Glutathione-related Enzymes Contribute to Resistance of Tumor Cells and Low Toxicity in Normal Organs to Artesunate

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Abstract. The anti-malarial artemesine (ART) also inhibits the growth of cancer cells. The active moiety is an endoperoxide bridge whose cleavage generates reactive oxygen species and free radicals. We analyzed whether glutathione-related enzymes contribute to tumor resistance to ART and to the low toxicity of ART towards normal organs. The microarray-based mRNA expression of dihydrodiol dehydrogenase, γ-glutamylcysteine synthase (γ-GCS), glutathione S-transferases GSTM4, GSTT2, GSTZ1, and microsomal glutathione S-transferase MGST3 showed significant relationships (p<0.05) to cellular response to ART in 55 cell lines of the National Cancer Institute, USA. A tendency for correlation (0.05<p<0.1) was observed for GSTA1, GSTA2, GSTP1 and MGST1. A further 12 glutathione-related genes were not linked to ART resistance. MSC-HL13 cells transfected with cDNAs for heavy and light subunits of γ-GCS were more resistant to ART than mock control vector-transfected MSV-PC4 cells. L-buthionine sulfoximine, a γ-GCS inhibitor that depletes cellular glutathione pools, completely reversed ART resistance in MSV-HL13 cells, while a partial reversion was obtained by ethacrynic acid, an inhibitor of GST. The expression of GST-P was analyzed immunohistochemically in normal rat organs. GST-P immunostaining was found in all organs analyzed, albeit with varying staining intensities and in different histological structures of the organs. GST expression in normal organs may, therefore, contribute to the good tolerability and minimal toxicity of ART in normal organs.

A wide variety of enzymes provide protection from harmful injury by toxic chemicals. In normal cells, these enzymes detoxify xenobiotics taken up from the environment, whereas in tumor cells they contribute to the development of resistance towards chemotherapeutic agents. Among them are enzymes associated with the glutathione detoxification system. The rate-limiting enzyme in glutathione synthesis is γ-glutamyleysteine synthase (γ-GCS), which catalyzes the peptide linkage between the γ-carbonyl group of glutamate and the amino group of cysteine. Glutathione synthase (GSS) catalyzes the condensation of the carbonyl group of cysteine with the amino group of glycine to form the tripeptide glutathione. Glutathione S-transferases (GST) conjugate electrophilic compounds and products of oxidative stress to reduced glutathione (1). In the presence of reducing equivalents from reduced glutathione, glutathione peroxidases detoxify hydrogen peroxide and lipid hydroperoxides produced by anti-cancer drugs (2). Esterase D/S-formylglutathione hydrolase (ESD) is an enzyme capable of hydrolyzing thiol esters of glutathione (3). Dihydrodiol dehydrogenase (DDH) catalyzes the detoxification of polycyclic aromatic hydrocarbons and other xenobiotics. Its expression is induced by the GST-inhibitor ethacrynic acid (4). The role of glutathione-related enzymes in resistance to established anti-cancer drugs has previously been analyzed in cell lines and human tumor biopsies (5-7).

Drugs from traditional Chinese medicine (TCM) have provoked increasing interest in recent years, since they may have cellular targets different from established anti-cancer drugs. A salient feature is their good tolerability and lack of severe, life-threatening side-effects, which are typical for many established cytostatic drugs. In recent years, we and others have investigated the anti-cancer activity of compounds from TCM (8-10). Recently, we focused our efforts on artesunate (ART). This is a semi-synthetic derivative of artemisinin, the active principle of Artemisia annua L., which has been used for more than 2000 years in China to treat fever and chills. Artemisinin and its derivatives are valuable drugs to treat multidrug-resistant Plasmodium falciparum and Plasmodium vivax infections (11). Interestingly, they reveal only minimal side-effects in malaria patients (12). The active moiety of artemisinins is an endoperoxide bridge. Its cleavage results in the generation of reactive oxygen...
controls were included for DMSO as solvent. The net outcome of cell proliferation and cell death. Vehicle after treatment with the drugs. The resulting growth data represent means of a growth inhibition assay as described (8). Aliquots of 5x10^4 cells/ml were seeded in 24-well plates and the drugs were added immediately at different concentrations. The drugs were applied in different concentrations to allow calculation of 50% inhibition concentration (IC_{50}) values. Cells were counted 7 days after treatment with the drugs. The resulting growth data represent the net outcome of cell proliferation and cell death. Vehicle controls were included for DMSO as solvent. The sulfonamide B assay for the determination of drug sensitivity in these cell lines has been reported (21). The response of the NCI tumor cell lines to ART has been determined in a previous collaboration with the NCI (15).

