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Modulation of *Anopheles gambiae* Epsilon glutathione transferase activity by plant natural products *in vitro*

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Abstract

Elevated glutathione transferase (GST) E2 activity is associated with DDT resistance in the mosquito *Anopheles gambiae*. The search for chemomodulators that inhibit the function of *Ag*GSTE2 would enhance the insecticidal activity of DDT. Therefore, we examined the interaction of novel natural plant products with heterologously expressed *An. gambiae* GSTE 2 *in vitro*. Five of the ten compounds, epiphyllocoumarin (Tral-1), knipholone anthrone, isofuranonaphthoquinones (Mr 13/2, Mr13/4) and the polyprenylated benzophenone (GG1) were shown to be potent inhibitors of AgGSTE2 with IC_{50} values of 1.5 μM, 3.5 μM, 4 μM, 4.3 μM and 4.8 μM respectively. Non-competitive inhibition was obtained for Tral 1 and GG1 with regards to GSH (K_i of 0.24 μM and 0.14 μM respectively). Competitive inhibition for Tral1 was obtained with CDNB (K_i = 0.4 μM) whilst GG1 produced mixed type of inhibition. The K_i and K_i' for GSH for Tral-1 and GG1 were 0.2 μM and 0.1 μM respectively. These results suggest that the novel natural plant products, particularly Tral-1, represent potent AgGSTE2 *in vitro* inhibitors.

Keywords: Anopheles gambiae, *glutathione* S-*transferases*, *malaria*, *natural plant products*, *chemomodulation*, *DDT-resistance*

Abbreviations: *GST, glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl β-D-thiogalactoside;EDTA; ethylenediammine tetraacetic acid, DDT,1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane; DDE,1,1-dichloro-2,2-bis-(p-chlorophenyl)ethane), DTT; dithiothreitol, AgGSTE2; Anopehels gambiae GSR epsilon 2, NaN_3; Sodium azide, BSA; bovine serum albumin, PMSF; phenyl- methylsulfonylfluoride, SDS-PAGE; sodium dodecyl sulphate polyacrylamide gel electrophoresis, Jb42c; 14-hydroxy, 8; 15-diacetoxy-1(10)4,11(13)germacratrien-12,6-olide, 2QG1; prinoidin, Mr13/4 and Mr11/2; isofuranonaphthoquinones, Tral-1 epiphyllocoumarin*

Introduction

Malaria affects the populations of tropical and subtropical areas of the world [1]. Each year, an estimated 0.7–2.7 million people die of malaria and 75% of them are African children [2]. Of the four species of *Plasmodium* that cause malaria in humans, *P. falciparum* is the most dangerous as the pathology it induces often leads to death [1]. Mosquitoes of the *Anopheles gambiae* complex are the most important malarial vectors in sub-Saharan Africa [3]. *Anopheles gambiae* is often the predominant vector during the wet season, when malaria is at its peak, whilst its sibling species *A. arabiensis* is better adapted to less humid environments, and is often the predominant or only member of the complex in the arid regions of West

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Africa, Ethiopia and Sudan. The problems of *Plasmodium* species resistance to antimalarial drugs and the spread of insecticide-resistance in *Anopheles* species have prompted the search for alternative, sustainable methodologies to control malaria [4].

Glutathione *S*-transferases (GSTs E.C. 2.5.1.18) are ubiquitous and versatile proteins capable of a range of enzymatic and non-enzymatic functions [5]. They help to protect from oxidative stress and chemical toxicants by aiding the excretion of electrophilic and lipophilic compounds from the cell [6]. Multiple forms of GST have been isolated in eukaryotic cells and they have differing catalytic activities to accommodate the wide range of functions of this enzyme family. In insects, glutathione transferases are an important enzyme system involved in the metabolism of a broad range of foreign and endogenous compounds [7, 8]. The GSTs play an essential role in herbivory through the detoxification of deterrent and toxic plant allelochemicals, and insecticides [9, 10]. Work on insect GSTs was initially motivated by their possible involvement in insecticide resistance. Elevated GST activity has been detected in strains of insects that are resistant to organophosphates and organochlorines [4,9]. Insect GSTs have been classified into six classes: δ, ε, σ, θ, ω, and ξ [11]. The δ and ε-class GSTs have been associated with detoxification of various insecticides [12]. *An gambiae* GSTs are of particular interest because of their involvement in resistance to DDT [1,1,1-trichloro-2,2-bis-*p*(-chlorophenyl)ethane]. DDT was widely used in the 1960s and 1970s to spray in houses as a defence against malaria, but since this period DDT-resistant strains of mosquitoes have arisen [11]. This has led to decreased effectiveness of this control measure. However, DDT is still being used today for malarial control in many parts of the world including Zimbabwe [3, 13]. In *A. gambiae* an increased rate of DDT dehydrochlorination (Figure 1) in the resistant strain is associated with an increase in GST expression [12]. It has now been shown that AgGSTE2 has very high DDT dehydrochlorinase activity by expressing this isoform in *Escherichia coli* [2].

