristics of Gstr1 obtained by molecular docking analyses;
- Mechanistic interactions of Gstr1 with GSH and endogenous and exogenous compounds revealed;
- Proposed role of Gstr1 in steroidogenesis, metabolism and/or physiological actions of androgens;
- Organophosphate insecticides and pharmaceuticals identified as potent Gstr1 inhibitors.

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[a] University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia

[b] Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia

[c] Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia

These authors contributed equally to this work

*Corresponding author

Tvrtko Smital, PhD

Laboratory for Molecular Ecotoxicology

Division for Marine and Environmental Research

Ruđer Bošković Institute

Bijenicka 54

10 000 Zagreb, CROATIA

Tel.: **385 1 45 61 039

E-mail: smital@irb.hr

Abstract

Glutathione S-transferases (GSTs) are multifunctional phase II detoxification enzymes with primary function of glutathione conjugation of various endogenous and exogenous compounds. Teleost-specific Gstr1 in zebrafish (*Danio rerio*) was previously shown to have high expression in toxicologically relevant tissues and high activity towards model substrates. The aim of this study was a detailed functional characterization of zebrafish Gstr1. Molecular docking analyses were used to get novel insight into structural characteristics of Gstr1 and elucidation the mechanistic interactions with both GSH and various Gstr1 substrates or inhibitors. An initial screening inhibition assay performed by using model fluorescence substrate monochlorobimane (MCB) revealed interactions of different endogenous compounds and environmentally relevant xenobiotics with zebrafish Gstr1. All interacting compounds were further analyzed to determine their inhibition type and K_i values. Our data revealed that pregnenolone, progesterone, testosterone, DHEAS and corticosterone competitively inhibited transformation of MCB by Gstr1 with the calculated K_i values in the range 14-26 μM, implying that these hormones are physiological substrates of zebrafish Gstr1. Estrogens had no effect on Gstr1 activity. Taurochenodeoxycholate (TCDC) expressed lower inhibition potency toward Gstr1 with the K_i value of 33 μM. Among tested xenobiotics tributyltin chloride and rifampicin non-enzymatically bound Gstr1 enzyme (the calculated K_i values are 0.26 μM and 65 μM, respectively) and inhibited its activity, showing that these compounds are reversible noncompetitive inhibitors of zebrafish Gstr1. Insecticide diazinon competitively inhibited Gstr1 activity with calculated K_i value of 27 μM, while others Gstr1-interacting insecticides, chlorpyrifos-methyl (CPF-methyl) and malathion, showed allosteric activation-like effect. Among tested pharmaceuticals, tetracycline, erythromycin and methotrexate demonstrated competitive type of inhibition with the calculated K_i values of 17.5, 36.5 and 29 μM, respectively. In summary, we suggest that zebrafish Gstr1 has an important role in steroidogenesis, metabolism and/or physiological actions of androgens, but not estrogens in fish. Finally, our results imply the role of Gstr1 in metabolism of xenobiotics and protection of fish against deleterious environmental contaminants such as organophosphate insecticides and pharmaceuticals.

Keywords: Glutathione-S-transferase r1; zebrafish; molecular docking, functional characterization, interaction screening, endogenous compounds, xenobiotics.

1. Introduction

Aquatic environment is under constant pressure of newly emerging environmental contaminants. Large portion of these contaminants persistently stays in the freshwater systems and can cause deleterious effects to aquatic organisms (Murray et al., 2010). Consequently, it is of great importance to have better insight into mechanisms evolutionary developed and present in aquatic organisms as protection against xenobiotic compounds. Cellular uptake, metabolism and elimination of xenobiotics is largely mediated by numerous phase 0 membrane transport proteins, following with the phase I oxidation reactions maintained mainly by the cytochrome P450 enzymes and various conjugation reactions of phase II, and finally extrusion mechanisms mediated by membrane extrusion transporters of the phase III of cellular detoxification. Together, these integral elements form absorption, distribution, metabolism and excretion system (ADME) which critically determine toxicity of environmental contaminants (ADME-Tox) and is directly or indirectly modulated by endo- and xenobiotics (Stegeman et al., 2010).

One of critical ADME-Tox elements are glutathione S-transferases (GSTs), multifunctional phase II detoxification enzymes with primary function of glutathione conjugation of various endogenous and exogenous compounds. GSTs are ubiquitously present in most living organisms, from bacteria to humans (Hayes and Pulford, 1995). Sheehan et al. (2001) proposed that GSTs evolved from primordial stress proteins and diverged into several classes. GST classes are organized into three families: soluble cytosolic GSTs, membrane-associated microsomal GSTs also known as MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism), and mitochondrial GSTs comprised of only one Kappa class (Hayes et al., 2005; Sheehan et al., 2001; Oakley, 2011). The most characterized are cytosolic GSTs, divided into six classes: alpha, mu, pi, omega, theta and zeta (Hayes and Pulford, 1995). There are several additional classes reported in non-mammalian species, such as rho class in teleosts and cephalochordates (Glisic et al., 2015).

Conjugation of GST substrates by reduced glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) is quantitatively the major reaction of phase II metabolism (Hodgson, 2010). GSTs enzymatic activity primarily consists of ability to bring a substrate to close proximity of GSH using the active site capable of binding both electrophilic substrate and GSH. Second part of GST enzymatic activity is activation of sulfhydryl group of GSH, allowing the nucleophilic attack to electrophilic substrate which results with more water soluble and less reactive compound (Armstrong, 1997). Additionally, GSTs are capable of several other enzymatic activities such as isomerization, opening of epoxide rings, nucleophilic aromatic substitutions, reversible Michael additions to α,β-unsaturated aldehydes and ketones, and peroxidase activity (Eaton and Bammler, 1999).

GSTs are active in monomeric and dimeric forms. Dimers usually consist of two identical chains, however heterodimers are also found. GST monomers are made of two distinct domains, C-terminal alpha helical domain

and N-terminal thioredoxin-like domain. Within the N-terminal domain there is a specific GSH-binding site, termed G-site, where the GSH cysteinyl side chain gets activated through hydrogen bonding. The C-terminal and N-terminal domain together shape the substrate binding site, termed H-site due to hydrophobic nature of substrates. The variability of structure and chemical properties of H-sites is the main reason for polyspecific interactions of GSTs with substrates (Oakley, 2011).

Some of well known deleterious endogenous and exogenous substrates which are conjugated by GSTs are 4-hydroxynonenal (4-HNE), an aldehyde product of lipid peroxidation that can damage proteins and DNA (Singhal et al., 2015), and acrolein, a reactive aldehyde used in various synthesis reactions in organic chemistry and as agricultural biocide. Numerous xenobiotics such as pesticides, pharmaceuticals, polyaromatic hydrocarbons (PAHs), persistent organic pollutants (POPs) and heavy metals are also reported to be substrates of GST conjugating activity (Higgins and Hayes, 2011). GSTs are also involved into detoxification processes of numerous epoxide carcinogens, such as aflatoxin B1, a toxic metabolic product of some *Aspergillus* species and environmental contaminant present in cereal crops. It is detoxified by GST conjugation and later eliminated through the mercapturic acid pathway (Dohnal et al., 2014). Furthermore, benzo(a)pyrene and trans isomer of stilbene oxide are reported as the GST substrates (Hu et al., 1997; Seidegard et al., 1989).

Due to GSTs role in multidrug cancer resistance, high number of GST inhibitors have been developed (Allocati, 2018). Additionally, the naturally occurring GST inhibitors in plants have been identified (Harshbarger et al., 2017). Some of the most characterized GST inhibitors are ethacrynic acid, an α,β-unsaturated ketone used as diuretic drug, and its derivatives (Sau et al., 2010). Ethacraplatin is GST inhibitor developed to overcome cisplatin resistance by adding two ethacrynic acid ligands (Johnstone et al., 2016). Some other frequently used GST inhibitors are 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), potent inhibitor of GSTP1-1, and auranofin, gold phosphine used as antiarthritic, with similar anticancer effects as cisplatin (Tentori et al., 2011; De Luca et al., 2013).

Recent advances achieved using zebrafish (*Danio rerio*) as model organism make zebrafish and ideal candidate for investigation of integral elements of ADME processes. In zebrafish, 27 Gst members are distributed within 3 major families, with 7 cytosolic Gst classes showing clear orthology relationships with human GST genes (Glisic et al., 2015). Tissue expression analyses of Gst genes in zebrafish revealed classes Gst Pi, Gstt1a, Gstz1, Gstr1, Mgst3a and Mgst3b as crucial elements of biotransformation of xenobiotics based on their high expression in barrier tissues such as liver, kidney, gills and intestine (Glisic et al., 2015). These GST members are even dominantly expressed in zebrafish embryos (Glisic et al., 2016). Additionally, tissue expression of adult zebrafish GSTs determined on the protein level showed comparable expression profile with mRNA levels (Tierbach et al., 2018).

Functionally, the most potent activities, with high affinities and turnover numbers in reactions with model substrates, were determined for members of Pi class and Gstr1 (Glisic et al., 2015).