**Materials and Methods**

**Drugs.** Artesunate (ART) was obtained from Saokim (Hanoi, Vietnam). L-buthionine sulfoximine and ethacrynic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany).

**Cell lines.** MSV-HL13 cells transfected with pcDNA3 expression vectors harboring cDNAs for heavy and light subunits of γ-glutamylcysteine synthase (γ-GCS) and mock control vector-transfected MSV-PC4 control cells with ART in combination with L-buthionine sulfoximine, an inhibitor of γ-GCS and glutathione biosynthesis, and ethacrynic acid, an inhibitor of glutathione S-transferases. Finally, we analyzed the glutathione S-transferase expression in normal rat organs.

**Immunohistochemistry.** A previously described method was used to detect glutathione S-transferase-P (the homologue of human GST-π) in crystalline sections of rat tissues (22). Briefly, after pre-incubation with hydrogen peroxide and protein blocking solution to suppress unspecific reactions, the primary antibody was applied for 16 h at 4°C. The rabbit polyclonal anti-GST-P antibody was kindly donated by Dr. K. Satoh (University School of Medicine, Hirosaki, Japan) After incubation with secondary antibody (goat anti-rabbit Ig), the streptavidin-biotinylated peroxidase complex was added and the peroxidase activity visualized with 3-amino-9-ethylcarbazole. Counter-staining was performed with hematoxylin. Both negative and positive controls were conducted. Negative controls were prepared by omitting the primary antibodies and by substituting irrelevant antibodies for the primary antibodies. The specificity of the reactions was proven by Western blots. To evaluate the protein expression, the staining intensity was determined semiquantitatively (-, absent; (+), weak, +, intermediate, ++, strong immunostaining).

**Statistical analyses.** The mRNA expression values of glutathione-related genes in the NCI cell lines were selected from the NCI's database, Bethesda, MA, USA (http://dtp.nci.nih.gov). The mRNA expression has been determined by microarray analyses as reported (18).

Objects were classified by calculation of distances according to the closeness of between-individual distances by means of hierarchical cluster analysis. All objects were assembled into a cluster tree (dendrogram). The merging of objects with similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relation. The distance of a subordinate cluster to a superior cluster represents a criterion for the closeness of clusters as well as for the affiliation of single objects to clusters. Thus, objects with tightly related features appear together, while the separation in the cluster tree increases with progressive dissimilarity. Recently, cluster models have been validated for gene expression profiling and for approaching molecular pharmacology of cancer (18, 23, 24). Cluster analyses applying the complete linkage method were done with the WinSTAT program (Kalma, Cambridge, MA, USA). Missing values were automatically omitted by the program, and the closeness of two joined objects was calculated by the number of data points they contained. In order to calculate distances between all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean = 0 and a variance = 1.

Kendall’s τ test was used to calculate significance values. This test was implemented into the WinSTAT Program (Kalma). Kendall’s τ test determines the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and are transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of Kendall’s τ test.