The appearance of mosquitoes resistant to the currently used insecticides and the emergence of multidrug resistant strains of parasites continue to frustrate efforts to eradicate the mosquitoes in the bid to control malaria [14]. The chemical defences of plants against insect predation make plants an attractive option to be considered as viable sources of insecticidal agents for the control of disease-transmitting insects and as sources of chemotherapeutic agents. Based on this premise, several research programmes have been initiated to search for potential medicinal and/or pest control agents from plants [15].

Chemomodulation involves the administration of a compound that is targeted to arrest the function of a protein that is involved in drug resistance [16]. Very little is known about inhibition of insect glutathione transferases. Only a few compounds have been identified to inhibit insect GSTs, including plant phenols (quercetin, ellagic acid, juglone, menadione, plumbagin), ethacrynic acid, organotin compounds, and hydroxyamic acids [7, 17]. Whilst some phytochemicals have been found to inhibit GSTs from rat liver, it is not known whether these phytochemicals inhibit insect GSTs since species differences in isozyme composition may affect their sensitivity towards an inhibitor. In this case the search for chemomodulators that would inhibit the functioning of *Ag*GSTE2 would enhance the insecticidal activity of DDT. The aim of this paper, therefore, is to study the effects of natural plant products on AgGSTE2 so that once identified to be inhibitory they can be employed as DDT-potentiating additives to enhance the effectiveness of this malaria control measure. The natural plant products (Figure 2) were selected on the basis of novelty and availability and also on containing diverse classes of secondary metabolites, including phenolic polyketides, terpenoids, and coumarin derivatives, many of which contain α,β-unsaturated carbonyl compounds which are known to be GST inhibitors.

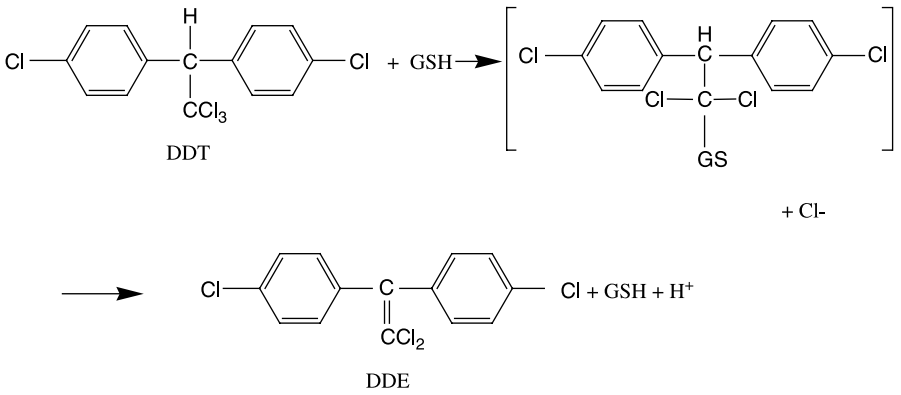

Figure 1. Dehydrochlorination of DDT to its non-insecticidal metabolite DDE by AgGSTE2. The two phenyl rings enter into the two pockets of the H-site during the dehydrochlorination reaction ([11]).