Consequently, due to high expression of teleost-specific Gstr1 in toxicologically relevant tissues, and high activity it shows towards model substrates, in this study we have characterized zebrafish Gstr1 in more detail. Our major goals were (1) to analyze interactions of zebrafish Gstr1 with different groups of endo- and xenobiotics, and (2) to determine the type of interaction of Gstr1 with tested substances in order to be able to perform initial evaluation of possible protective and/or physiological role of Gstr1. Furthermore, our intention was to provide novel insight into structural characteristics of this teleost-specific GST protein and elucidate the mechanistic interactions with both GSH and various Gstr1 substrates or inhibitors. Therefore, by using recombinant zebrafish Gstr1 protein we performed a screening inhibition assay with different endogenous and exogenous compounds to identify interacting compounds, and to define their inhibition type and K_i values. By molecular docking analyses we obtained data on the mechanistic interactions of Gstr1 with GSH and different endogenous and exogenous compounds.

2. Materials and methods

Chemicals

All tested compounds were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Alfa Aesar (Ward Hill, MA, USA) unless stated otherwise**.**

Inhibition assay

Gstr1 was cloned and purified as described before by Glisic et al., 2015. The inhibition assay was based on previously described fluorometric assay for GST activity toward monochlorobimane (MCB). The assay was performed at 25 ℃ in black, flat bottom 96-well plates with final reaction volume of 250 µL. The reaction mixture consisted of phosphate buffer (pH 6.5) in concentration of 100 mM, Gstr1 recombinant protein in final concentration of 1.5 µg per well, inhibitor of desired final concentration, GSH co-substrate in final concentration of 1 mM, and model substrate (MCB) in final concentration of 100 µM. The resulting fluorescence was measured with microplate readers (Infinite M200, Tecan, Salzburg, Austria or Fluoroskan Ascent FL Microplate Reader, Thermo Fisher, Helsinki, Finland) at 355 nm excitation and 460 nm emission wavelengths for 10 min in 15 or 30 s intervals. For blank control the reaction mixture was prepared without Gstr1 recombinant protein with 100 µL of phosphate buffer. Reaction mixture without tested compound was used as positive control, with 100 µL of phosphate buffer.

An initial Gstr1 inhibition screening was performed first, using single 100 µM concentration of tested compounds. Then, for compounds which showed the Gstr1 inhibition above 50% the K_i values and type of reversible interaction

were determined. Inhibition assay for determination of K_i values of tested compounds was based on Michaelis-Menten kinetics of MCB, which was determined using MCB in the range of 5 - 600 µM. MCB dose response was inhibited with three concentrations of inhibitors based on the percentage (%) of inhibition observed by single concentration inhibition screening. The strength of inhibition of the tested compounds was considered to be very strong for K_i < 1 µM, strong for K_i 1-20 µM, moderate for K_i 20-40 µM, and weak for K_i > 40 µM.

Modeling the three-dimensional structure of Danio rerio Rho class, Gstr1, and molecular docking studies

Biovia Discovery Studio Client v17.2 (Accelrys, San Diego, CA, USA) implemented Build Homology Models protocol was used to construct Gstr1 homology models as based on alignment of the model sequence and the template structure. Build Homology Models protocol uses MODELER (Sali and Blundell, 1993) automodel to build homology models. To build Gstr1 homology model, the input sequence alignment between the model sequence of Gstr1 monomer and the sequence of chain A from Gst from Antarctic clam (*Laternula elliptica*) was used (Fig. 5) (Park et al., 2013). Rest of the parameters in the Parameters Explorer of Build Homology Models protocol were set as described previously (Mihaljević et al., 2017). Ligands to be docked in the homology model of Gstr1 were created with ChemBio3D Ultra 13.0 (PerkinElmer, Inc., Waltham, MA, USA) and minimized using the MMFF94 force field implemented in ChemBio3D Ultra 13.0.

Biovia Discovery Studio Client v17.2 implemented Dock Ligands (CDOCKER) protocol was used for the docking study. CDOCKER is a grid based molecular docking method that employs CHARMm force field (Brooks et al., 1983; Momany and Rone, 1992). The model with the highest overlay similarity was used as the rigid receptors while the ligands were allowed to flex during the refinement. Binding site within the homology models was defined by a sphere (r = 13.0 Å) surrounding the amino acids that have been located within the identified G- and H-site. Rest of the parameters included in the CDOCKER protocol were set as described elsewhere (Maraković et al., 2016).

The binding affinities of docked ligands in the poses generated by Dock Ligands (CDOCKER) protocol were estimated using the scoring functions as implemented in the Biovia Discovery Studio Client v17.2 Score Ligand Poses protocol. Together with POSE_NUMBER, -CDOCKER_ENERGY, -CDOCKER_INTERACTION_ENERGY, following scoring functions were calculated. LigScore1_Dreiding and LigScore2_Dreiding (Krammer et al., 2005) are fast, simple scoring functions for predicting receptor-ligand binding affinities which are computed in units of pKI (-logKI). PLP1 (Gehlhaar et al., 1995) and PLP2 (Parrill and Reddy, 1999) are fast and simple docking functions that have been shown to correlate well with protein-ligand binding affinities. PLP scores are measured in arbitrary units. Higher PLP scores indicate stronger receptor-ligand binding (larger pKI values). Jain (Jain, 1996) is an empirical scoring function developed through an evaluation of the structures and binding affinities of a series of protein-ligand complexes. The Jain score is a sum of five interaction terms. These terms describe: lipophilic interactions, polar attractive interactions, polar repulsive interactions, solvation of the protein and ligand and an

entropy term for the ligand. The PMF (Muegge and Martin, 1999) and PMF04 (Muegge, 2006) scoring functions were developed based on statistical analysis of the 3D structures of protein-ligand complexes. They were found to correlate well with protein-ligand binding free energies while being fast and simple to calculate. The PMF04 score is an updated version of the original PMF score. The PMF scores are reported in arbitrary units. A higher score indicates a stronger receptor-ligand binding affinity.

To identify the poses of docked ligands that score high in more than one scoring function, the Biovia Discovery Studio Client v17.2 Consensus Score protocol was used. The Consensus Score protocol calculates the consensus scores of a series of docked ligands for which other scores have been previously computed. For each selected scoring function, the ligands are listed by score in descending order. The consensus score for a ligand is an integer between zero (none of the scores are in the top-ranking percentile) and the total number of scores (all of the scores are in the top-ranking percentile) listed in Input Properties. Thus, in the Parameters Explorer of Consensus Score protocol, the following parameters were set. In Input Properties score properties -PLP2, -PMF, -PMF04, Jain, -CDOCKER_ENERGY, -CDOCKER_INTERACTION_ENERGY, LigScore2_Dreiding, LigScore1_Dreiding, and POSE_NUMBER were chosen to calculate the consensus score. Consensus Percentage was set to 20 to specify the percentage of top molecules to include in the consensus. Use Best Pose only was set to False.

The representative poses of docked ligands with the highest consensus score were minimized using Biovia Discovery Studio Client v17.2 Minimization protocol, as described elsewhere (Maraković et al., 2016).

Data analysis

All assays were performed in 3 independent experiments run in triplicates. Data shown on related figures represent mean ± standard errors of mean (SEM). All calculations were performed using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, California, USA) as described below. The kinetic parameters, *Km* and *Vmax* values were calculated using the Michaelis-Menten equation (1):

$$V = \frac{Vmax \times [S]}{[S] + Km} \quad (1)$$

where *V* is velocity (fluorescence units per milligram of protein per minute), *Vmax* is maximal velocity, [*S*] is substrate concentration and *Km* is Michaelis–Menten constant.

For purpose of K_i determination, measured data were analyzed by nonlinear regression, mixed model enzyme inhibition. The used model also provided the alpha value (α) which was used to determine the type of interaction of tested compounds with Gstr1. Alpha value determines the level to which the inhibitor changes the affinity of the enzyme for substrate. In case of alpha equals 0, the inhibitor does affect the binding of substrate to the

enzyme, which results in noncompetitive inhibition. When alpha equals higher number, binding of inhibitor blocks binding of the substrate resulting in competitive inhibition. If alpha equals less than 1, binding of the inhibitor additionally stimulates binding to the enzyme, which results in uncompetitive inhibition. In addition, Lineweaver-Burk plots (or double reciprocal plots), a graphical representation of the Lineweaver-Burk equation of enzyme kinetics, were used to illustrate the type of interactions. Intersecting lines that converge at the y-axis is diagnostic signature for the competitive inhibition modality. Intersecting lines that converge to the left of the y-axis and on the x-axis is diagnostic signature for noncompetitive (α = 1) inhibition modality (Copeland, 2005).

3. Results

3.1 Inhibitory potential of tested compounds

To elucidate possible interaction of different endogenous compounds (Fig. 1) and environmentally relevant xenobiotics (Fig. 2) with zebrafish Gstr1, we performed an initial screening inhibition assay with 100 μM concentration of selected chemicals, except carbaryl and probenecid which were tested in concentration of 50 μM due to their low solubility. The selection of xenobiotics was based on the prioritization of trace pollutants in surface waters (Murray et al., 2010). In the second step, all compounds that inhibited Gstr1 enzyme activity at 100 μM by more than 50% compared to the control were further analyzed to define their inhibition type and to determine K_i values as it is shown in Table 1. To determine the type of interaction with Gstr1, we compared kinetic parameters (*Km* and *Vmax*) of transformation of fluorescence substrate MCB in the absence and in the presence of different interacting compounds using nonlinear regression, and mixed model enzyme inhibition. All of the inhibition curves obtained are given in the Supplementary Data, while representatives of exemplary interactors are shown in Figs. 3 and 4.