The χ^2 test was used as implement of the WinSTAT program (Kalma) to prove bivariate frequency distributions for pairs of nominal scaled variables for dependencies.
The objective of this investigation was to evaluate the profiles of mRNA expression of glutathione-related genes involved in resistance to artesunate (ART). For this reason, the mRNA expression of 22 genes in 55 cell lines of the NCI panel was correlated with the IC50 values for ART by Kendall’s $\tau$ test. The mRNA expression values were reported earlier and deposited in the database of the NCI (18; http://dtp.nci.nih.gov). The IC50 values for ART were reported earlier by us (15). Out of 22 genes, 10 showed a correlation between mRNA expression and cellular response to ART (Table I). The mRNA expression of dihydrodiol dehydrogenase, $\gamma$-glutamylcysteine synthase ($\gamma$-GCS), glutathione S-transferase isoenzymes GSTM4, GSTT2 and GSTZ1, and microsomal glutathione S-transferase MGST3 showed significant relationships to cellular response to ART at $p<0.05$ (Table I). A tendency for correlation ($0.05<p<0.1$) was observed for GSTA1, GSTA2 and GSTP1, and for MGST1. For comparison, an established alkylating agent, carmustine (BCNU), was analyzed. The mRNA expression of GSTA1, GSTA2, GSTT2, GSTZ1, MSTG1 and MGST3 correlated significantly with the IC50 values for BCNU. The mRNA expression of GSTA3, GSTA4, GSTM1, GSTM3, GSTM5, GSTT1, GPX1, GPX2, GPX3, GPX4 (glutathione peroxidases 1-4), GSS (glutathione synthetase) and ESD (esterase D/formylglutathione hydrolase) neither correlated with the IC50 values for ART nor with those for BCNU.

The mRNA values of the genes correlating with the IC50 values for ART at $p<0.1$ were then subjected to hierarchical cluster analysis, in order to find out an expression profile indicative for resistance to ART (Figure 1). We divided the dendrogram into three clusters and correlated them with the IC50 values data for ART, which were not included as a parameter into the cluster analysis (Table II). Clusters 1 and 3 were enriched with cell lines sensitive to ART ($n=13$ and $n=19$, respectively), while cluster 2 was enriched with resistant ones ($n=23$). This relationship was significant ($p=0.034$), indicating that glutathione-related enzymes are relevant for resistance of tumor cells to ART.

To analyze this hypothesis in more detail, we used MSV cells transfected with cDNAs for heavy and light subunits of $\gamma$-glutamylcysteine synthetase ($\gamma$-GCS) (MSV-HL13) and mock control vector (MSV-PC4), respectively. For

<table>
<thead>
<tr>
<th>Table I. Correlation of microarray-based mRNA expression of glutathione-related genes to IC50 values for ART and carmustine (BCNU) in 60 cell lines of the NCI panel as analyzed by Kendall’s $\tau$ test.</th>
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<tbody>
<tr>
<td>Symbol</td>
</tr>
<tr>
<td>DDH</td>
</tr>
<tr>
<td>$\gamma$-GCS</td>
</tr>
<tr>
<td>GSTA1</td>
</tr>
<tr>
<td>GSTA2</td>
</tr>
<tr>
<td>GSTM4</td>
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<tr>
<td>GSTP1</td>
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<tr>
<td>GSTZ1</td>
</tr>
<tr>
<td>MGST1</td>
</tr>
<tr>
<td>MGST3</td>
</tr>
</tbody>
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* borderline significant ($0.05<p<0.1$)

n.s., not significant

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<tr>
<th>Table II. Separation of clusters of NCI cell lines obtained by hierarchical cluster analysis shown in Figure 1 in comparison to sensitivity to ART.</th>
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<tbody>
<tr>
<td>Cluster</td>
</tr>
<tr>
<td>Cluster 1</td>
</tr>
<tr>
<td>Cluster 2</td>
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<tr>
<td>Cluster 3</td>
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</tbody>
</table>

The median log$_{10}$IC50 value (-5.335 M) was used as cut-off to separate tumor cell lines as being 'sensitive' or 'resistant'.

Figure 1. Dendrogram of hierarchical cluster analysis (complete linkage method) obtained from mRNA expression of glutathione-related genes correlating with IC50 values for artesunate. The dendrogram shows the clustering of 60 NCI cell lines.
combination treatments, we used a concentration of 0.2 μg/ml BSO (0.9 μM) and 2 μg/ml EA (6.6 μM), respectively, which caused only fair inhibition of growth of both cell lines if applied alone (data not shown). The cytotoxicity of control treatments with these concentrations of BSO or EA alone in combination with ART was set as 100% to allow direct comparison with the dose-response curve of ART alone.