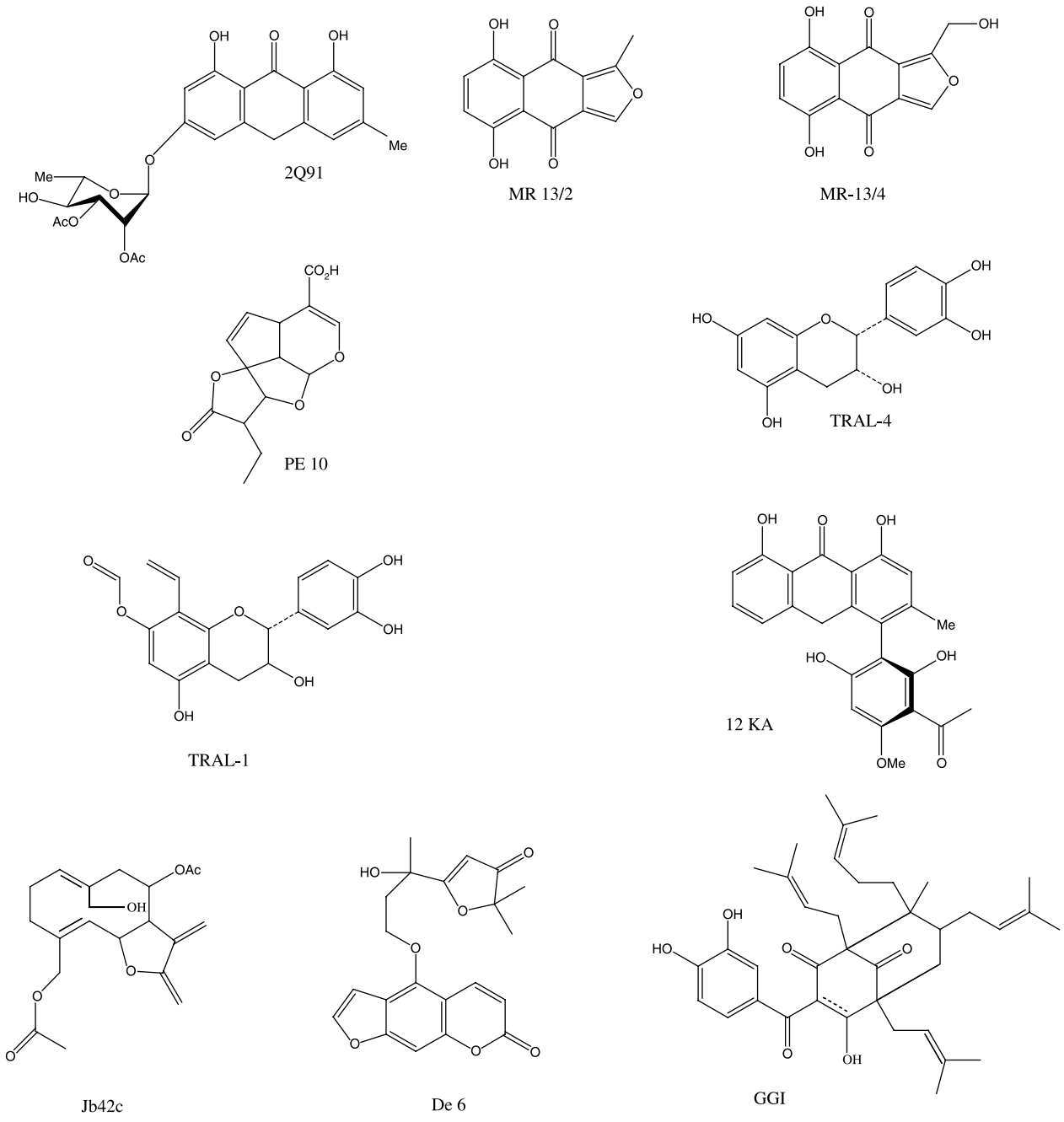

Figure 2. Chemical structures of the novel plant compounds used in this study.

Materials and methods

Chemicals

All reagents used were of analytical grade. The following chemicals and biochemicals were obtained from Sigma-Aldrich Chemical Companies, (St. Louis MO, USA): 1-chloro-2, 4-dinitrobenzene (CDNB), glutathione (GSH), hexylglutathione, ethacrynic acid, EDTA, DDT, NaN_3, BSA, tris-(hydroxymethyl) amino methane (Trizma base), yeast extract, glycerol, tryptone, ampicillin, isopropyl thiogalactoside (IPTG), chicken egg white lysozyme, molecular weight markers.. The isofuranonaphthoquinones (Mr13/4 and Mr11/2) were extracted from *Bulbine* species [18], 14-hydroxy, 8, 15-diacetoxy-1(10)4, 11(13)germacratrien-12,6-olide (Jb42c) was extracted from *Dicoma anomala*, knipholone anthrone, (12KA) was extracted from *Knipholfia foliosa*, prinoidin (2QG1) and geshoidin were extracted from *Rhamnus prinoides* [19], PE10 was extracted from *Plumera rubra* [20], DE6 was from *Dorstenia elliptica* [21], GGI, Tral-1 and Tral-4 were extracted from *Garcinia* species. The natural products GG1, JB42c, Tral1 and Tral-4 were extracted from the above mentioned plants using the following general protocol. The sun-dried plant material (ca 1 kg) was soaked in a mixture of dichloromethane-methanol

(1:1) and pure methanol for 24 h and 2 h, respectively at room temp. Concentration of the combined organic extract gave a residue (ca 50–65 g). Part of this residue was chromatographed on a silica gel column eluting with hexane-ethyl acetate mixtures, to give fractions of 250 mL each. The fractions were concentrated and monitored by TLC and ^{1}H NMR and similar fractions were combined. The first fractions examined by TLC (hexane-ethyl acetate; 9:1) contained mainly mixtures of hydrocarbons and phytosterols, which were not investigated further. More polar fractions were passed through Sephadex LH-20 column ($CHCl_3$/methanol, 2:1). The post chlorophyll fractions were subjected to repeated silica gel CC and PTLC to yield the various metabolites. Pure metabolites molecular structures were established by spectroscopic techniques such as NMR, MS, and IR.

