J Mol Model (2011) 17:2651–2657
DOI 10.1007/s00894-010-0952-9

ORIGINAL PAPER

Studies on filarial GST as a target for antifilarial drug development—in silico and in vitro inhibition of filarial GST by substituted 1,4-naphthoquinones

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Received: 8 September 2010 / Accepted: 29 December 2010 / Published online: 26 January 2011
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Abstract Eleven 1,4-naphthoquinone analogues with different amino substitutions at position 3 of the quinone ring earlier reported for macrofilaricidal activity were selected and screened against purified cytosolic GST isolated from the bovine filarial worm *Setaria digitata* and IC_{50} values were determined. Of the 11 compounds tested, 8 showed good inhibition against *S. digitata* GST. The IC_{50} values of the most effective macrofilaricidal compounds—**11** [2-(4-methylpiperazin-1-yl)naphthalene-1,4-dione] and **9** {2-[(1,3-dimethylbutyl)amino]naphthalene -1,4-dione}—were 0.872 and 0.994 mM, respectively. Compounds **9** and **11** were further studied for type of enzyme inhibition and found to exhibit competitive and uncompetitive inhibition kinetics, respectively, with respect to substrate GSH. All 11 compounds were in agreement with Lipinski's rule of five and passed through the FAFDrugs ADME/tox filter. Molecular docking was carried out using the modeled 3D structure of *wb*GST PDB ID:1SFM as receptor and substituted naphthoquinones as ligands using AutoDock 4.0. The binding energy of nine compounds varied from −9.15 to −6.58 Kcal mol^{-1}, whereas compounds **8** and **10** did not show any binding to the receptor. Among the compounds studied, compound **7** {2-[3-(diethylamino) propyl]aminonaphthalene-1,4-dione} showed maximum affinity towards *wb*GST as it exhibited the lowest binding energy, followed by compounds **11** and **9**. However compound **7** was not macrofilaricidal while **11** and **9** exhibited macrofilaricidal activity. The results of in silico and in vitro studies with the synthesized 1,4 - naphthoquinone analogues on filarial GST and in vitro macrofilaricidal activity against adult bovine filarial worm *S. digitata* open up a promising biochemical target for antifilarial drug development.

Keywords 1,4-naphthoquinone · Antiparasitic · Macrofilaricidal · Lymphatic filariasis · GST inhibition · AutoDock

Electronic supplementary material The online version of this article (doi:10.1007/s00894-010-0952-9) contains supplementary material, which is available to authorized users.

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Introduction

Lymphatic filariasis (LF)—a parasitic infection spread by mosquitoes—is caused by thread-like worms that damage the human lymphatic system. One of the world's most disabling and disfiguring diseases, LF afflicts the poorest of the community. The disease is estimated to infect over 120 million people, with more than 40 million disfigured with inflammation of the limbs and breasts (lymphoedema), and genitals (hydrocele), or with swollen limbs with dramatically thickened, hard, rough and fissured skin (elephantiasis) [1]. Currently available drugs, such as diethylcarbamazine and ivermectin, are active mainly against circulating microfilariae. There is at present no safe and consistent chemotherapeutic agent active against adult filarial worms. In spite of advances in vector control methods and chemotherapy, LF, particularly the infection caused by *Wuchereria bancrofti* (*wb*), continues to be a major cause of clinical morbidity in tropical countries. Thus, there is an urgent need for antifilarials against adult

worms. Presently, identification of novel therapeutic targets from numerous parasite enzymes, receptors, genome data and metabolic pathways [2], and development of new active chemical entities against these enzymes are the key goals of the drug discovery process [3, 4].