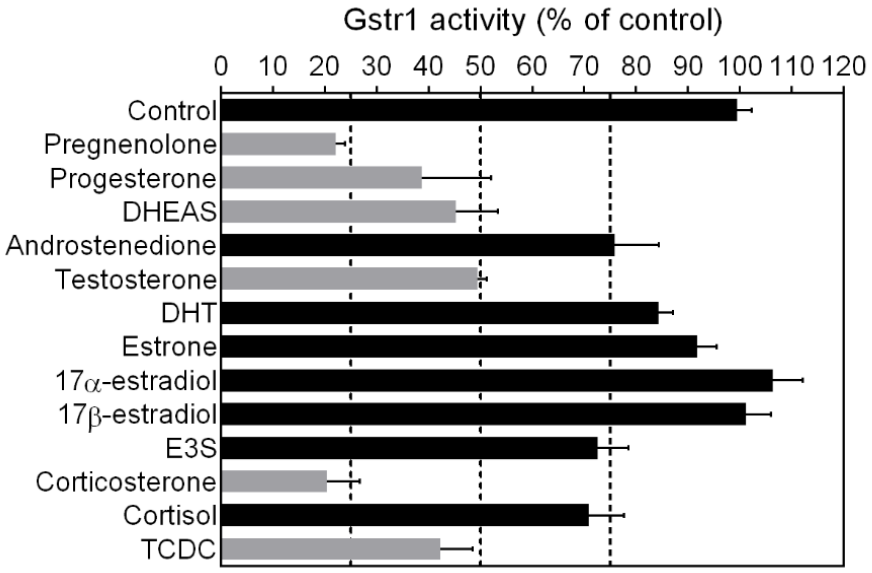

Figure 1. Interaction of zebrafish Gstr1 with endogenous compounds. Data are expressed as percentage (%) of the Gstr1 activity toward model fluorescence substrate monochlorobimane (MCB; 100 µM) in presence of each modulator (100 µM) and co-substrate glutathione (GSH; 1 mM) relative to Gstr1 activity in absence of a modulator (control, set to 100%). Abbreviations: dehydroepiandrosterone sulfate (DHEAS), dihydrotestosterone (DHT), estrone-3-sulfate (E3S), taurochenodeoxycholate (TCDC). Data represent mean ± SEM of triplicates from three independent experiments.

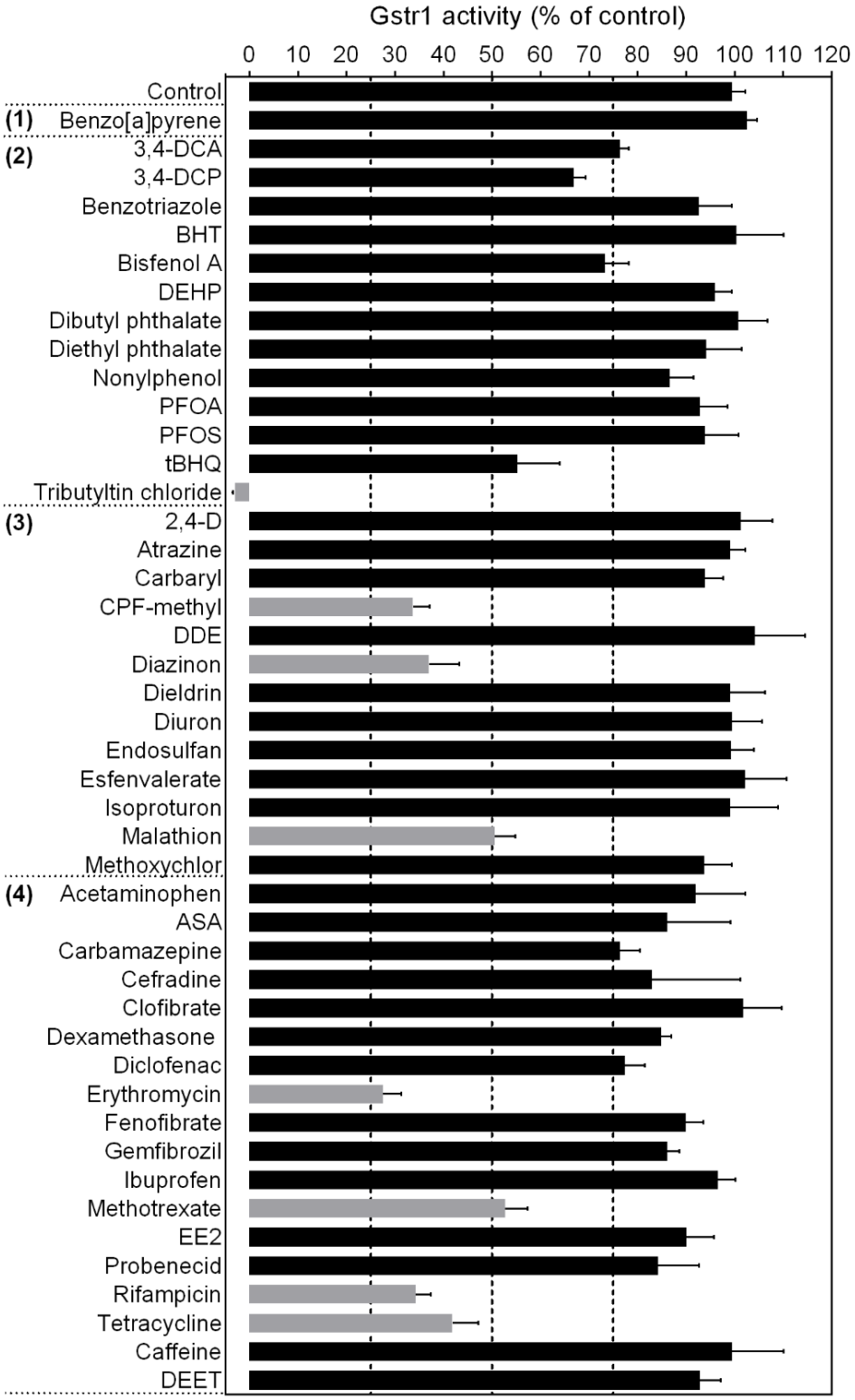

Figure 2. Interaction of zebrafish Gstr1 and environmental contaminants. Data are expressed as percentage (%) of the Gstr1 activity toward model substrate monochlorobimane (MCB; 100 µM) in presence of each modulator

(100 µM) and co-substrate glutathione (GSH; 1 mM) relative to Gstr1 activity in absence of a modulator (control, set to 100%). Carbaryl and probenecid are tested at 50 µM concentration due to their low solubility. Xenobiotic interactors are divided into four groups: (1) polycyclic aromatic hydrocarbon, (2) industrial chemicals, (3) pesticides, and (4) pharmaceuticals and personal care products (PPCPs). Abbreviations: 3,4-dichlorophenol (DCP), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,4-dichloroaniline (DCA), 17α-ethynilestradiol (EE2), acetylsalicylic acid (ASA), bis(2-ethylhexyl) phthalate (DEHP), butylated hydroxytoluene (BHT), chlorpyrifos-methyl (CPF-methyl), dichlorodiphenyldichloroethylene (DDE), N,N-diethyl-meta-toluamide (DEET), perfluorooctane sulphonate (PFOS), perfluorooctanoic acid (PFOA) and *tert*-Butylhydroquinone (tBHQ). Data represent mean ± SEM of triplicates from three independent experiments.

Table 1. Inhibition constants (K_i) determined for a series of endogenous compounds and xenobiotic interactors of zebrafish Gstr1. Type of interactions, alpha (α) values, 95% confidence intervals and coefficients of determination are shown. Interactors are divided into four groups separated by doted lines: endogenous compounds, industrial chemicals, pesticides and pharmaceuticals.

