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Proposed Reductive Metabolism of Artemisinin by Glutathione Transferases *in vitro*

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Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge. It is a promising new antimalarial and is particularly useful against the drug resistant strains of *Plasmodium falciparum*. It has unique antimalarial properties since it acts through the generation of free radicals that alkylate parasite proteins. Since the antimalarial action of the drug is antagonised by glutathione and ascorbate and has unusual pharmacokinetic properties in humans, we have investigated if the drug is broken down by a typical reductive reaction in the presence of glutathione transferases. Cytosolic glutathione transferases (GSTs) detoxify electrophilic xenobiotics by catalysing the formation of glutathione (GSH) conjugates and exhibit glutathione peroxidase activity towards hydroperoxides. Artemisinin was incubated with glutathione, NADPH and glutathione reductase and GSTs in a coupled assay system analogous to the standard assay scheme with cumene hydroperoxide as a substrate of GSTs. Artemisinin was shown to stimulate NADPH oxidation in cytosols from rat liver, kidney, intestines and in affinity purified preparations of GSTs from rat liver. Using human recombinant GSTs heterologously expressed in *Escherichia coli*, artemisinin was similarly shown to stimulate NADPH oxidation with the highest activity observed with GST M1-1. Using recombinant GSTs the activity of GSTs with artemisinin was at least two fold higher than the reaction with CDNB. Considering these results, it is possible that GSTs may contribute to the metabolism of artemisinin in the presence of NADPH and GSSG-reductase We propose a model, based on the known reactions of GSTs and sesquiterpenes, in which (1) artemisinin reacts with GSH resulting in oxidised glutathione; (2) the oxidised glutathione is then converted to reduced glutathione via glutathione reductase; and (3) the latter reaction may then result in the depletion of NADPH via GSSG-reductase. The ability of artemisinin to react with GSH in the presence of GST may be responsible for the NADPH utilisation observed *in vitro* and suggests that cytosolic GSTs are likely to be contributing to metabolism of artemisinin and related drugs *in vivo*.

Keywords: Glutathione transferases, metabolism, artemisinin, reduction, NADPH

INTRODUCTION

Artemisinin is a sesquiterpene lactone endoperoxide antimalarial that was isolated from

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Artemisia annua L. (sweet wormwood, annual wormwood) by Chinese scientists.[1] It acts through the generation of free radicals that alkylate parasite proteins.[2] Since its isolation, artemisinin and its derivatives such as dihydroartemisinin (Figure 1), have been widely used for the therapy of malaria in China, Vietnam, Burma and some parts of Africa.[3,4] Despite the proven efficacy of artemisinin against the asexual stage of the parasite life cycle in erythrocytes, its precise mode of action remains uncertain.[5,6] The endoperoxide moiety has also been reported to be responsible for toxic effects in the host.[7–10]

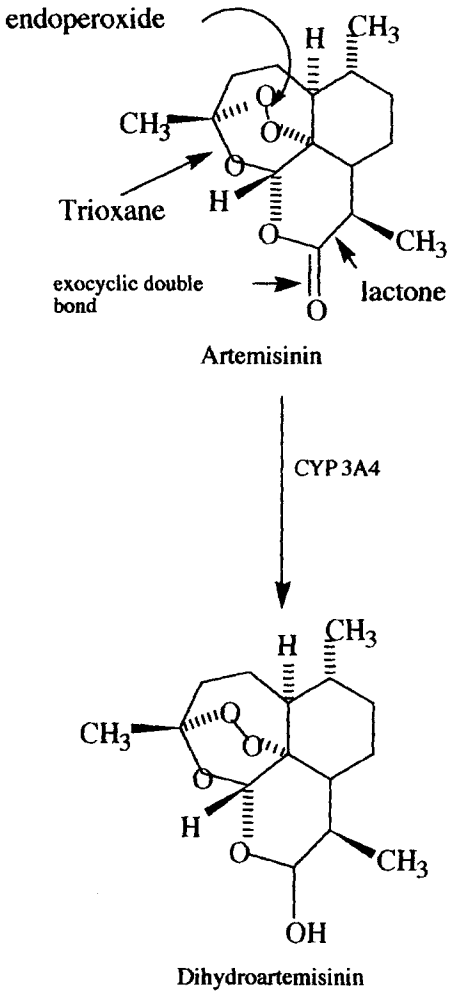

FIGURE 1 Chemical structures of artemisinin and its derivative dihydroartemisinin (DQHS).