Glutathione S-transferase (GST, EC 2.5.1.18) is a major phase-II detoxification enzyme comprised of multifunctional proteins. Helminths have limited detoxification enzymes and appear to lack the important cytochrome P-450 dependent detoxification reaction [5]. GSH is proposed to constitute the antioxidant system responsible for the long-term existence of filarial worms in mammalian hosts by protecting them from the reactive oxygen species (ROS) produced by normal metabolism and by the immune cells of the host [6, 7]. GST has been detected in a range of helminths [8], where it may be one of the major detoxification enzymes, and plays a role in the survival of these parasites within the host environment. Adult filarial worms can survive up to 15 years in man. During their development in man, these parasites encounter an immune response directed against them. However, the parasites survive, probably due to a variety of mechanisms. It has been proposed that GST enzymes may contribute to these persistence mechanisms [9]. The ability of helminth GSTs to effectively neutralise cytotoxic products arising from ROS attack on cell membranes provides evidence that GSTs have the potential to protect the parasite against the host immune response. The inhibition of parasite GST(s) thus deprives the parasite of its major defense against oxidative stress, making them unable to survive [8, 10–12]. GST is considered as one of the biochemical targets for antifilarial drug development. Rational drug design requires the three dimensional (3D) crystalline structure of the target protein. In the absence of the crystal structure of *wb*GST, a computationally modeled 3D structure (PDB ID: 1SFM) was constructed earlier in our laboratory using bioinformatic tools [13].

The bovine filarial parasite *Setaria digitata* (family: Onchocercidae, subfamily: Setariinae), which resembles the human parasite in its nocturnal periodicity and antigenic pattern [14], was used as a model organism in place of the human filarial parasite (adult *W. bancrofti* worms are unavailable for drug screening and other experimental purposes) for drug development research. The easy availability of the adult worms makes them more convenient for preliminary screening of antifilarials. *Setaria* GST has been reported as a target for GST inhibition studies leading to the identification of antifilarials [15]. The *S. digitata*–Mastomys coucha model has been found to be amenable to chemotherapeutic and immunobiological investigations in experimental filariasis [16].

Information acquired from chemical structure and molecular modeling of target proteins, as well as bioinformatics and various theoretical approaches, can offer many valuable and timely approaches to drug discovery. We reported earlier the antifilarial potential of substituted 1,4-naphthoquinones [17] and the filarial GST inhibition of 5-hydroxy-2-methyl-1,4-naphthoquinones [15]. We also carried out molecular modeling of *wb*GST [13]. Stimulated by the results of these studies, here we studied the inhibition of *S. digitata* GST (*sd*GST) by substituted naphthoquinones and carried out a comparative study with the in silico results of *wb*GST inhibition as a 3D model of *sd*GST is not available for in silico studies.

Materials and methods

GST inhibition studies

Adult *S. digitata* (Nematoda: Filariodea) female worms were collected from the peritoneal cavity of freshly slaughtered naturally infected cattle and washed with phosphate buffered saline (PBS) pH 7.4 before using for extraction. Each time, a 10% crude homogenate was prepared by crushing worms (100 mg/ml) in ice-cold conditions in PBS (pH 7.4) containing 1% Triton X-100, 1 mM PMSF (phenylmethylsulfonylfluoride) and 1 mM EDTA. The homogenate was centrifuged (Beckman Coulter, Optima max-XP Ultracentrifuge, Rotor-MLA-150) at 10,000 *g* for 15 min at 4°C to remove the mitochondrial fraction. The supernatant was further centrifuged at 100,000 *g* for 60 min to remove the microsomal fraction, and the final supernatant containing the cytosolic fraction was subjected to GSH-agarose affinity column chromatography (GST purification kit, GeNei, India) (1×1 cm) . The affinity purified cytosolic fraction was used for GST inhibition studies. Eleven substituted 1,4-naphthoquinones (compounds **1**–**11**) that were reported earlier from this laboratory [17] for their macrofilaricidal activity against adult *S. digitata* (see Table 1) were selected for the study. GST assay was conducted as per Habig's method [18] with and without the presence of inhibitor compounds. The enzyme was incubated with varying concentrations of each compound for 10 min at room temperature in a reaction mix containing DPBS (Dulbecco's phosphate buffered saline) and 1 mM GSH (glutathione). The reaction was initiated by the addition of 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and absorbance was recorded at 340 nm using a Spectra MAX Plus (Molecular Devices, Sunnyvale, CA). The results were expressed in the form of IC_{50} values (concentration of inhibitor required to inhibit 50% of enzyme activity), and compounds showing more GST inhibition were further studied to determine the type of inhibition by incubating the GST enzyme with varying concentrations of inhibitor at different substrate concen-