Compound	Inhibition type	α	K_i (µM)	95% c.i.	R^2
Pregnenolone	Competitive	α » 1	13.55 ± 1.49	10.60-16.50	0.98
Progesterone	Competitive	α » 1	26.24 ± 2.59	21.10-31.39	0.98
DHEAS[a]	Competitive	α » 1	19.50 ± 1.61	16.31-22.69	0.98
Testosterone	Competitive	α » 1	19.91 ± 2.23	15.54-24.29	0.94
Corticosterone	Competitive	α » 1	20.49 ± 4.89	10.79-30.19	0.89
TCDC[b]	Competitive	α » 1	32.99 ± 2.74	27.55-38.44	0.98
Tributyltin chloride	Noncompetitive	1.2 ± 0.6	0.26 ± 0.02	0.21-0.31	0.93
Chlorpyrifos-methyl	nd	0.1 ± 0.0	nd	-	-
Diazinon	Competitive	α » 1	27.03 ± 2.06	22.93-31.12	0.98
Malathion	nd	0.7	nd	-	-
Erythromycin	Competitive	α » 1	36.47 ± 3.29	29.94-43.00	0.97
Methotrexate	Competitive	α » 1	29.29 ± 3.04	23.25-35.33	0.98
Rifampicin	Noncompetitive	1.8 ± 0.4	64.83 ± 2.48	59.96-69.70	0.96
Tetracycline	Competitive	α » 1	17.48 ± 0.95	15.59-19.37	0.98

nd – not determined.
[a] Dehydroepiandrosterone sulfate;
[b] Taurochenodeoxycholate

3.1.1 Endogenous compounds

Among 10 steroid gonadal hormones screened for their interaction with Gstr1, 4 hormones showed over 50% inhibition of enzyme activity compared to the control (Fig. 1) which implies possible physiological interaction of Gstr1 with those steroids. All of them demonstrated competitive type of inhibition of Gstr1 activity (Table 1, Fig.

3). The calculated K_i values are in the micromolar range (14-26 µM), and the pregnenolone expressed the highest inhibition potency (K_i of 13.55 ± 1.49 µM) and is classified as strong inhibitor. All other endogenous compounds that showed interactions with Gstr1 are classified as moderate inhibitors. Testosterone and DHEAS showed similar potency with K_i (approx. 20 µM, Table 1, Fig. 3, Supplementary Data Fig. S1), while none of the tested estrogenic hormones expressed activity toward Gstr1 (Fig. 1). Corticosterone showed similar inhibition potency as androgens with calculated K_i of 20.49 ± 4.89 µM (Table 1, Fig. 3), while cortisol was without effect. Taurochenodeoxycholate (TCDC) expressed lower inhibition potency toward Gstr1 with K_i value of 33 µM (Table 1, Fig. 3).

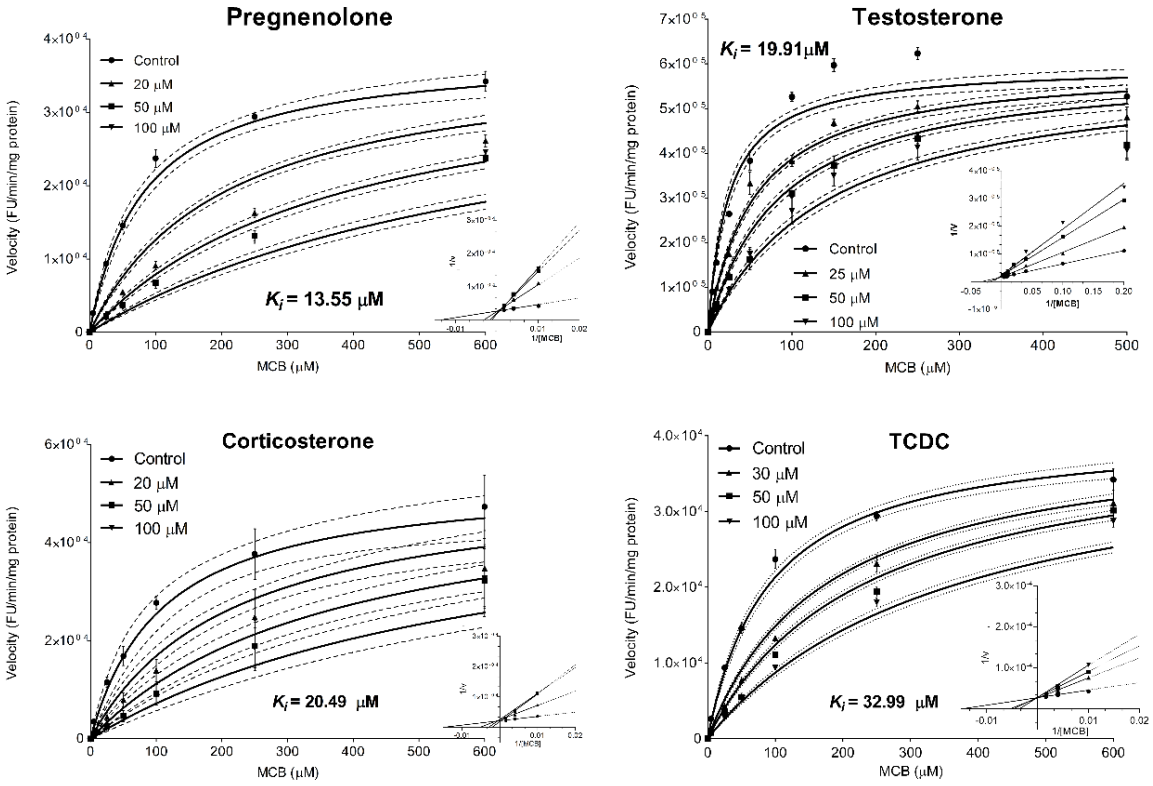

Figure 3. Competitive inhibition of zebrafish Gstr1 activity by selected endogenous compounds (pregnenolone – the lowest K_i value of tested endogenous compounds; testosterone – the lowest K_i value of tested androgens; corticosterone – representative of glucocorticoids; taurochenodeoxycholate (TCDC) – representative of bile salts). Results are shown as concentration dependence of Gstr1 mediated monochlorobimane (MCB) transformation expressed in fluorescence units normalized to time and protein concentrations (FU/min/mg proteins) over MCB concentration (µM) at 25 °C in presence of 1 mM GSH co-substrate. Inserted figures: Lineweaver-Burk plots that show the type of inhibition. Data were fitted in the GraphPad Prism 6. Mean, SEM and confidence interval (c.i.) were calculated from 4-6 replicates of three independent experiments.

3.1.2 Industrial chemicals

Among tested industrial chemicals only tributyltin chloride showed a very strong noncompetitive inhibition of Gstr1 activity, with K_i value of 0.26 µM (Table 1, Fig. 4). Phthalates (DEHP, dibutyl phthalate and diethyl phthalate), and perfluorinated compounds (PFOA and PFOS) showed no interaction with Gstr1 enzyme. Other tested industrial chemicals showed limited (inhibition of enzyme activity <50%) to no interaction with Gstr1 enzyme (Fig. 2).

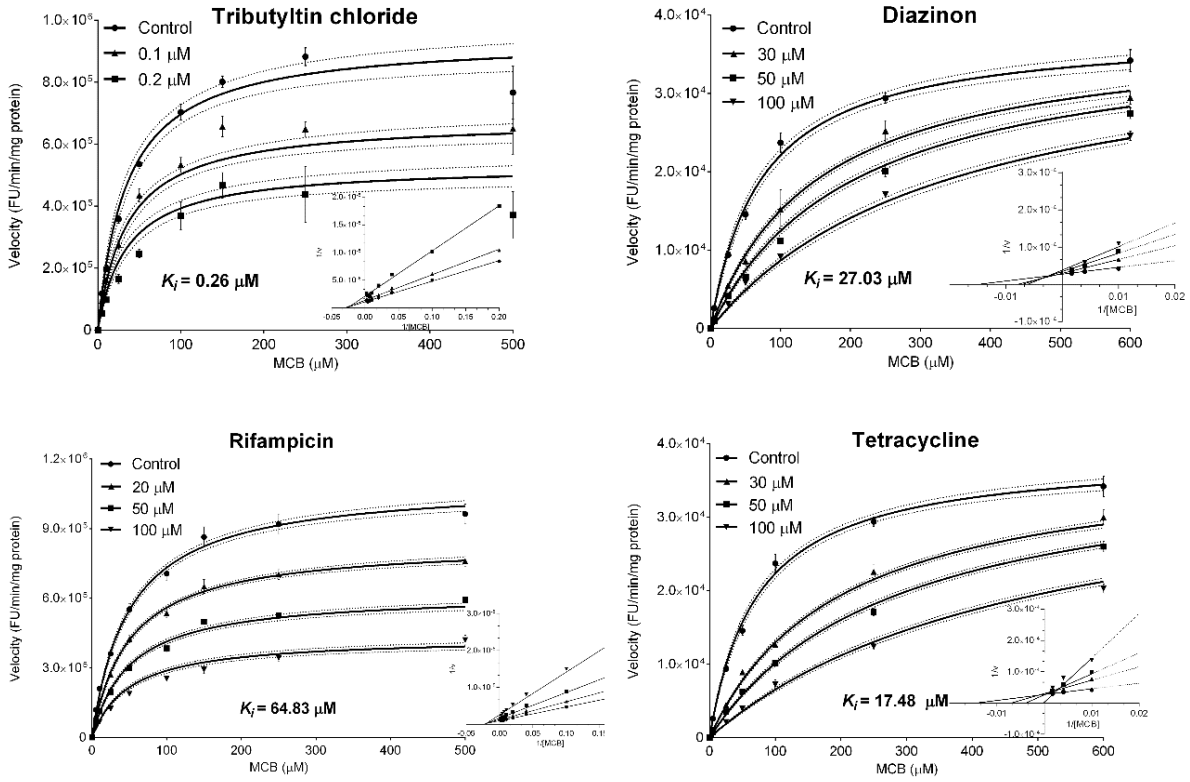

Figure 4. Inhibition of zebrafish Gstr1 activity by industrial chemical (tributyltin chloride – the lowest K_i value of all tested compounds), pesticide representative (diazinon) and pharmaceuticals (rifampicin – as a noncompetitive inhibitor; tetracycline – the lowest K_i value among tested pharmaceuticals). Concentration dependence of Gstr1 mediated monochlorobimane (MCB) transformation is expressed as fluorescence unit normalized to time and protein concentrations (FU/min/mg proteins) over MCB concentration (µM) at 25 °C in presence of 1 mM GSH co-substrate. Inserted figures: Lineweaver-Burk plots that show type of inhibition. Data were fitted in the GraphPad Prism 6. Mean, SEM and confidence interval (c.i.) were calculated from 4-6 replicates of three independent experiments.