Cells were treated with different concentrations of ART in combination with or without BSO or EA. As shown in Figure 2, ART inhibited cell growth in a dose-dependent manner. Again, the dose-response curves were used to calculate IC₅₀ values (0.94 and 3.13 μM, respectively). MSV-HL13 cells were 3.3-fold resistant to ART as compared to MSV-PC4 cells (Table III). The addition of BSO led to a complete reversion of ART resistance in MSV-HL13, while ART sensitivity in MSV-PC4 cells remained unaltered upon BSO exposure (Figure 2A, Table III). ART sensitivity was increased by the addition of EA both in MSV-HL13 and MSV-PC4 cells. The combination of ART plus EA resulted in a higher IC₅₀ value (1.85 μM) in MSV-HL13 cells compared to treatment of MSV-PC4 cells with ART alone (0.94 μM), indicating that EA only partially modulated ART resistance in MSV-HL13 cells (Figure 2B, Table III).

To investigate whether glutathione S-transferases might also play a role in the resistance of normal tissues to ART, we examined the expression of GST-P (the homologue of human GST-π) in cryostat sections of normal rat organs by means of immunohistochemistry. GST-P expression was detected with varying staining intensities in all organs investigated, albeit in different histological structures (Table IV). In the digestive tract, the epithelium, glandulae oesophageae and muscles of the esophagus were GST-P-positive, while the tunica propria, muscularis mucosae and tunica submucosa were negative. In the stomach, small and large intestine, the epithelium but not the smooth muscles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MSV-PC4</th>
<th>MSV-HL13</th>
<th>Resistance factora</th>
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<tbody>
<tr>
<td>ART alone</td>
<td>0.94*</td>
<td>3.13</td>
<td>3.3</td>
</tr>
<tr>
<td>ART + BSO</td>
<td>0.86</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td>ART + EA</td>
<td>0.60</td>
<td>1.85</td>
<td>3.08</td>
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* μM
*I₅₀ of MSV-HL13 divided by I₅₀ of MSV-PC4

Figure 2. Growth inhibition of MSV-HL13 transfected with cDNAs for heavy and light subunits of ß-GCS and mock-vector-transfected MSV-PC4 cells by combination treatments with artesunate (ART) and L-buthionine sulfoximine (BSO) or ethacrynic acid (EA). (A) ART plus BSO; and (B) ART plus EA.

Table III. 50% inhibition concentration (IC₅₀) of MSV-PC4 and MSV-HL13 cells after treatment with ART, BSO and EA in growth inhibition assays.

showed strong GST-P immunostaining. The bile ducts of the liver expressed GST-P, while the parenchyma did not. The pancreas and salivary glands were also GST-P-positive. In the respiratory tract, the cartilage cells of the trachea and bronchi(oli) and alveolar macrophages of the lung were stained for GST-P. Positive immunostaining in the lung is shown in Figure 3A. A strong immunostaining was observed in the kidney, but only a faint staining was apparent in the endometrium of the uterus (Figure 3B). In the circulatory system, the myocard but not the endocard of the heart stained positive for GST-P. Furthermore, the tunica intima, but not the tunica media and the tunica adventitia, of blood vessels expressed GST-P. While the red pulpa of the spleen was strongly GST-P-positive, the white pulpa was negative. Lymph nodes were also GST-P-positive (Figure 3C). The adrenal showed a strong GST-P expression in the zona glomerulosa and a weak GST-P expression in the zona fasciculata and zona reticularis. The capsule of the adrenal did not express GST-P. The brain capillaries stained positive for GST-P, while the brain parenchyma did not.

**Discussion**

In the present investigation, we showed that artesunate (ART) is a substrate of the glutathione detoxification system. The correlations of the mRNA expression of glutathione-related genes with the IC50 values for ART in the NCI tumor cell lines provide a clue that the glutathione system affects the cytotoxicity of ART.

To analyze whether the observed correlations between ART's cytotoxicity and the expression of glutathione-related enzymes are causative, we treated cells transfected with a cDNA for α-GCS with ART. Since α-GCS is the rate-limiting enzyme in the biosynthesis of glutathione, it is to be expected that cells transfected with α-GCS are resistant towards ART, if glutathione is of relevance for cellular response to ART. Indeed, we found a 2.5-fold resistance of MSV-HL13 cells transfected with cDNAs for heavy and light subunits of α-GCS in comparison to mock control vector-transfected MSV-PC4 cells. Moreover, we used L-buthionine sulfoximine (BSO), which is a specific inhibitor of α-GCS and thereby depletes cellular glutathione pools (25). BSO sensitized MSV-HL13 cells for ART to a comparable level as the MSV-PC4 cells. Therefore, we conclude that glutathione plays a causative role in protecting against ART-induced cytotoxicity.