Table 1 Effect of substituted napthoquinones on filarial GST

Compound No	Substitution R	IC_{50} (mM)
1	$-NHCH_2CH_3$	>10
2	$-NHCH_2CH_2CH_3$	3.039
3	$-NHCH (CH_3)_2$	2.845
4	$-NHCH_2CH_2CH_2CH_3$	3.994
5	$-NHCH (CH_3)CH_2CH_2$	2.631
6	$-NHCH_2CH_2CH_2N(CH_3)_2$	3.847
7	$-NHCH_2CH_2CH_2N(CH_2CH_3)_2$	1.5
8	$-NHCH_2CH_2CH_2N(CH_2CH_2CH_2CH_3)_2$	>10
9	$-NHCH(CH_3)CH_2CH(CH_3)_2$	0.994
10	$-NHCH_2CH_2$–(p-phenylene)–Cl	>10
11	-N(piperazine)N—CH_3	0.872

trations (0.25, 0.5, 1.0 and 1.5 mM GSH) for 10 min at room temperature.

ADME/tox filtering and docking studies

Absorption, distribution, metabolism, excretion and toxicity (ADME/tox) are key properties that need to be considered early on any drug development project. FAFDrugs is an online service that allows users to process their own compound collections via simple ADME/tox filtering rules such as molecular weight, polar surface area, logP, or number of rotatable bonds [19, 20]. Compounds **1**–**11** were transformed to SMILES coordinates to generate ADME properties. The parameters were either in compliance with Lipinski's rule of five or set as default.

Molecular docking was carried out using the modeled 3D structure of *wb*GST PDB ID:1SFM [13] as receptor and substituted naphthoquinones as ligands using AutoDock 4.0 [21]. To calculate the binding energy using AutoDock, polar hydrogens were added to the receptor 1SFM coordinates using the AutoDockTools interface [22]. The 3D affinity grid fields were created using the auxiliary program AutoGrid. The amino acid residue TYR 116, a central residue in the active site, was chosen as the grid center. At this stage, the protein was embedded in the 3D grid and a probe atom was placed at each grid point. The affinity and electrostatic potential grid was calculated for each type of atom in the ligand molecule. Grid maps were prepared using the AutoGrid utility with 66.7×15.8×20 points and grid spacing set to 0.375 Å. The same grid was used for all the compounds **1**–**11**. Docking parameters modified were: number of individuals in the population (set to 150), maximum number of energy evaluations (set to 250,000), maximum number of generations (set to 27,000).

Docking calculations were carried out using Autodock. Three binding energy terms were taken into account in the docking step: the van der Waals interaction, represented as a Lennard-Jones 12–6 dispersion/repulsion term; hydrogen bonding, represented as a directional 12–10 term; and the Coulombic electrostatic potential. At the end of a docking job with multiple runs, AutoDock performed cluster analysis. Docking solutions with ligand all-atom root mean square deviations (RMSDs) within 2.0 Å of each other were clustered together and ranked by the lowest energy representative. The lowest-energy solution of the lowest ligand all-atom RMSD cluster was accepted as the calculated binding energy.