Artemisinin and its derivatives do not bind DNA but they alkylate proteins.[11,12] In addition, there is iron-mediated activation of the endoperoxide that may then result in the generation of free radicals.[8]

Artemisinin and β-arteether, a related derivative, are metabolised in the liver by cytochrome P-450 isoforms to a biologically active metabolite, dihydroartemisinin.[13–15] Dihydroartemisinin is eliminated through the kidneys or can be metabolised further to other metabolites. Chemically it has been observed that other sesquiterpene lactones react with the sulphydryl group of cysteine or glutathione via nucleophilic attack leading to sulphydryl adducts through the lactone ring.[16,17] In addition artemisinin hydroperoxide is a metabolite which can result from the nucleophilic attack of artemisinin at the lactone ring.[18] This reduction reaction from the hydroperoxide has in fact been proposed *in vivo* for the metabolism of other sesquiterpene lactones.[17] This reduction reaction is analogous to the glutathione transferase reaction with cumene hydroperoxide.[19]

Glutathione transferases (GSTs) (EC 2.5.1.18) are a group of multi-functional proteins found in most aerobic organisms.[20] The cytosolic isoforms are grouped into the Alpha (A), Mu (M), Pi (P), Theta (T) and Zeta (Z) classes according to structural and catalytic properties.[21,22] The enzymes are widely expressed in mammalian tissue cytosols and they catalyse the conjugation of reduced glutathione (GSH) to a wide variety of electrophilic agents.[23] The substrates include carcinogens as well as various compounds that are products of oxidative stress including DNA and lipid hydroperoxides.[24]

By generation of free radicals, artemisinin is a compound that is capable of exerting oxidative stress in the cell. On the other hand, glutathione transferases are proteins that are capable of protecting the cell from compounds that can cause oxidative damage to cellular macromolecules.[24] In this context we have investigated the possibility of an interaction between artemisinin and GSTs *in vitro*. We propose that

artemisinin might undergo reductive metabolism mediated by GSTs according to the scheme shown in Figure 2. In support of this proposal, we report here a study of the putative glutathione peroxidase activity of GSTs with artemisinin in a coupled assay system based on the oxidation of NADPH by glutathione reductase and analogous to the standard GST assay scheme with cumene hydroperoxide as a substrate.

MATERIALS AND METHODS

Reagents

Artemisinin, cumene hydroperoxide (CuOOH), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase (Type III, Bakers Yeast), and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were all