3.1.3 Pesticides

Gstr1 showed interaction with insecticides chlorpyrifos-methyl (CPF-methyl), diazinon and malathion, and inhibition of enzyme activity was in the range of 49-66% (Fig. 2). Diazinon expressed moderate competitive type of inhibition with calculated K_i value of 27 µM (Table 1, Fig. 4). However, CPF-methyl and malathion showed allosteric activation-like effect. In fact, in the presence of the lowest concentration of both pesticides the enzyme activity was increased in comparison to control, while in the presence of the highest concentration (100 µM) the enzyme activity was similar to control values (Supplementary Data, Fig. S2). Other tested pesticides showed no interaction with Gstr1 enzyme (Fig. 2).

3.1.4 Pharmaceuticals and personal care products (PPCPs)

Initial screening inhibition assay showed that pharmaceuticals erythromycin, methotrexate, rifampicin and tetracycline inhibited Gstr1 activity in the range of 47-72% (Fig. 2). Among them tetracycline demonstrated strong competitive type of inhibition with the lowest calculated K_i value of 17.5 µM (Table 1, Fig. 4), while erythromycin and methotrexate, moderate competitive inhibitors, have higher K_i values of 36.5 and 29.29 µM, respectively (Table 1, Supplementary Data, Fig. S3). On the contrary, rifampicin showed weak noncompetitive inhibition of Gstr1 activity with K_i values of 65 µM (Table 1, Fig. 4). Other tested PPCPs showed limited (inhibition of enzyme activity <25%) to no interaction with Gstr1 enzyme (Fig. 2).

3.2 In silico analysis of zebrafish Gstr1 structure and binding sites

3.2.1 Homology modeling

Prior to the docking studies, the three-dimensional model structure of *Danio rerio* Rho class Gstr1 was constructed by homology modeling. For template structure used in homology modeling, crystal structure of a glutathione S-transferase from Antarctic clam *Laternula elliptica*, chain A (PDB ID: 3QAV) (Park et al., 2013; Espinoza et al., 2013) was chosen based on high indexes of sequence identity (39.1) and sequence similarity (63.9), and sequence alignment was carried out (Fig. 5). To further improve the obtained model structure, energy minimization was performed as described in the Materials and methods section. However, prior to defining and editing binding site of the Gstr1 model structure we needed to obtain the full dimer of model structure, as it is known for binding site to be outlined with amino acid residues from counterpart B monomer to considerable extent. This was done through structural alignment of two copies of generated Gstr1 model structure with crystal structure of dimer of human class pi glutathione S-transferase P1-1 (hGSTP1-1) (PDB ID: 1MD3), followed by manual rotation of one monomer of modeled structure until the same symmetry between the two monomers was obtained as the one

observed in crystal structure of hGSTP1-1. Finally, binding site was defined and edited using software feature that automatically identifies cavities within the receptor and knowledge of different GSTs active sites.

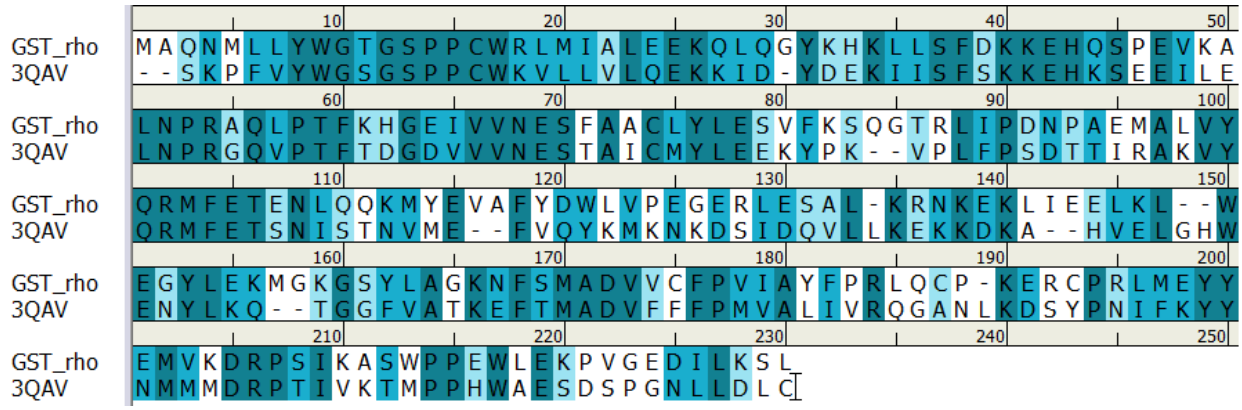

Figure 5. Sequence alignment between 3QAV and Gstr1.

3.2.2 Molecular docking

To confirm our experimental data that showed different modes of interaction between substrates and inhibitors of rho class GST, binding studies were carried with GSH and compounds identified as substrates (testosterone and corticosterone) or inhibitors (tributyltin chloride and rifampicin) using molecular docking approach. Since amino acid residues that constitute glutathione-binding site (G-site) and hydrophobic substrate binding site (H-site) are largely conserved through different classes of GSTs, by sequence alignment of zebrafish Gstr1 with different GSTs (GSTP1, GSTP2, 6GST, GST4_4) the residues forming G-site (Tyr8, Trp9, Ser13, Pro15, Lys40, Glu42, His43, Glu47, Lys49, Gln56, Leu57, Glu69, and Ser70) and H-site (Thr11, Pro14, Cys16, Glu107, Gln111, Lys112, Tyr114, Glu115, Val116, Phe118, Pro124, Gly219, and Glu220) were identified. Also, to obtain starting structure for docking studies with GSH occupying G-site, structural alignment between model structure of Gstr1 and crystal structure of a glutathione S-transferase from Antarctic clam *Laternula elliptica* in a complex with GSH (PDB ID: 3QAW) was conducted to hard dock the GSH to the G-site of Gstr1 model structure. Moreover, hard docking of GSH was used to confirm the positions of G-site forming amino acid residues above identified via sequence alignment. Indeed, GSH was found to be in close proximity to Ser13, Pro15, His43, Gln56, Leu57, Glu69, and Ser70 (Fig. 6).

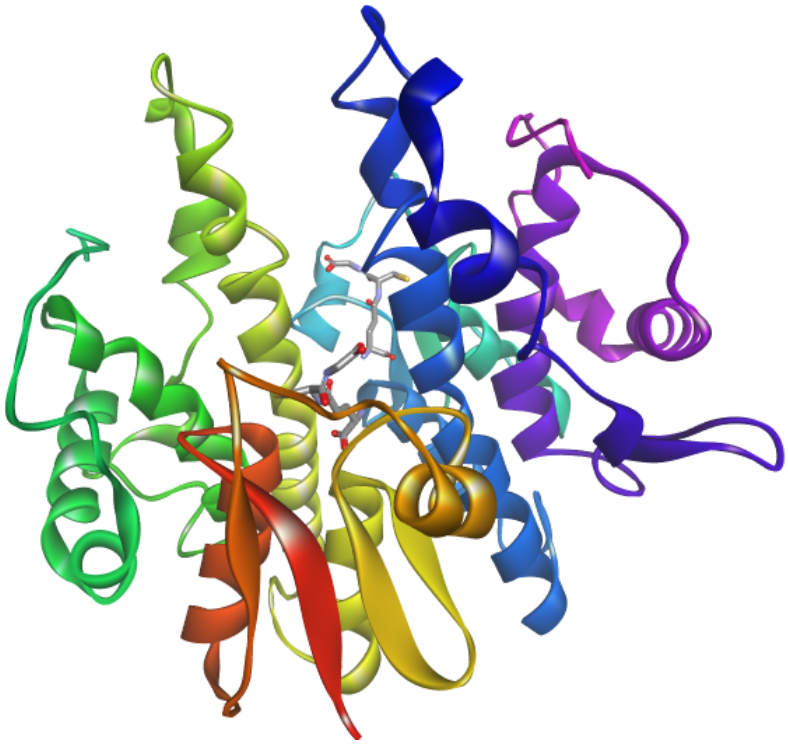

Figure 6. Final 3D structure of zebrafish Gstr1 with docked GSH in each monomer G-site.