Results and discussion

The results of GST inhibition studies are given in Table 1. It was observed that out of the 11 substituted naphthoquinones screened for affinity purified *sd*GST inhibition, 8 could inhibit *sd*GST in different concentration ranges in a dose-dependent manner. The IC_{50} values of the most effective compounds, **11** and **9**, were 0.872 and 0.994 mM,

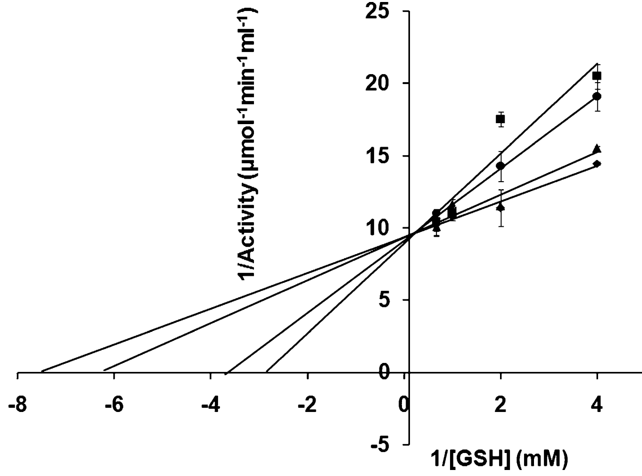

Fig. 1 Double reciprocal plots of the velocities of *sd*GST in the presence of compound **9**. Competitive inhibition was observed by incubating the *sd*GST enzyme with varying concentrations of compound **9** at different substrate concentrations (0.25–1.5 mM GSH) for 10 min at room temperature. Experiments were performed in duplicate. ♦ No inhibitor, ■ 0.25 mM inhibitor, ▲ 0.5 mM inhibitor, ● 0.75 mM inhibitor

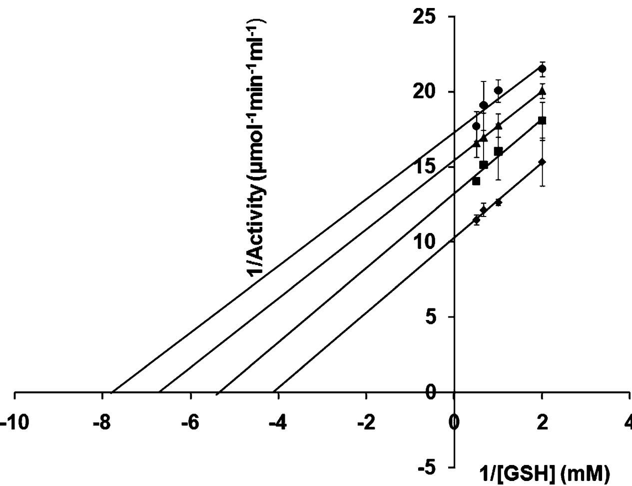

Fig. 2 Double reciprocal plots of the velocities of *sd*GST in the presence of compound **11**. Un-competitive inhibition was observed by incubating the *sd*GST enzyme with varying concentrations of compound **11** at different substrate concentrations (0.25–1.5 mM GSH) for 10 min at room temperature. Experiments were performed in duplicate. ♦ No inhibitor, ■ 0.25 mM inhibitor, ▲ 0.5 mM inhibitor, ● 0.75 mM inhibitor

respectively. The remaining compounds (**7**, **5**, **3**, **2**, **6** and **4**) showed a decreasing trend in GST inhibition with IC_{50} values of 1.5, 2.63, 2.85, 3.04, 3.85 and 3.99 mM, respectively, whereas compounds **1**, **8** and **10** did not show any inhibition towards *sd*GST up to 10 mM. The most effective compounds (**9** and **11**) were studied for type of inhibition and were found to exhibit competitive (Fig. 1) and non-competitive (Fig. 2) inhibition kinetics, respectively, with respect to the substrate GSH.

The results generated for the ADME/tox properties of compounds **1**–**11** using FAFDrugs ADME/tox filtering are listed in Table 2. All compounds were in agreement with Lipinski's rule of five and passed through the filter.