Escherichia coli

Expression and purification of recombinant AgGSTE2. The strain Origami (DE3) pLysS *Escherichia coli* cells transfected with a pET3a plasmid containing *AgGSTE2* was prepared as described in Ranson et al. [2] and used to inoculate a 100 mL starter culture of 2TYA medium (54 g tryptone, 40.5 g yeast extract, 13.5 g NaCl and 27 g glycerol in 2700 mL water) containing 13.5 μL ampicillin (1 M stock). The culture was incubated in a shaking incubator (Labcon, Labotec, South Africa) operating at 170 rpm and 37°C overnight for 20 h. Three 2000 mL conical flasks containing 500 ml 2tya medium and 67.5 μL ampicillin (1 M stock) were inoculated with 5 mL of the culture and incubated in the shaking incubator at the same settings. When an A_{600} of 0.2–0.3 was obtained, isopropylthiogalactoside (IPTG) was then added to a final concentration of 0.2 mM in each flask to induce expression of the *AgGSTE2* gene and the cells were grown overnight. The bacteria were sedimented by centrifugation using a Sigma 3K15centrifuge (Germany) at 3 500 rpm for 10 min at 15°C using an A6.9 rotor. The cells were resuspended in an equal volume of lysis buffer consisting 10 mM Tris-HCl pH 7.8, 50 mM EDTA, 15% (w/v) anhydrous glucose, 0.2% (w/v) sodium azide, 0.2 mM dithiothreitol (DTT) and 1 mg/mL chicken egg white lysozyme. The suspensions were incubated on ice for 1 h and then the cells were disrupted by sonication 3 times (Dawex, Soniprobe, England) at setting 5 for 30 s, on ice. Phenyl- methylsulfonylfluoride (PMSF) was added to a final concentration of 170 μM to inhibit proteases and cellular debris was removed by centrifugation at 105 000 × g for 1 h 15 min at 4°C, using a Beckman Optima TM-80k centrifuge to obtain the cell supernatant fraction.

Glutathione sepharose 4B (Pharmacia, Uppsala, Sweden) affinity gel was used for affinity chromatography purification of AgGSTE2. The gel was stored in buffer A (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.2 mM DTT and 0.02% (w/v) NaN_3. The cell supernatant was combined with the gel and the mixture was kept on ice with gentle periodic swirling for 1 h to allow the GST to bind. The gel was packed into a column and the non-bound protein fraction was collected. The column was then washed with buffer A fortified with 0.2 M NaCl. The AgGSTE2 was eluted with 10 mM glutathione in buffer A. The fractions collected from the column were tested for GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The fractions that exhibited activity were pooled (affinity pool fraction) and concentrated using a PLGC membrane (Millipore, NMLW 10 000) under vacuum on ice to give a concentrated affinity pool. The concentrated protein was dialysed against 9 L of buffer A using 3 × 3 L buffer changes. The various fractions collected during purification i.e., the cell supernatant, non bound, affinity pool concentrated pool and dialysate fractions, were tested for GST activity [22] and protein content using the Lowry procedure [23].

The molecular weight of the purified *Ag*GSTE2 was determined by SDS-PAGE on 15% slab gels with the help of molecular weight markers (14 200 to 66 000 Da, Sigma, St. Louis, MO, USA) using a BioRad Protean system (BioRad Laboratories, California, USA). The protein bands were stained using Coomassie G stain (0.025% Coomassie G250, 40% methanol, 7% acetic acid) overnight, destained for 1 h in 50% methanol, 10% acetic acid and then further destained in 5% methanol, 7% acetic acid solution.

Screening for GST Inhibition using natural plant products

A range of natural products were screened for inhibition of AgGSTE2. The incubation mixture contained recombinant GST, 1 mM CDNB, 1 mM GSH, natural product dissolved in appropriate solvent and 0.1 M sodium phosphate buffer pH 6.5, 1 mM EDTA in 96-well plates. The blanks did not contain enzyme. The incubation temperature was 30°C. GST activity was monitored by measuring absorbance at 340 nm with time, using a SpectraMax 340 microplate spectrophotometer equipped with a kinetics mode (Molecular Devices, Sunnyvale, California, USA). The final concentration of organic solvent in the inhibition assays was 2.5%. Initially the natural plant compounds were tested at arbitrary concentrations of 33 μM and 100 μM. These concentrations were chosen so as to cover potent as well as moderate inhibitors. Stock solutions of the natural compounds were prepared in DMSO. Once a compound was found to be a potent inhibitor, various concentration ranges of natural product were tested to generate inhibition curves from which IC_{50} values could be determined, the IC_{50} value being the concentration required for 50% inhibition of enzyme activity. The IC_{50} value was determined by plotting sigmoidal

dose response curves of enzyme activity *vs*. log natural product concentration using GraphPad Prism version 3.00 for Windows, (GraphPad™ Software Inc., San Diego, California, USA).