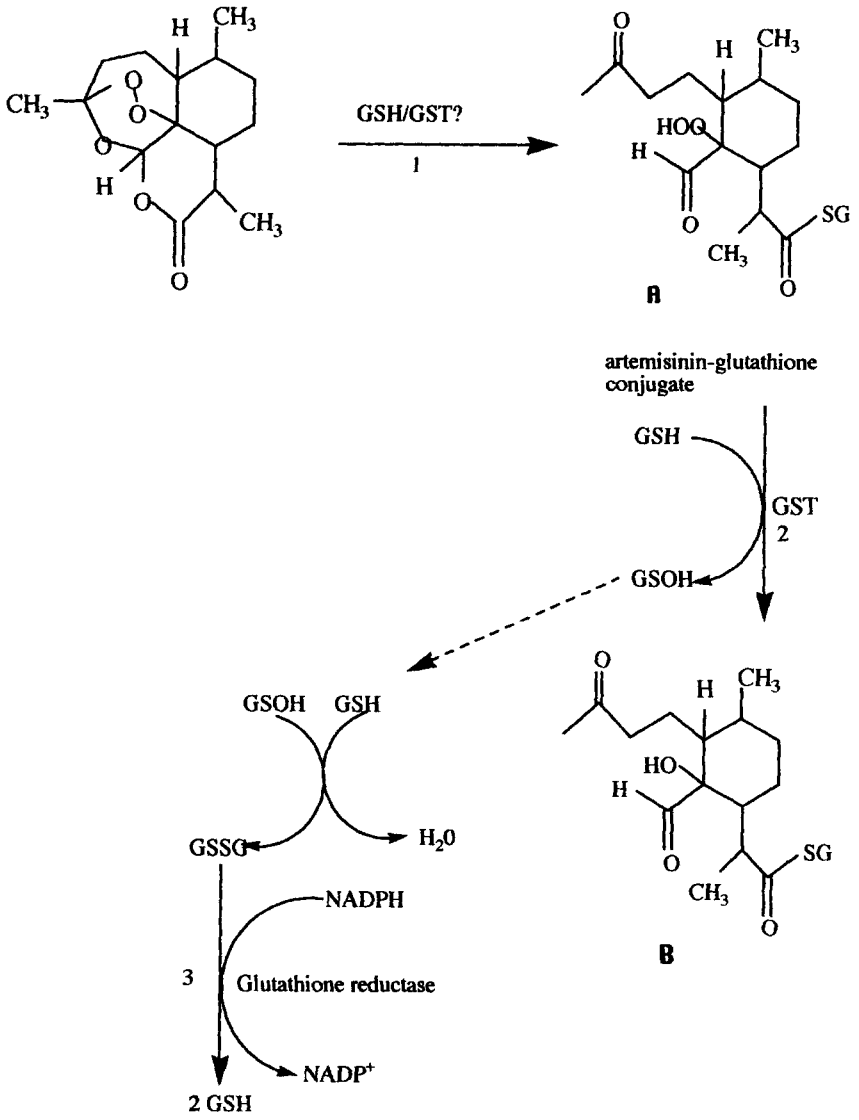

FIGURE 2 Proposed scheme for the reaction of artemisinin and glutathione through a GST catalysed reaction. Reaction 1 is proposed to be enzyme-catalysed or non-enzymatic. Reaction 2 is catalysed by GSTs resulting in the formation of GSSG. GSSG is reduced to GSH through a glutathione reductase pathway as indicated in Reaction 3.

analytical reagent grade obtained from various sources.

Preparation of Cytosolic Fractions from Rat Tissue

Eight-week-old male Sprague-Dawley rats (255 ± 17 g) were obtained from an in-house laboratory animal colony (Animal House, University of Zimbabwe). The animals received commercial food pellets (Mouse Combroids™, National Foods (PVT), Ltd., Harare, Zimbabwe) and water *ad libitum*. The animals were sacrificed by cervical dislocation. Perfusion with ice-cold saline was performed *in situ* to remove erythrocytes which are a high source of intracellular iron known to activate artemisinin to produce free radicals.[13] The livers, kidneys and intestines were excised immediately and were rinsed in ice-cold saline. Tissues were homogenised in three times volumes of phosphate buffered saline pH 7.4. Homogenates were centrifuged for 15 minutes at $10,000 \times g$ at 4 °C and the resultant supernatants were centrifuged for 1 hour at $100,000 \times g$ at 4 °C. The supernatant was taken as the cytosolic fraction. Protein was measured by the method of Lowry.[25]

Preparation of Purified Rat GST

Cytosolic fractions prepared from pooled livers or kidneys of rats were purified on a hexylglutathione affinity column according to Warholm *et al.*[26] The equilibration and washing buffer consisted of 10 mM Tris-HCl buffer, pH 7.8 containing 1 mM EDTA and 0.2 mM DTT (buffer A). Glutathione transferases were eluted from the column by addition of buffer A fortified with 5 mM hexylglutathione and 0.2 M NaCl (buffer B). Fractions containing GST activity were pooled and dialysed overnight at 4 °C. Dialysis was carried out in 100 times volume of buffer A and then concentrated by microfiltration (Millipore, NMWL 10,000) and protein concentration was measured by the method of Lowry *et al.*[25]

Expression and Purification of Recombinant Human GST P1-1

Human GSTs A1-1, P1-1 and M1-1 (allelic variant b) were expressed in *Escherichia coli* (JM103 and XL1-Blue strains) containing the appropriate plasmids and purified according to Stenberg *et al.*,[27] Kolm *et al.*[28] and Comstock *et al.*,[29] respectively, with some minor modifications. The bacterial cells were harvested by centrifugation for 10 minutes at $3,000 \times g$ and the pellet resuspended in an equal volume of lysis buffer containing 10 mM Tris-HCl, pH 7.8, 50 mM EDTA, 15% glucose and 1 mg/ml chicken egg white lysozyme. After 1 hour incubation on ice, the cells were disrupted by sonication (Soniprobe, Dawex, USA). After the addition of phenylmethylsulfonyl fluoride (PMSF) to 170 μM, the soluble fraction was obtained by centrifugation at $100,000 \times g$ for 1 hour. The supernatant was combined with 40 ml S-hexylglutathione-Sepharose 6B or glutathione affinity matrices in buffer A (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.2 mM dithiothreitol, 0.02% w/v NaN_3). The mixture was kept on ice with gentle manual agitation for 1 hour. The gel was packed into a column and washed with buffer A and then buffer A fortified with 0.2 M NaCl (buffer B). GSTs were eluted with either glutathione or 50 mM glycine, pH 10. The eluted protein was concentrated using a PLGC membrane™ NMLW 10,000 (Pharmacia, Uppsala, Sweden). The concentrated protein was dialysed against buffer A. Protein was determined by the Lowry method[25] using BSA as a standard.