The results of docking of the target protein *wb*GST (PDB ID: ISFM) with 11 substituted 1,4- naphthaquinones are given in Fig. 3. The binding energy of the nine compounds varied from −9.15 to −6.58. Among the compounds studied, compound **7** showed maximum affinity towards *wb*GST as it exhibited the lowest binding energy (−9.15 Kcal mol^{-1}) followed by compound **11** (−8.68 Kcal mol^{-1}) and **9** (−8.31Kcal mol^{-1}). Compounds **8** and **10** showed least affinity towards *wb*GST. The remaining compounds **5, 3**, **2**, **4**, **1** and **6** bound to the active site and showed the following decreasing trend with increase in binding energies: −8.08, −8.02, −7.97, −7.74, −7.65 and −6.58 Kcal mol^{-1} respectively. Figures 4 and 5 illustrate the binding of compound **9** and **11**, respectively, to the modeled *Wb*GST. The hydroxyl group of Tyr-7 is within hydrogen bond distance of the amino group of **9**, as evidenced by the hydrogen bond interaction seen in Fig. 4.

Table 2 Absorption, distribution, metabolism, excretion and toxicity (ADME/tox) properties of compounds **1**–**11**

Compound	MW[a]	Drs[b]	Ars[c]	FB[d]	RB[e]	C[f]	nC[g]	Chrg[h]	LogP[i]	PSA[j]
1	201.1	1	3	2	13	12	3	0	2.26	46.17
2	215.1	1	3	3	13	13	3	0	2.62	46.17
3	215.1	1	3	2	13	13	3	0	2.72	46.17
4	229.2	1	3	4	13	14	3	0	3.19	46.17
5	229.2	1	3	3	13	14	3	0	3.08	46.17
6	258.2	1	4	5	13	15	4	0	2.05	49.41
7	286.2	1	4	7	13	17	4	0	2.9	49.41
8	342.2	1	4	11	13	21	4	0	4.75	49.41
9	257.2	1	3	4	13	16	3	0	4.16	46.17
10	311.7	1	3	4	19	18	4	0	4.33	46.17
11	256.2	0	4	1	19	15	4	0	1.62	40.62

[a] Molecular weight
[b] Hydrogen donors
[c] Hydrogen acceptors
[d] Flexible bonds
[e] Rigid Bonds
[f] Carbons
[g] Non carbons
[h] Number of charges
[i] LogP (octanol / water)
[j] Polar surface area

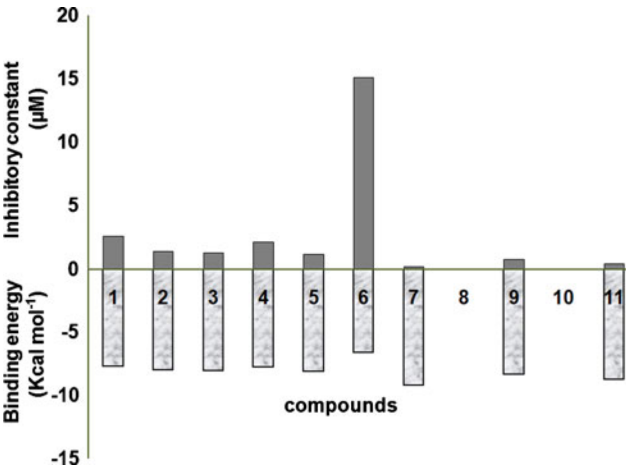

Fig. 3 Binding energies and inhibitory constants resulted from docking 1SFM with compounds **1**–**11** as ligands. The binding energy of the nine compounds varied from −9.15 to −6.58 whereas compounds **8** and **10** showed no binding to the receptor. Compound **7** showed maximum affinity towards *wb*GST followed by compounds **11** and **9**