Using molecular docking of compounds previously established as substrates (testosterone or corticosterone) we were able not only to confirm its susceptibility to nucleophilic addition of the GSH, but also to confirm the positions of above identified H-site forming amino acid residues. From the 30 generated conformations of docked ligand the representative pose was chosen based on the shortest distance between the thiol sulfur of GSH and β-carbon atom from α,β-unsaturated carbonyl group of ligand. Namely, testosterone and corticosterone are supposed to undergo conjugate addition of GSH to their α,β-unsaturated carbonyl group. Figure 7 depicts model complex between corticosterone and GSH-Gstr1 obtained via molecular docking. The distance between thiol sulfur and β-carbon atom of corticosterone is estimated to be 4.43 Å, a distance close enough for reactivating groups in pre-reactionary complex for nucleophilic addition to proceed. Another important feature predicted by model complex is the 3.41 Å distance between thiol sulfur and hydrogen atom of the hydroxyl group of Ser13, a highly conserved residue known to catalyze nucleophilic addition of the GSH to substrates electrophilic groups, which is sufficient for hydrogen bond to occur between these two atoms. Also, corticosterone is found to be in close proximity of Tyr114 and Glu115, amino acid residues above identified to form H-site, but also in close proximity of Gln110, pointing at this residue as another H-site forming residue. Interestingly, corticosterone is found to be forming alkyl-type hydrophobic interaction with Val116, another H-site forming residue, but from the counterpart B

monomer. Other H-site residues from the B monomer in close proximity of corticosterone include Gln111 and Glu115. This finding emphasizes the importance of using dimeric model structures of GSTs for more accurate binding studies.

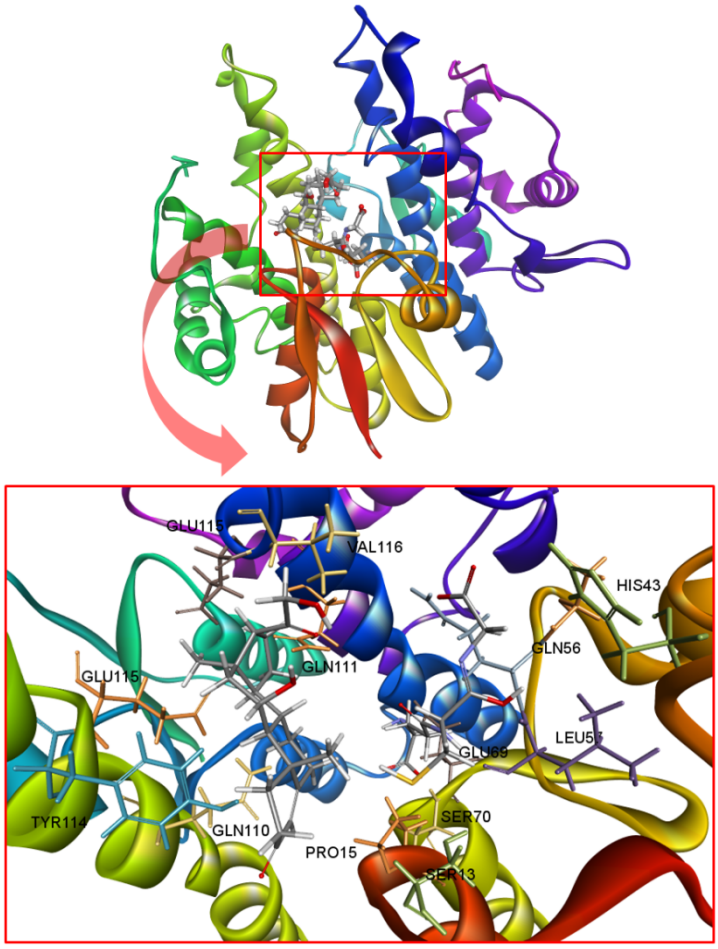

Figure 7. Final docking pose of the corticosterone-GSH-Gstr1 complex, and close-up view of the active site residues.

On the other hand, with molecular docking of compounds established as reversible noncompetitive inhibitors (tributyltin chloride or rifampicin) we were able to elucidate the binding mode responsible for its inhibitory mode of action. The representative pose was chosen based on the highest score when implementing Consenus Score protocol from Biovia Discovery Studio Client v17.2 (for more details, please see Materials and methods section). Indeed, as can be seen in Figure 8, binding studies suggest that known reversible inhibitor rifampicin occupies G-site, thus blocking the approach of GSH. In doing so, rifampicin interacts with neighboring residues via different types of non-covalent interactions.

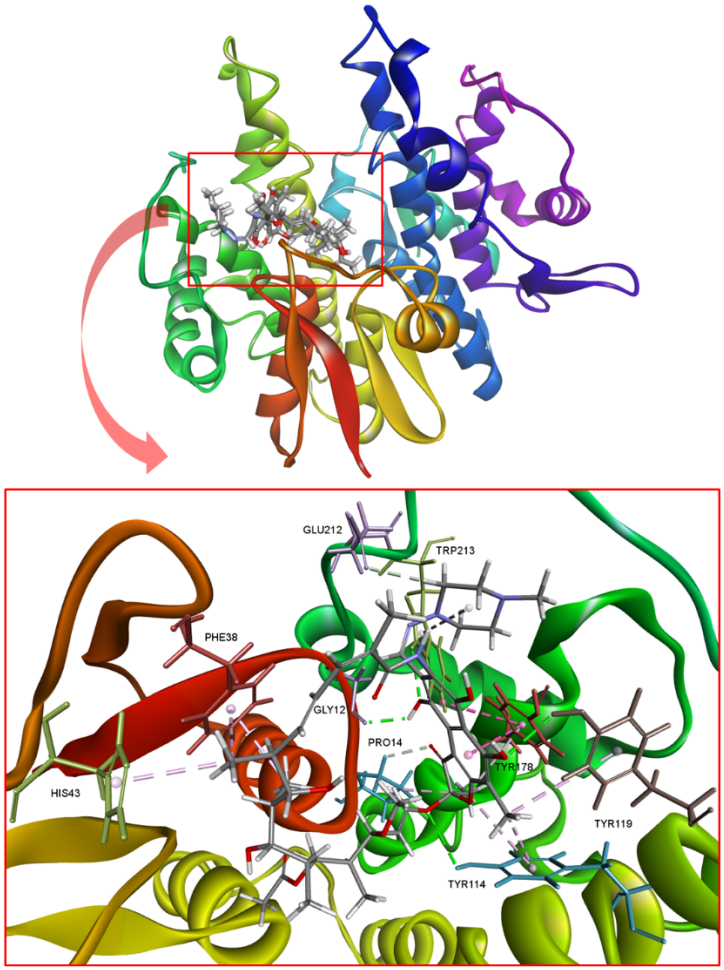

Figure 8. Final docking pose of the rifampicin-GSH-Gstr1 complex, and close-up view of the active site residues. Dotted lines represent non-covalent interactions.

4. Discussion

Zebrafish (*Danio rerio*) is a powerful model organism for the study of vertebrate biology. In recent years it has emerged as a popular model for use in pharmacological and toxicological studies. To understand the disposition of endo- and xenobiotic compounds in zebrafish, it is important to identify and characterize in detail critical elements of the absorption, distribution, metabolism and excretion (ADME) processes. GST superfamily, an integral element of ADME, still awaits to be fully elucidated in fish species. In our previous study (Glisic et al., 2015) we performed a comprehensive characterization of the GST superfamily in zebrafish, revealing a great diversity of fish GSTs (in total 27 members found in zebrafish), as well as clear orthology relations with human and other species Gsts. Furthermore, our tissue expression profiling, the initial functional characterization of nine

cytosolic Gst enzymes, and their functional similarity to the human orthologs in respect to the xenobiotic metabolism, pointed to involvement of Gst pi, Gstt1a, Gstz1, Gstr1, and Mgst3a and 3b in biotransformation of xenobiotics. Gstr1 is highly expressed in toxicologically relevant tissues and, together with members of Pi class, it functionally showed the most potent activities, with high affinities and turnover numbers in reactions with model substrates (Glisic et al., 2015). Based on those insights in this study we aimed at detailed characterization of a teleost-specific GST member, Gstr1.

Using the recombinant enzyme and the screening assay, we have been able to identify endo- and xenobiotic compounds that interact with zebrafish Gstr1. In the next step of our study, by using inhibition enzyme assays we have determined type and K_i values of observed interactions. Finally, by *in silico* analysis of zebrafish Gstr1 structure and binding sites we have confirmed the positions of identified G- and H-site forming amino acid residues (Fig. 6) and substrates susceptibility to nucleophilic addition of the GSH (Fig. 7). In addition, we elucidated the binding mode responsible for reversible noncompetitive inhibitor mode of action (Fig. 8).