Characterisation of Rat Liver Purified GSTs and Heterologously Expressed GSTs

The human recombinant GSTs were purified to homogeneity and a single band was obtained on SDS-PAGE analyses with molecular weights of

25,200, 27,100 and 24,100 for GSTs A1-1, M1-1 and P1-1 respectively. Isoelectric points were 9, 6.2 and 4.5 for GSTs A1-1, M1-1 and P1-1 respectively and are in agreement with other published findings.[20,23] For the rat affinity pools, 3 bands were obtained with molecular weights 25,400, 26,000 and 27,000 which correspond to the Ya, Yb and Yc subunits found in the rat.[24] The isoelectric points were also in agreement with other workers for purified GSTs from rat liver.[31] The effects of standard inhibitors on GST activity with CDNB were also determined. The inhibition characteristics (IC_{50} values) of recombinant GSTs from *E. coli* were comparable with those reported previously[20] (data not shown).

Assay of GST Activities

Enzyme activity was assessed by measuring the conjugating activity with 1-chloro-2,4-dinitrobenzene (CDNB).[20] Activity with CDNB and CuOOH was determined using a Shimadzu double beam UV spectrophotometer, UV1601 (Shimadzu Corp., Columbia, Maryland). Activity with CuOOH was measured in the presence of NADPH, GSH and glutathione reductase as described before.[19,20] For the human recombinant GSTs the final concentrations of GSH and CDNB were both 1 mM.

Glutathione Peroxidase Activity of GST with Artemisinin: Assay of NADPH Oxidation

NADPH oxidation was determined in 0.1 M phosphate buffer pH 7.0 containing 1 mM EDTA as described by Lawrence and Burk.[19] A 900 µl mixture containing 1 unit GSSG-reductase (50 µl), 0.2 mM NADPH (50 µl), 1 mM GSH (50 µl), 1 mM sodium azide (50 µl), 0.025 mg cytosol protein (50 µl), 0.5 µg of affinity chromatography-purified rat liver GST (50 µl) or 0.5–1.0 µg of human recombinant GST. The mixture was allowed to incubate for 5 minutes at room temperature (25 °C) before initiation of the reaction by addition of 50 µl of artemisinin to give a final concentration of 1.5 mM and 5% ethanol. The rate of oxidation of NADPH was assayed spectrophotometrically at 340 nm for 60 seconds in a thermostated (30 °C) Shimadzu double beam UV1601 spectrophotometer. The reference cuvette contained the reaction mixture with either heat-treated enzyme (97 °C) or with buffer instead of enzyme and artemisinin at a final 1.5 mM and ethanol at 5% final concentration. The activity was calculated as µmoles NADPH oxidised/minute/mg of protein ($\varepsilon = 6.20\,mM^{-1}\,cm^{-1}$).

RESULTS

NADPH Oxidation

The artemisinin-induced NADPH oxidation was observed to be dependent on the presence of artemisinin, GSH and glutathione reductase. Table I shows the rates of oxidation of NADPH by glutathione reductase in a coupled assay system for the putative reduction of artemisinin by cytosolic fractions and affinity chromatography-purified GSTs. Values of GST activity with CDNB and CuOOH are also shown for comparative purposes. The proposed reaction with artemisinin is essentially similar to that of CuOOH, the difference being the electrophilic substrate. It can be seen that using liver and intestine cytosolic fractions, NADPH oxidation in the reaction mixture with artemisinin was comparable with activity with CDNB whilst for the kidney fraction, the activity with artemisinin was three fold higher than that of CDNB. To exclude the possibility of other cytosolic enzymes being involved affinity purified rat liver GSTs and human recombinant GSTs were used and the values obtained for artemisinin activity (NADPH oxidation) were at least two fold higher. The reaction was found to be non-specific compared to CuOOH but comparable to the lack of specificity seen for CDNB. The rates were 2.5 fold higher compared with CDNB for human recombinant GSTs. When one considers the ratio of activity

TABLE I Artemisinin-induced NADPH oxidation by glutathione reductase in the presence of different GST enzyme preparations