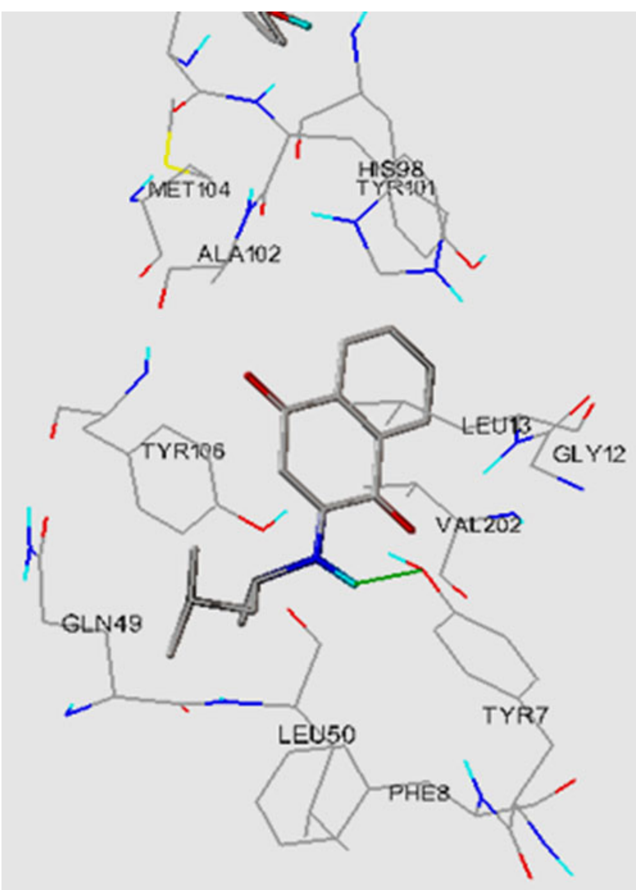

Fig. 4 Illustration of the interaction between compound **9** and the modeled 3D structure of *wb*GST. The H-bonding interaction of Tyr-7 compound **9** is depicted

Tyr-7 is in the GSH binding site of *Wb*GST. The residues seen near the binding site of **9** are Tyr-7, Leu-13, Gly-12 and Gln-49. Compound **11** binds GST at a different site (Fig. 5) compared to **9**, with neighboring residues Arg-95, Gly-64, Phe-152, His-98 and Tyr-101.

Correlation of the results of GST inhibition studies with in silico docking revealed that most of the compounds exhibited low binding energy, and inhibitory constants in docking were effective in filarial GST inhibition. The IC_{50} values for compounds **1**–**11** were in the order of **11**<**9**<**7**<**5**<**3**<**2**<**6**<**4**<**1**≤**8**≤**10**. The binding energies were in the order of **7**<**11**<**9**<**5**<**3**<**2**<**4**<**1**<**6**<**8**≤**10**. It can be seen that compounds **8** and **10** did not bind to the active site while docking. In filarial GST inhibition studies up to 10 mM, these two compounds showed no inhibitory properties. With the exception of compounds **1**, **6** and **7**, all the compounds showed almost similar trends in in silico binding energies and GST inhibitory properties.

We have previously reported the screening of compounds **1**–**11** for macrofilaricidal activity against adult female *S. digitata* worms by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay and worm motility assay. Of the 11 compounds investigated for antifilarial activity, 7 had promising macrofilaricidal activity against the adult *S. digitata*, with ED_{50} values ranging from 0.91 to 298 μM [17]. No macrofilaricidal activity was observed for compounds **6**, **7**, **8**, and **10**. Compounds **9**, **11**, **5**, **3**, **2**, **4** and **1** showed macrofilaricidal activity with ED_{50} (dose that gives a 50% response) values of 0.91, 1.2, 3.1, 3.6, 36, 41.6 and 298 μM, respectively, at 48-h incubation. It was observed that most of the compounds showing good GST inhibition were effective in killing adult filarial worms, except for compounds **6** and **7**, for which some other factors may be responsible for the reduced macrofilaricidal activity. The ED_{50} values for the two most promising macrofilaricidal compounds, **9** and **11**, were 0.91 and 1.2 μM, respectively. These compounds exhibited good GST inhibition, with IC_{50} values 0.994 and 0.872 mM, respectively. The results were in agreement with the results of molecular docking studies with modeled filarial GST as the target and substituted 1,4-naphthoquinones as ligands.