There is a high degree of conservation of endocrine processes and roles of steroid hormones between zebrafish and terrestrial vertebrates (Tokarz et al., 2013). As a discrepancy, it was shown that 11-ketotestosterone instead of testosterone is the prominent circulating androgen in zebrafish (Waal et al., 2007). We showed that zebrafish Gstr1 activity is competitively inhibited by four steroid hormones: progesterone, pregnenolone, DHEAS and testosterone, with calculated K_i values in range of 14-26 μM (Tab. 1, Fig. 3, Supplementary Data Fig. S1). Molecular docking of testosterone, experimentally identified as Gstr1 substrate, confirms its susceptibility to nucleophilic addition of the GSH by Gstr1. These findings revealed steroid hormones as probable physiological substrates of zebrafish Gstr1. It was shown that mammalian GSTs have the ability to bind hormones, and particularly sexual steroids, and influence their transport, metabolism, and physiological action (Listowsky et al., 1988). A high binding capacity for steroids was previously displayed by certain isoenzymes of GST (Eliasson et al., 1999; Homma et al., 1986). Particularly, testosterone and progesterone have the ability to bind mammalian GSTs with moderate (10^{-6} M < K_d < 10^{-4} M) or high (K_d < 10^{-6} M) affinity, respectively (Listowsky et al., 1988). This is in line with our findings. Another study (Remoué et al., 2002) demonstrated a specific binding between testosterone and a parasite *Schistosoma haematobium* GST enzyme, with higher affinity of binding (K_d = 5.7 x 10^{-7} M). Functionally, cytosolic alpha class of GST enzymes has role in the GSH dependent obligatory double-bond isomerization of delta(5)-androstene-3,17-dione and delta(5)-pregnene-3,20-dione, precursors to testosterone and progesterone, respectively (Johansson and Mannervik, 2001; Tars et al., 2010). Demonstrated interactions of 4 steroid hormones and zebrafish Gstr1 enzyme suggest possible involvement of Gstr1 in steroidogenesis, metabolism and/or physiological action of these androgens. On the contrary, none of the tested estrogenic hormones showed activity toward Gstr1 (Fig. 1). As we previously showed, Gstr1 expression in ovary is 5-fold lower than in testes (Glisic et

al., 2015) which could be possibly explained by absence of interactions between Gstr1 enzyme and estrogenic hormones.

GSTs have glucocorticoid-binding properties and, thereby, may influence transport, metabolism, and action of steroids (Homma and Listowsky, 1985). While corticosterone is no hormone naturally occurring in teleosts (Balm et al., 1989), it is almost identical to cortisol lacking only the 17α-hydroxyl group. Furthermore, it was found to bind to and activate the teleostean glucocorticoid receptor (Mommsen et al., 1999). We showed that zebrafish Gstr1 activity is competitively inhibited by corticosterone, with calculated K_i value of 20.5 μM (Table 1, Fig. 3). This finding showed that corticosterone could be a physiological Gstr1 substrate. As previously showed, Gstr1 is highly expressed in zebrafish brain of both genders (Glisic et al., 2015). Interestingy, in brain of rats a corticosterone-induced decline of GST activity that may strongly promote neurodegeneration was reported (Zafir and Banu, 2009). Molecular docking of corticosterone, identified in this study as a substrate, confirms its susceptibility to nucleophilic addition of the GSH by Gstr1. We showed that corticosterone forms alkyl-type hydrophobic interaction with Val116, another H-site forming residue, but from the counterpart B monomer. In addition, H-site residues from the B monomer are in close proximity of corticosterone (Fig. 7). These findings emphasize the importance of using dimeric model structures of GSTs for more accurate binding studies. However, although cortisol is the major stress hormone in zebrafish that acts as both glucocorticoid and mineralocorticoid (Tokarz et al., 2013), it does not interact with Gstr1 (Fig. 1).

One of the two major primary bile salts in many ray-finned fish is chenodeoxycholyltaurine (taurochenodeoxycholic acid), while zebrafish is the only teleost model species that synthesizes mainly 5α (*trans* A/B ring) bile salts (Hagey et al., 2010). Herewith we show that taurochenodeoxycholate (TCDC) is a competitive inhibitor, i.e. probable physiological substrate of zebrafish Gstr1 (Table 1, Fig. 3) with calculated K_i value of 33 μM. Therefore, we suggest that Gstr1 activity could influence physiological actions of TCDC in zebrafish.

Among tested industrial chemicals (Fig. 2) only tributyltin chloride, an organometal used as an antifouling biocide, demonstrated a strong noncompetitive inhibition of Gstr1 with calculated K_i value of 0.26 μM (Table 1, Fig. 4). These findings, together with molecular docking results, revealed reversible inhibitor character of tributyltin chloride toward Gstr1 enzyme. Our results are similar to some previous observations for inhibitory effect of tributyltin on GST activity in fish. A very high potency of organotin compounds (tributyltin, triphenyltin) for inhibition of plaice cytosolic GST activity *in vitro* was shown by George and Buchanan (1990). Also, organotins including tributyltin are reported to inhibit GST activity in fish (Al-Ghais and Ali, 1999; Padrós et al., 2003; WU et al., 2007), and it was speculated that organotin-GSH complexes bind to the first and the second catalytic sites of GST. On the contrary, it was shown that long-term exposure to sub-lethal concentrations of tributyltin, in a range of K_i value we calculated, caused ROS stress in the liver of common carp and significantly induced GST activity (Li

et al., 2015). Also, Wang et al. (2006) showed that GST activity increased after exposure to lower dose of tributyltin in liver of *Sebastiscus marmoratus*, and opposite effect was noted with higher dose. Studies with rats confirmed that tributyltin induces oxidative stress by inhibiting GST which results in apoptosis (Ishihara et al., 2012; Mitra et al., 2013). GST activity inhibition leads to increase lipid peroxidation as its substrates inside the cells are organic hydroperoxides and 4-hydroxyalkenals. Generally, inhibition of the GST activity by tributyltin reduced the capacity of cells/organs to detoxify other chemicals and increase the vulnerability to oxidative stress.

Of all tested pesticides, group of organophosphate insecticides, chlorpyrifos-methyl (CPF-methyl), diazinon and malathion caused inhibition of Gstr1 activity in the range of 49-66% in comparison to control (Fig. 2). The absence of interactions of Gstr1 enzyme with other tested pesticides is in line with data published so far. Actually, Trute et al. (2007) showed that major hepatic coho salmon (*Oncorhynchus kisutch*) GST isoforms, belonging to Pi and a Rho-class, have no activity towards the pesticides, including atrazine. However, Booth and O'Halloran (2001) revealed that GST catalyses the conjugation of glutathione with xenobiotics, including organophosphorus pesticides. Our results revealed that diazinon competitively inhibits Gstr1 activity with calculated K_i value of 27 μM (Table 1, Fig. 4). Gstr1 activity changes caused by different concentration of CPF-methyl and malathion did not reveal clear and consistent inhibition pattern and it was not possible to calculate K_i values (Table 1, Supplementary Data Fig. S2). Because of that, the nature of interactions of these pesticides with Gstr1 remains unclear. However, their environmental loads (Murray et al., 2010) are not sufficient to reach effective concentrations required to modulate zebrafish Gstr1 activity (Fig. 2). Based on presented results, we propose that diazinon is possible Gstr1 substrate, which could qualify zebrafish Gstr1 as a diazinon-metabolizing enzyme. Diazinon-metabolizing GST enzyme was revealed for silkworm, suggesting that it may detoxify diazinon (Yamamoto and Yamada, 2016). Another study revealed that diazinon exposure to common carp (*Cyprinus carpio*) induces GST enzyme activities in liver, which were assumed to have resulted from the defense against the toxicity of diazinon (Oruc, 2011). Previous studies on effect of chlorpyrifos on GST activity showed statistically significant decrease in the GSTs activity in the zebrafish larvae, common carp and rats (Jin et al., 2015; Xing et al., 2012; Mansour and Mossa, 2009). Interestingly, in fish species *Labeo rohita* and *Carassius auratus gibelio*, malathion caused modulation of GST activity in time-dependent and tissue-specific manner (Thenmozhi et al., 2011; Huculeci et al., 2008). Similarly, increase and decrease of GST activity across different organs were observed after exposure of neonatal rats to malathion (Timur et al., 2003). Overall, according to our results and previously published data, Gstr1 probably belongs to group of enzymes capable of metabolizing organophosphate insecticides, thus protecting fish against those deleterious environmental contaminants.

Screening assay performed in this study revealed that from all tested pharmaceuticals and personal care products (PPCPs), only erythromycin, methotrexate, rifampicin and tetracycline interact with zebrafish Gstr1 (Fig. 2).