Determination of kinetic properties

Epiphyllocoumarin (Tral-1) and the polyprenylated benzophenone (GG1) were used as inhibitors for the determination of kinetic properties of AgGSTE2 using the SpectraMax 340 microplate spectrophotometer equipped with the kinetics mode (Molecular Devices, California, USA). Since GSTs have two substrate binding sites, kinetic parameters were determined for each site. In one case, the concentration of CDNB was varied from 0.05–1.5 mM at a fixed concentration of GSH of 5 mM and in the second case, the concentration of GSH was varied from 0.05-5 mM at a fixed concentration of CDNB of 1.5 mM. Michaelis-Menten plots were used to determine the kinetic parameters K_m and V_{max} using GraphPad Prism 4 software.

The effects of Tral-1 on the kinetics of GSTE2 were determined as described by [24]. The $K_{m(app)}$ and $V_{max(app)}$ were determined using GraphPad Prism™ version 4.00 for Windows, (GraphPad™ Software Inc., San Diego, California, USA). The K_i values with respect to GSH and CDNB, as well as the type of inhibition were determined. The type of inhibition was deduced by determination of trends of K_m and V_{max} values with increase in natural product concentration. To determine the trend, the means of the K_m (or V_{max}) values with increase in inhibitor concentration were compared by performing a one-way ANOVA with Dunnett's post test using GraphPad InStat™ version 3.00 for Windows 95, (GraphPad™ Software, Inc.). The inhibition constant, K_i, was determined by means of re-plots [24]. The type of re-plot depends on the type of inhibition, for example, plotting $1/V_{max}$ versus inhibitor concentration for non-competitive inhibition will give K_i as the intercept on the baseline [25].

Results

Purification of AgGSTE2

A. gambiae recombinant *Ag*GSTE2 was over expressed and isolated from *E. coli* cells and purified by affinity chromatography. Purified *Ag*GSTE2 had a specific activity of 1.86 units compared to literature value of 2.879 units [2]. Using SDS-PAGE, the molecular weight of *Ag*GSTE2 was found to be about 24 kDa (data not shown).

Effect of some natural products on AgGSTE2

The effect of various natural products on the CDNB conjugating activity of AgGSTE2 was determined (Figure 3). A typical plot for the determination of theIC_{50} value is shown in Figure 4. The results are summarized in Table I. The most potent inhibitors were found to be epiphyllocumarin (Tral-1), knipholone anthrone, the isofuranonapthoquinones (Mr11/2, Mr13/4) and the polyprenylated benzophenone (GG1) with IC_{50} values of 1.5 μM, 3.5 μM, 4 μM, 4.3 μM and 4.8 μM respectively. JB42c, DE6, Tral-4, prinoidin and geshoidin were also inhibiting to a lesser extent. Further studies were carried out to determine the type of inhibition with respect to both GSH and CDNB on the compounds found to be potent inhibitors.

Steady state kinetics

The effects of epiphyllocumarin (Tral-1), and the polyprenylated benzophenone (GG1) on the kinetics of the AgGSTE2 were determined. The trend in changes of $K_m^{GSH/CDNB}$ and $V_{max}^{GSH/CDNB}$ values with increase in the natural product concentration was used to determine the type of inhibition, (Figure 5 and Table II). The type of inhibition with respect to the G site (GSH) was non-competitive and for the H site (CDNB) was competitive and mixed with respect to Tral-1 and GG1, respectively. Figure 6 shows the secondary plots for determination of K_i values. A summary of the kinetic properties in the absence and presence of Tral-1 are shown in Table III.

Discussion

With the resurgence of malaria as a killer disease in Africa, countries have resorted to the use of DDT. However, the advent of DDT resistant strains of mosquitoes has decreased the effectiveness of this control measure [11]. A DDT resistant strain of *A. gambiae* has been found to have elevated levels of multiple epsilon class GSTs. The isoform AgGSTE2 encodes an enzyme that has the highest levels of DDT dehydrochlorinase and hence has been implicated in resistance to the organochlorine insecticide [9]. In order to restore the effectiveness of DDT, it may

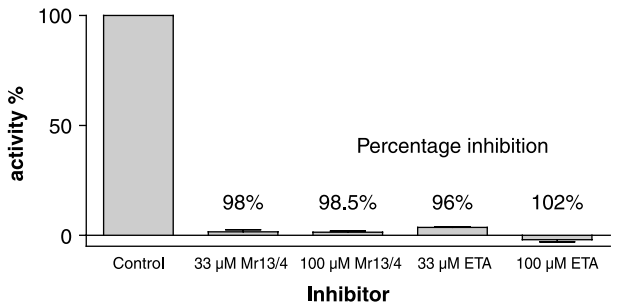

Figure 3. The effect of the natural plant product Mr13/4 on the activity of AgGSTE2. Two concentrations of 30 and 100 μM of the inhibitors were used. Ethacrynic acid (ETA) is shown here as a standard GST inhibitor.