An analysis of the inhibition of *S. digitata* GST at various concentrations of GSH showed that compound **9** exhibited the characteristics of a linear competitive inhibitor (i.e., plots made with varying amounts of a competitive inhibitor will cross at the same *y*-intercept and V_{max} is unaffected). The competitive type of inhibition indicates that the inhibitor binds to the active site of *S. digitata* GST, and its linear dependence on inhibitor concentration implies that only one molecule of inhibitor is involved per active site [23]. A competitive inhibitor raises the K_m , indicating that the affinity of the enzyme for the substrate is lower in the presence of the inhibitor. The effect of a competitive

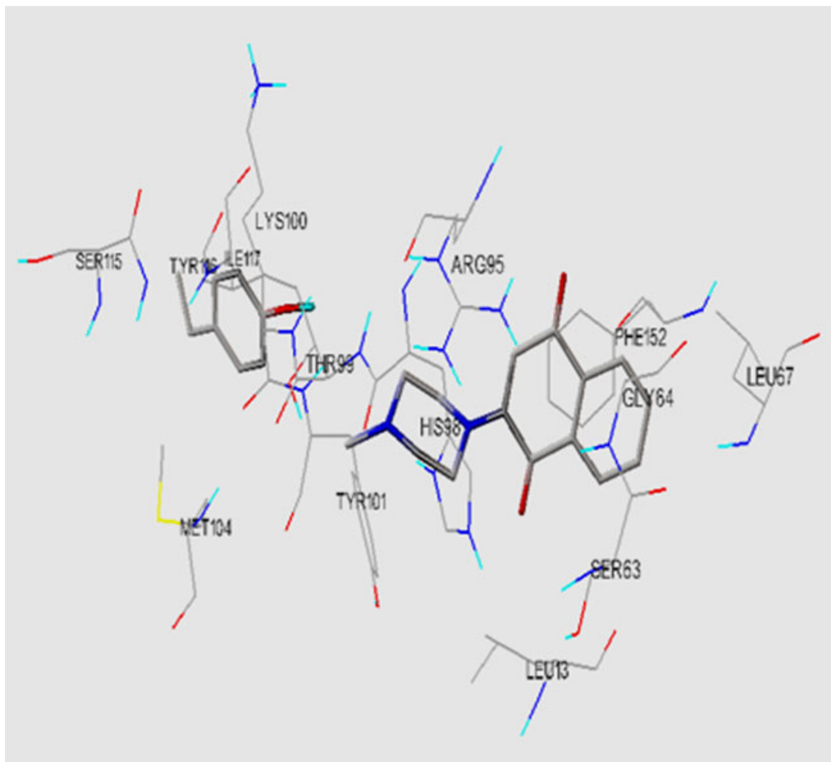

Fig. 5 Illustration of the interaction between compound **11** and the modeled 3D structure of *wb*GST

inhibitor in a Lineweaver-Burk plot is to move the *x*-intercept and increase the slope. Inhibition studies of *S. digitata* GST by compound **11** clearly revealed un-competitive inhibition towards GSH (un-competitive inhibition leads to different intercepts on both the *y*- and *x*-axes but the same slope), which indicates that compound **11** binds preferentially to a preformed GST–GSH complex [24].

Comparison of the results of docking, GST inhibition and in vitro macrofilaricidal activity showed that, with a few exceptions, the majority of compounds exhibiting low binding energy inhibitory constants in docking were effective in filarial GST inhibition and effective against adult filarial worms. Among the 11 compounds studied, compounds **9** and **11** showed better target affinity in docking, *Sd*GST inhibition and in vitro macrofilaricidal activity. These two compounds bind to two different binding sites, which accounts for the observed competitive (filarial GST binds **9** at the active site through Tyr-7, which is in the GSH substrate binding site) and un-competitive (the binding site of **11** is further away from the GSH binding site) types of inhibition exhibited by these compounds.