Furthermore, we applied the diuretic drug ethacrynic acid (EA), which is a potent inhibitor of rat and human glutathione S-transferase isoenzymes α, μ and π, respectively, by reversible covalent binding (26). Upon treatment of MSV-PC4 and MSV-HL13 cells with ART plus EA, we found that the cell growth of both cells lines was more inhibited compared to treatment with ART alone.
However, the modulatory effect was weaker that that seen with BSO. This indicates that depletion of glutathione by BSO is more important than inhibition of glutathione S-transferases by EA in MSV cells.

Our results are in accord with a report of Mukanganyama and colleagues, who have shown that the activity of glutathione S-transferases was inhibited by artemisinin, the mother drug of ART, and that glutathione S-transferases may contribute to the metabolism of artemisinin (27, 28).

The authors proposed a model in which (i) artemisinin reacts with GSH resulting in oxidized glutathione; (ii) the oxidized glutathione is then converted to reduced glutathione via glutathione reductase; and (iii) the latter reaction may then result in the depletion of NADPH by glutathione reductase.

BSO significantly increases the neurotoxic effect of non-toxic concentrations of artemether and dihydroartemisinin (29), suggesting that endogenous glutathione participates in the prevention of the neurotoxicity of artemisinins. This is consistent with a role of artemisinins in mediating damage of redox-sensitive proteins by free radicals and/or reactive oxygen species.

Secondary plant compounds make plants unpalatable or poisonous to herbivores and protect against microbial pathogens. Animals as well as microorganisms have developed detoxification mechanisms against such plant compounds during evolution. Glutathione-related enzymes are part of this detoxification system. It is, therefore, worth hypothesizing that the expression of these enzymes protects normal organs from the poisonous and otherwise detrimental effects of natural products such as ART. We used immunohistochemistry to analyze GST-P expression in normal organs. The advantage of immunohistochemistry over Western blotting, Northern blotting, or polymerase chain reaction is the ability to determine which cells in each tissue express GST-P. Its presence in normal tissues with secretory, excretory, or respiratory functions (gastro-intestinal tract, urogenital tract, glands, respiratory tract) suggest a role in detoxification of potentially harmful compounds taken up by foods or breath. Their expression in blood vessels may have a barrier function protecting tissues from circulating xenobiotics, and the GST expression in brain capillaries suggests a role in the blood-brain barrier. The endogenous generation of aggressive reactive oxygen species in cells of the immune system may explain the need for high GST expression in spleen and lymph nodes. The frequent expression of glutathione S-transferase in normal organs may contribute to the good tolerability and the minimal toxicity of ART in normal organs, as previously reported in a large meta-analysis with 9,241 malaria patients (12). Experiments with laboratory animals, however, show that very high doses and prolonged exposure to artemisinins provoke neurotoxicity (30). Glutathione S-transferase expression in brain capillaries represents a constituent of the blood-brain barrier (31) and may protect brain tissue from the detrimental effects of artemisinins. At high concentrations, the detoxification capability of GST might be overloaded and neurotoxic symptoms appear. It still remains open whether treatment of cancer patients with ART is also free of severe side-effects as found for malaria treatment.

Figure 3. Immunohistochemical detection of GST-P in normal tissues of the rat. (A) lung, (B) endometrium, (C) lymph node.
While a protective role has been suggested in the present investigation only for glutathione-related enzymes, other antioxidant proteins may also contribute to this phenomenon. Recent experiments with cell lines transfected with cDNAs for catalase or thioredoxin have shown that these genes also counteract the cytotoxic action of ART (32). A role of GSTs for detoxification is also conceivable with the frequently found co-expression with P-glycoprotein, an important transport protein for many natural products. This was found by us for rat organs (33) and by others for human organs (34). Different protective mechanisms may be co-regulated as a general protection machinery against a wide array of harmful substances.

In conclusion, it is reasonable to hypothesize that glutathione-related enzymes contribute (i) to resistance of tumor cells to ART and (ii) to the lack of severe toxicity in normal organs.

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References


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