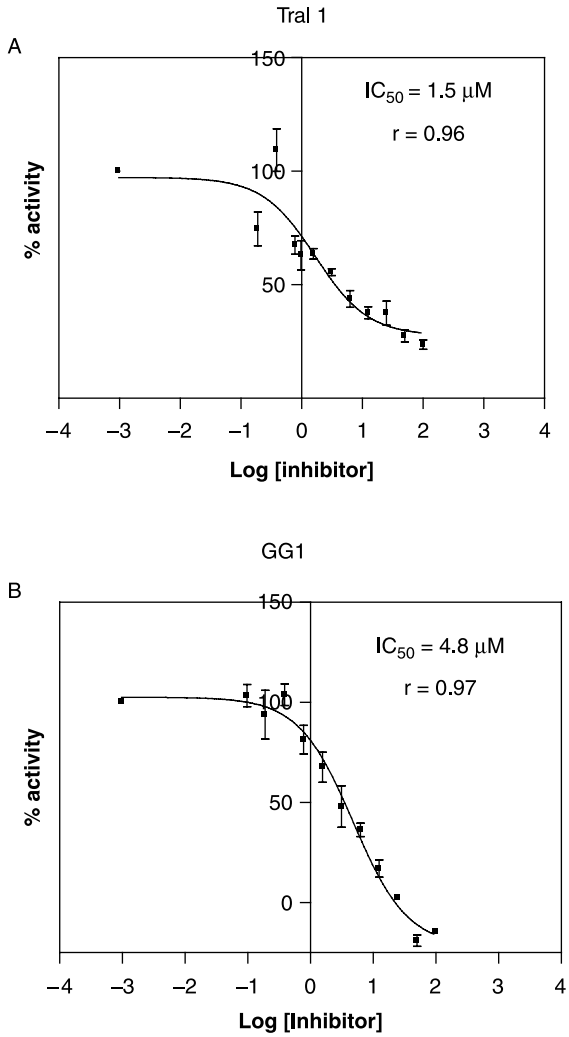

Figure 4. Inhibition of AgGSTE2 by Tral-1 and GG1 with CDNB as substrate. The IC_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity. Data are the mean ± standard deviation of quadruplicate experiments each performed twice.

Table I. Effect of some natural products on AgGSTE2 activity.

Inhibitor	33 μM % inhibition	100 μM % inhibition	IC_{50} (μM)
Mr11/2	75	85	4
Mr 13/4	95	95	4.3
Tral-1	90	95	1.5
GG1	95	90	4.8
KA	90	95	3.5
Jb42c	55	30	ND[a]
De6	25	45	ND
Tral-4	65	85	ND
Prinoidin	65	70	ND
Geshoidin	70	85	ND
Ethacrynic acid[b]	ND	ND	1.4

The extent of inhibition is shown at the two concentrations of 33 μM and 100 μM. Values are mean values for quadruplicate determinations. Data are the means of quadruplicate determination as shown in Figure 3. The IC_{50} values were calculated from the sigmoidal- dose response curve as shown in Figure 4.
[a]Not determined; [b]Ethacrynic acid is used as a standard GST inhibitor.

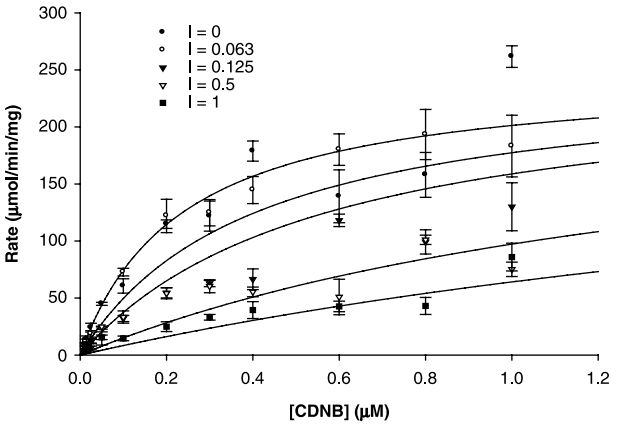

Figure 5. The effect of increasing the concentration of Tral-1 on the rate of reaction catalysed by AgGSTE2 with respect to the CDNB-binding site.

be possible to co-administer DDT and a chemomodulator to inhibit the action of AgGSTE2.