Filarial nematodes have adopted a number of strategies to evade, modify or neutralize the host's defense mechanisms to survive in a hostile environment. Increasing evidence shows that a parasite's enzymatic pathways play an important role in effectively implementing these strategies [25, 26]. The attractiveness of enzymes as drug targets results not only from their essential catalytic activity but also from the fact that enzymes, by their very nature, are highly amenable to inhibition by low-molecular-weight drug-like molecules. Because of their susceptibility to inhibition by small drug molecules, enzymes are common targets of new drug discovery.

The various functional properties of GSTs make these enzymes key targets for distinct therapeutic areas. The diverse functions, including catalytic GSH conjugation, passive ligandin-type binding and modulation of signal transduction, may be targeted selectively by different inhibitors [27]. GST activity in relation to these strategies has been identified in filarial nematodes and other helminths [10].

Poor pharmacokinetics and toxicity information have been important causes of costly late-stage failures in drug development. There is an increasing need to predict ADME/tox properties to serve two key aims: (1) to reduce the risk of late-stage attrition, and (2) to optimize screening and testing by looking at only the most promising compounds [28]. The properties that make drugs different from other chemicals are spelled out by Lipinski's 'rule-of-five' [29], which identifies several critical properties that should be considered for compounds with oral delivery in mind. These properties include reduced molecular flexibility, as measured by the number of rotatable bonds, and low polar surface area or total hydrogen bond count (sum of donors and acceptors) are found to be important predictors of good oral bioavailability, independent of molecular weight. Oral bioavailability measurements have allowed us to analyze the relative importance of molecular properties considered to influence that drug

property. The fact that, on average, both the number of rotatable bonds and the polar surface area or hydrogen bond count tend to increase with molecular weight may explain, in part, the success of the molecular weight parameter in predicting oral bioavailability. Earlier observations suggest that compounds that meet only the two criteria of (1) ten or fewer rotatable bonds, and (2) polar surface area equal to or less than 140 A° (or 12 or fewer H-bond donors and acceptors) will have a high probability of good oral bioavailability in the rat [30]. Reduced polar surface area correlates better with increased permeation rate than lipophilicity, and increased rotatable bond count has a negative effect on the permeation rate. A threshold permeation rate is a prerequisite of oral bioavailability. Lipophilicity is the key physicochemical parameter linking membrane permeability, and hence drug absorption and distribution, with route of clearance [28]. The extent to which drug molecules cross from the blood into the brain is governed by two physiologically and anatomically related systems: the blood brain barrier and the blood-cerebral spinal fluid barrier, which form two pathways by which drug compounds partition between plasma and brain tissue [31]. All 11 compounds mentioned here were filtered by the FAFDrugs ADME/tox filter.

The GST system of parasites represents the main detoxification mechanism of hydrophobic and electrophilic compounds. Parasites lack cytochrome P-450 activity, and part of the function of this enzyme system has been taken over by other enzymes including GSTs. Cytosolic GSTs are found in this system and constitute a versatile and numerous group that, in parasites, displays many peculiarities compared to mammalian cytosolic GSTs [23]. The information available on crystal structures and activity properties of parasite cytosolic GSTs are suitable for the rational design of specific inhibitors for these enzymes. For example, specific inhibitors for *Pf*GST and *Sj*26GST have already been developed and established as good targets for antischistosomal [32] and antimalarial [33] drug development. When a structure is not available, an in silico generated model may help to test several inhibitors against the enzyme, as proven with parasite cytosolic GSTs from *W. bancrofti* [13].

In conclusion, the results of in silico and in vitro studies of the interaction of 11 synthesized 1,4 -naphthoquinone analogues with filarial GST and in vitro screening of these compounds for macrofilaricidal activity against adult bovine filarial worm *S. digitata* reveal that filarial GST is a promising biochemical target for antifilarial drug development.

Acknowledgments N.M. expresses sincere gratitude to the Department of Science and Technology (DST), Government of India for funding [Grant SR/SO/HS-83/2005] to conduct this study. The authors are grateful to Dr. P. Jambulingam, Director, Vector Control Research Center, Pondicherry, for his encouragement during the study. The technical assistance of Mrs. Anila Kumari and Mr. Srinivasan is also acknowledged.

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