GSTs	ATN (NADPH Oxidised)	CDNB (Conjugate formed)	CuOOH (NADPH Oxidised)	ATN / CDNB	ATN / CuOOH
		μmoles/min/mg protein			
Rat liver[a] cytosol	23.14 ±1.0	28.31 ±2.5	3.92 ±0.4	0.8	6
Rat kidney[a] cytosol	16.67 ±4.5	5.34 ±0.4	1.17 ±0.3	3	14
Rat intestine[a] cytosol	1.60 ±0.4	1.70 ±0.3	0.45 ±0.1	0.95	4
Rat liver[b] affinity pool	305.62 ±62	344.2 ±13	5.21 ±0.1	0.89	59
Human[b] GST A1-1	390 ±12	159.6 ±2.3	10[c]	2.4	39
Human[b] GST M1-1	467 ±26	172.4 ±16	0.6[c]	2.6	778
Human[b] GST P1-1	282 ±3	106.82 ±11	0.03[c]	2.6	9400

[a] Values for activity in the cytosolic fractions are represented as mean ± SD ($n = 6$). [b] Values for GST activity using affinity preparations are represented as mean ± SD for triplicate determinations. [c] Values for CuOOH activity for recombinant human GSTs were obtained from Mannervik and Widersten.[20]

with artemisinin to CuOOH activity, it was observed that the cytosolic fractions had 4–14 times higher activity with artemisinin. The increase in GST activity for NADPH utilisation vs. CuOOH activity was higher for the affinity purified GSTs.

DISCUSSION

The major objective of this study was to investigate the possible reduction of artemisinin by glutathione transferases *in vitro*. Glutathione is a nucleophile and artemisinin can be attacked by nucleophiles in a number of ways. The exocyclic double bond has been reported to open upon nucleophilic attack to give disulphide derivatives[18] (Figure 2). A hydroperoxide of artemisinin has been reported to be formed from artemisinin by a nucleophilic opening of the lactone ring, similar to compound A in Figure 2 and it has been reported to be an active artemisinin metabolite.[18] It has also been reported that the sesquiterpenes undergo a Michael-type addition reaction with the sulphydryl group of cysteine e.g. as in GSH[16] (compare Figure 2). We propose that our results for NADPH oxidation by glutathione reductase in the presence of artemisinin and GSTs may be explained in the following way (Figure 2):

Reaction 1: Glutathione enzymatically or non-enzymatically reacts with artemisinin at the lactone ring to form a derivative with a hydroperoxide moiety.
Reaction 2: The hydroperoxide could react with more GSH in a type II glutathione peroxidase reaction which is GST mediated[19,32] and
Reaction 3: To replenish the levels of reduced glutathione, glutathione reductase utilises NADPH to convert GSSG to GSH and this results in the decreases in absorbance at 340 nm.

The proposal for the possible non-enzymatic reaction of GSH with artemisinin in the reaction above was suggested by the work of Mitchell *et al.*,[33] in which it was shown that GSTs were able to react with haloenol lactones. Glutathione was shown to protect GST P1-1 from haloenol

inactivation and this was attributed to the non-enzymatic reaction of GSH with the lactone ring.

Many observations of other workers led us to postulate that GSTs could catalyse the reductive metabolism of sesquiterpenes such as artemisinin. Sy *et al.*,[34] reported that the reduction of artemisinin *in vitro* to the hydroperoxide derivative occurs in the presence of reducing agents. Schimdt and Chung[17] showed that the S9 fraction of a mouse liver preparation enhanced the oxidation of NADPH in a reaction mixture containing NADPH, cytosolic protein and sesquiterpene lactones at 25 °C and pH 7.4. These workers proposed that these sesquiterpene lactones could react with GSH either non-enzymatically or enzymatically through a GST catalysed reaction[17] and that a ketone/lactone reductase was responsible for a direct reaction of NADPH with sesquiterpene lactones. These workers used S9 fractions that may contain GSH (up 10 mM in some cells) and glutathione reductase. It is, therefore, possible that the proposed oxidation of NADPH may be explained by the activity of a glutathione reductase and GST rather than by a ketone reductase as shown in Figure 2. This means that what these workers observed may be a GST/GSSG-reductase coupled reaction that can occur in the presence of GSH, NADPH and an appropriate xenobiotic. Glutathione reductase may be playing the same role as the proposed ketone/lactone reductase but the reaction may be occurring at the second stage in our reaction scheme (Figure 2). The existence of the GSH-artemisinin conjugate, however, still needs to be confirmed by isolation and characterisation. Our results together with those observed by Schimdt and Chung[17] suggest that GSTs are involved in the metabolism of artemisinin.

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