GSTs are able to interact covalently and non-covalently with various compounds that are not substrates for enzymatic activity [7]. GST activity has been shown to be modulated by natural plant products [26]. Flavonoids have been shown to inhibit GSTs in human blood platelets as well as in cancer cell lines [27]. Our study has revealed that a range of natural products inhibited heterologously expressed *A. gambiae* GST *in vitro*. The most potent inhibitors of AgGSTE2 were epiphyllocumarin, knipholone anthrone, the isofuranonapthoquinones (Mr11/2, Mr13/4) and the polyprenylated benzophenone (GG1) with IC_{50} values of 1.5 μM, 3.5 μM, 4 μM, 4.3 μM and 4.8 μM respectively. The five compounds that exhibited a significant inhibitory effect on the activity of AgGSTE2 had their IC_{50} values determined and these are shown in Table I. Of these five compounds that were successful on the initial screening, epiphyllocoumarin (Tra-1) proved to be the most potent inhibitor of AgGSTE2 as shown by its very low IC_{50} value relative to the IC_{50} values of the other four compounds. The α,β-unsaturated carbonyl configuration which is present in the structure of Tral-1resembles that which is found in the structure of ethacrynic acid, a well known inhibitor of GSTs [27]. This could explain why Tral-1 was a potent inhibitor of AgGSTE2.

The effect of each of the five potential inhibitors on the kinetic parameters of the enzyme was investigated. Generally each of these compounds reduced the K_{cat}/K_m of the enzyme for both CDNB and GSH at concentrations greater than 0.063 μM. K_{cat}/K_m, describes the catalytic efficiency of the enzyme. Just as expected, the natural plant products that exhibited inhibitory effects on the enzyme lowered this value with increase in concentration. Tral-1 resulted in lowest values of the catalytic efficiency of the enzyme

Table II. The effects of Tral-1 on kinetic properties of AgGSTE2 with CDNB as electrophilic substrate.

[GG1] (μM)	K_m CDNB (μM)	K_m GSH (μM)	V_{max} CDNB (μmol/min/mg)	V_{max} GSH (μmol/min/mg)	K_{cat} CDNB	K_{cat} GSH	K_{cat}/K_m CDNB	K_{cat}/K_m GSH
0	0.26 ± 0.08	2.19 ± 0.48	236 ± 25	475 ± 39	0.098	0.20	0.38	0.09
0.063	0.32 ± 0.09	0.19 ± 0.08	217 ± 22	137 ± 0.19	0.09	0.06	0.28	0.3
0.125	0.63 ± 0.25	0.07 ± 0.07	175 ± 31	32.07 ± 5	0.08	0.01	0.12	0.19
0.5	0.05 ± 0.08	−0.64 ± 0.07	13.36 ± 4.06	−0.99 ± 1.9	0.005	−0.0004	0.12	0.0006
1	0.18 ±0.4	−0.33 ± 0.05	17.03 ± 10	3.28 ± 2.1	0.007	0.0014	0.04	−0.004

for both GSH and CDNB, compared to the other four compounds, Knipholne anthrone, Mr11/2, Mr13/4and GG1. This is because of the low IC_{50} value of Tral-1. The kinetic data is in concurrence with the results from IC_{50} determination, that Tral-1 is the most potent inhibitor of AgGSTE2 of the ten compounds being studied. Tral-1 was found to be a non-competitive inhibitor with respect to the GSH binding site. In this type of inhibition V_{max} is decreased but the Michaelis-Menten constant, K_m is not changed. The binding of inhibitor and substrate do not overlap i.e. the inhibitor binds to a site other than the substrate binding site. This form of inhibition cannot be overcome by large amounts of the substrate. Tral-1 was shown to be a competitive inhibitor with respect to the CDNB-binding site. This increases the likelihood for competition with electrophilic substrates of AgGSTE2 like DDT, which binds to the CDNB-binding site. Interaction of Tral-1 with the CDNB-binding site of AgGSTE2 suggests that this natural compound may be a substrate for the enzyme, also supported by the α,β-unsaturated carbonyl group on the compound.

Since there is elevated expression of GSTs in many cases of insecticide resistance [10], it is possible that compounds that inhibit the functioning of GSTs might actually restore the utility of these insecticides. In order to restore the effectiveness of DDT, it may be possible to co-administer DDT and a chemomodulator to inhibit the action of AgGSTE2. Thus, studies of insect GSTs from insecticide-resistant strains will help in the understanding the mechanism of resistance to important pesticides and guide the design of novel inhibitors to overcome insecticide resistance. The results obtained in the present study showed that Tral-1 is an effective inhibitor of AgGSTE2 *in vitro* suggesting that inhibition of AgGSTE2 *in vivo* may be an effective role for this compound as chemomodulator against DDT resistance *A. gambiae* mosquitoes.