However, these pharmaceuticals interact with Gstr1 differently. E.g., macrocyclic antibiotic rifampicin is a noncompetitive reversible inhibitor of Gstr1 activity with calculated K_i value of 65 μM (Table 1, Fig. 4). Molecular docking confirmed that rifampicin is reversible noncompetitive inhibitor of zebrafish Gstr1 enzyme and elucidated the binding mode responsible for its inhibitory action. Binding studies suggest that rifampicin occupies G-site of Gstr1, thus blocking the approach of GSH. Actually, rifampicin interacts with neighboring residues via different types of non-covalent interactions (Fig. 8). Although several previous studies have shown induction of expression of GST transcripts by rifampicin in fish and human hepatocytes (Corcoran et al., 2012; Rae et al., 2001), and induction of GST activity in rats (Adachi et al., 1985), there are no reports describing rifampicin-dependent modulation of GSTs enzyme activity in fish. To the best of our knowledge, this is the first report describing interaction of rifampicin to GST enzyme. On the other hand, pharmaceuticals erythromycin, methotrexate and tetracycline competitively inhibit Gstr1 activity with calculated K_i values in a range of 17.5-36.5 μM (Table 1, Fig. 4, Supplementary Data Fig. S3). Competitive type of inhibition categorizes these drugs as possible substrates which are metabolized by zebrafish Gstr1 enzyme. There is evidence that erythromycin, a macrolide antibiotic, inhibits GST activity in liver of crucian carp (*Carassius auratus*), where the inhibition rate decreased with exposure time and concentration. Among all tested xenobiotics, tetracycline expressed the most potent competitive inhibition (K_i = 17.5 μM) of Gstr1 enzyme activity (Table 1, Fig. 4). This polyketide antibiotic inhibits human recombinant GSTP1-1 and GSTM3-3 enzymes with IC_{50} values of 13 μM and 47 μM, respectively (Mukanganyama et al., 2002).

5. Conclusion

Our study provides the first functional characterization of a teleost-specific GST Rho member protein in zebrafish, Gstr1. Combining experimental data obtained using the described *in vitro* inhibition assays, insights based on the obtained 3D structure of zebrafish Gstr1, and finally molecular docking studies, we have shown that pregnenolone, progesterone, testosterone, DHEAS and corticosterone are probable physiological substrates of zebrafish Gstr1. Based on the obtained data we hypothesize that Gstr1 probably has an important role in steroidogenesis, metabolism and/or physiological actions of androgens, but not estrogens in fish. In addition, reversible inhibitors of fish Gstr1 were identified among environmental contaminants, and our results imply the role of Gstr1 in metabolism of xenobiotics and protection of fish against deleterious environmental contaminants such as organophosphate insecticides and pharmaceuticals.

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Supplementary Data

Molecular characterization of zebrafish Gstr1, the only member of teleost-specific glutathione S-transferase class

Authors:

Branka Bašica[a], Ivan Mihaljević[b], Nikola Maraković[c], Radmila Kovačević[a], Tvrtko Smital[b,*]

Affiliations:

[a] University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovica 2, 21000 Novi Sad, Serbia

[b] Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenicka 54, 10000 Zagreb, Croatia

[c] Instiute for Medical Research and Occupational Medicine, Ksaverska cesta 2, 10000 Zagreb, Croatia

These authors contributed equally to this work

*Corresponding author

Tvrtko Smital, PhD

Laboratory for Molecular Ecotoxicology

Division for Marine and Environmental Research

Ruđer Bošković Institute

Bijenicka 54

10 000 Zagreb, CROATIA

Tel.: **385 1 45 61 039

E-mail: smital@irb.hr

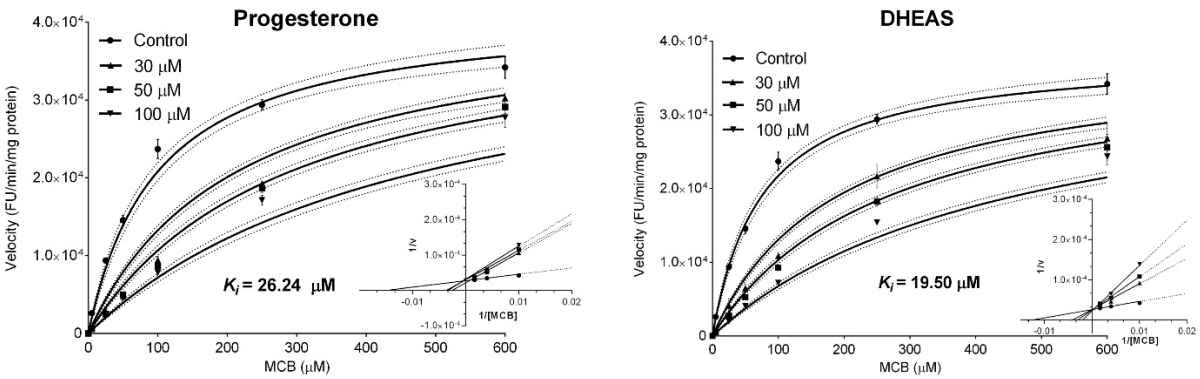

Figure S1. Inhibition of zebrafish Gstr1 activity by endogenous compounds (progesterone and DHEAS). Concentration dependence of Gstr1 mediated monochlorobimane (MCB) transformation is expressed in fluorescence units normalized to time and protein concentration (FU/min/mg proteins) over MCB concentration (μM) at 25 °C in presence of 1 mM GSH co-substrate. Inserted figures: Lineweaver-Burk plots that show the type of inhibition. Data were fitted in the GraphPad Prism 6. Mean, SEM and confidence interval (c.i.) were calculated from 4 replicates of two independent experiments.

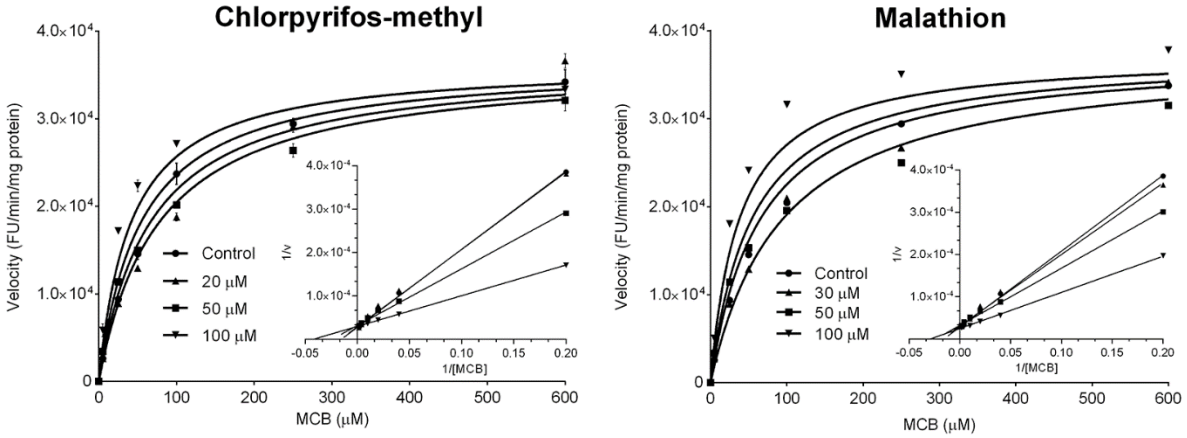

Fig. S2. Inhibition of zebrafish Gstr1 activity by organophosphate insecticides (chlorpyrifos-methyl and malathion). Concentration dependence of Gstr1 mediated monochlorobimane (MCB) transformation is expressed in fluorescence units normalized to time and protein concentration (FU/min/mg proteins) over MCB concentration (μM) at 25 °C in presence of 1 mM GSH co-substrate. Inserted figures: Lineweaver-Burk plots. Data were fitted in the GraphPad Prism 6. Mean, SEM and confidence interval (c.i.) were calculated from 4 replicates of two independent experiments.

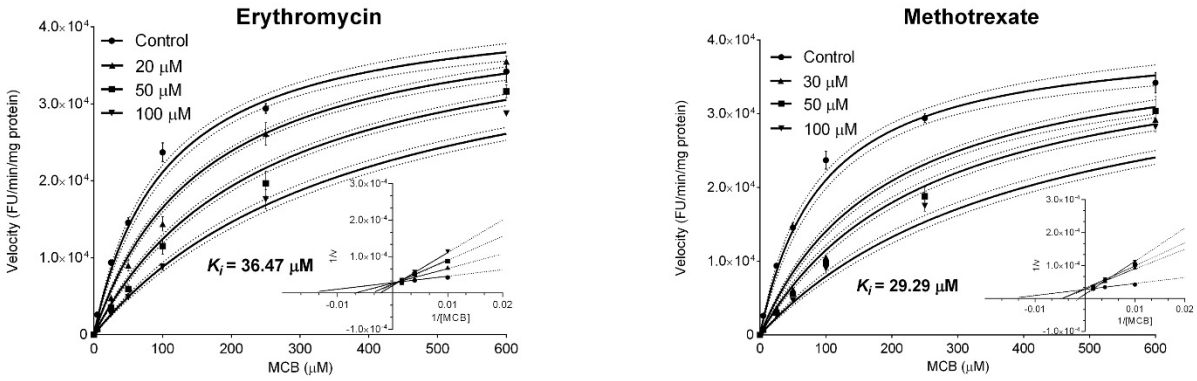

Fig. S3. Inhibition of zebrafish Gstr1 activity by pharmaceuticals (erythromycin and methotrexate). Concentration dependence of Gstr1 mediated monochlorobimane (MCB) transformation is expressed in fluorescence units normalized to time and protein concentration (FU/min/mg proteins) over MCB concentration (μM) at 25 °C in presence of 1 mM GSH co-substrate. Inserted figures: Lineweaver-Burk plots that show the type of inhibition. Data were fitted in the GraphPad Prism 6. Mean, SEM and confidence interval (c.i.) were calculated from 4 replicates of two independent experiments.