Control of malaria has relied on plant-drugs for centuries. Most have been used in traditional medicines and some have subsequently found their way into conventional pharmacies. Insecticides alternatives are generally more expensive which limits their use in poor resource nations and in situations where antimalarial efforts are already under funded [29]. The most cost-effective way of fighting malaria is to spray the insides of houses with DDT. This either kills the mosquito, or drives them away. The recommended alternatives, pyrethroids, are 4 times as expensive as DDT and also less effective. DDT sprayed on house walls (2 g/m^2) has been reported to exert a powerful control over indoor transmission of malaria [30]. The mosquitoes that transmit malaria best *A. funestus* and *A. gambiae* dominate in Africa. By 1984, a world survey showed that 233 species, mostly insects were resistant to DDT [31]. This study has identified Tral-1 and GG1 as natural plant compounds that inhibit the AgGSTE2 *in vitro* and might possibly restore sensitivity to DDT in DDT resistant species. It may be recommended that Tral 1 be a primary candidate for initial *in vivo* trials. Compared to other plant products investigated Tral-1 is attractive as a chemomodulator as it had an IC_{50} value close to that of ethacrynic acid, a standard mammalian GST inhibitor. Also as a chemomodulator as Tral 1 would be expected to be biodegradable being a natural plant product itself and, therefore, co-spraying it with DDT would augment the action of DDT either as an insecticide or as a repellent. Studies would still need to be undertaken to investigate the possible repellent activities of Tral-1 itself as well as its potential insecticidal activities. Technically it should be possible

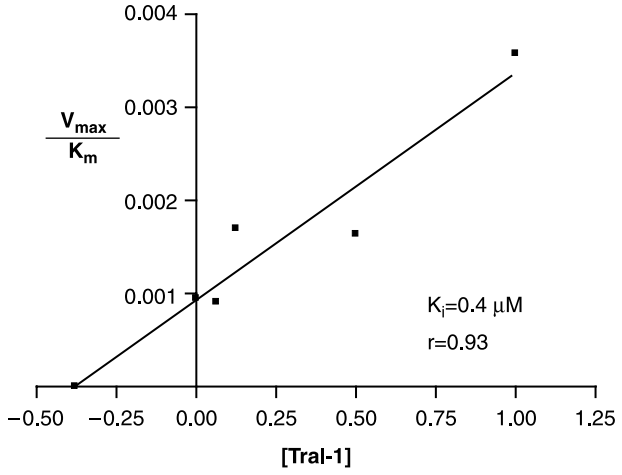

Figure 6. Determination of K_i for GSH and CDNB for cDNA expressed *A. gambiae* GST in the presence of Tral-1. The V_{max} were obtained from a series of experiments in which the AgGSTE2 was exposed to increasing concentrations of Tral-1. The rate of formation of the CDNB conjugate was determined on an ELISA reader equipped with a kinetics function (Spectra Max340, Molecular Devices, California, USA).

Table III. Mode of inhibition and inhibition parameters for Tral-1 and GG1.

Compound	G-site	H-site
Tral-1	Non-competitive (K_i = 0.24 μM)	Competitive (K_i = 0.4 μM)
GG1	Non-competitive (K_i = 0.14 μM)	Mixed (K_i = 0.19 μM, K_i' = 0.1 μM)

to co-spray DDT with Tral-1 but other studies would have to be carried out first to determine whether the effects of Tral-1 are time-dependent; the concentration of Tral -1 that is actually achieved inside the mosquito when the mixture of DDT and Tral 1 are co-sprayed on the walls; the feasibility of mass production of this compound from the plant sources; the environmental impact with regards to sustainability of extracting this compound from the *Garcinia* species. Because there is a danger of mosquitoes developing resistance to the currently used synthetic pesticides such as DDT, natural products that work by restoring the sensitivity to the current insecticides may help in the prevention and or/ eradication of malaria. Since many of the medicinal plants are grown under forest cover and are shade tolerant, agroforestry would offer a convenient strategy for promoting the cultivation and conservation of medicinal plants.

Further work needs to be done to evaluate the use of natural compounds as reversal agents in resistance to insecticide during malarial vector control. There is need to determine whether these compounds might interact with other macromolecules in the insects. The ubiquitous expression of GSTs and particularly GST Epsilon in different mosquito species [32] suggests that the addition of reversing agents such as natural products could enhance the efficacy of a variety of insecticidal agents.

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