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Original Contribution

ENHANCEMENT OF CYTOTOXICITY OF ARTEMISININS TOWARD CANCER CELLS BY FERROUS IRON

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Abstract—Iron(II) heme-mediated activation of the peroxide bond of artemisinins is thought to generate the radical oxygen species responsible for their antimalarial activity. We analyzed the role of ferrous iron in the cytotoxicity of artemisinins toward tumor cells. Iron(II)–glycine sulfate (Ferrosanol) and transferrin increased the cytotoxicity of free artesunate, artesunate microencapsulated in maltosyl-β-cyclodextrin, and artemisinin toward CCRF-CEM leukemia and U373 astrocytoma cells 1.5- to 10.3-fold compared with that of artemisinins applied without iron. Growth inhibition by artesunate and ferrous iron correlated with induction of apoptosis. Cell cycle perturbations by artesunate and ferrous iron were not observed. Treatment of p53 wild-type TK6 and p53 mutated WTK1 lymphoblastic cells showed that mutational status of the tumor suppressor p53 did not influence sensitivity to artesunate. The effect of ferrous iron and transferrin was reversed by monoclonal antibody RVS10 against the transferrin receptor (TfR), which competes with transferrin for binding to TfR. CCRF-CEM and U373 cells expressed TfR in 95 and 48% of the cell population, respectively, whereas TfR expression in peripheral mononuclear blood cells of four healthy donors was confined to 0.4–1.3%. This indicates that artemisinins plus ferrous iron may affect tumor cells more than normal cells. The IC_{50} values for a series of eight different artemisinin derivatives in 60 cell lines of the U.S. National Cancer Institute were correlated with the microarray mRNA expression of 12 genes involved in iron uptake and metabolism by Kendall's τ test to identify iron-responsive cellular factors enhancing the activity of artemisinins. This pointed to mitochondrial aconitase and ceruloplasmin (ferroxidase).

Keywords—Artemisinin, Artesunate, Transferrin, Iron(II)–glycine sulfate, Free radicals

INTRODUCTION

Artemisinin (ARS), also known as Qinghaosu, is the active principle of the Chinese plant qinghao (*Artemisia annua L.*) used for centuries in traditional Chinese medicine as a remedy for chills and fever [1]. Its antimalarial properties were discovered in China only in 1972 [2]. ARS is a sesquiterpene trioxane lactone containing an endoperoxide bridge, which is essential for its activity [3].

The lactone can easily be reduced, resulting in the formation of dihydroartemisinin, the active intermediate. The derivatives of ARS are active against both *Plasmodium falciparum* and *Plasmodium vivax*, showing broad stage specificity and rapid action against early blood forms of the parasite [1], with artesunate (ART) as the most potent derivative in vitro [4]. ART is a semisynthetic derivative of ARS (hemisuccinate of dihydroartemisinin) that is active against chloroquine- and mefloquine-resistant strains of *P. falciparum*, with a very good tolerability and very few side effects [5].

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Artemisinins exert their antimalarial activity by iron-mediated cleavage of the endoperoxide bridge and generation of organic free radicals [6]. These radical molecules cause macromolecular damage and cell death of the parasites [7,8]. In erythrocytes, iron is present in large excess bound to hemoglobin. Artemisinins are first activated by intraparasitic heme-iron, which catalyzes the cleavage of this endoperoxide. It has been proposed that the transfer of an oxygen atom from the peroxide group of artemisinins to a chelated iron ion generates a Fe(IV)═O species. A resulting free radical intermediate may then kill the parasite by alkylating and poisoning one or more essential malarial protein(s) [9,10]. Recently, studies have shown that artemisinins also reveal profound cytotoxic activity against tumor cells [11–15] and antiviral activity [16]. The role of iron in the toxicity of artemisinins toward tumor cells is far less understood.

As the iron storage of tumor cells is generally much less than of erythrocytes, on the one hand, but is greater in tumor cells than in normal cells [17], we were intrigued by whether iron may also play a role in the inhibitory action of artemisinins toward tumor cells.

To address this question, we used human CCRF-CEM leukemia and human U373 astrocytoma cells and treated them with ART and ARS in combination with iron(II)–glycine sulfate (Ferrosanol) or transferrin. ART was used either as the free drug or microencapsulated with maltosyl-β-cyclodextrin. As a control drug, we used doxorubicin (DOX), as the modulating effect of iron on cellular DOX sensitivity has been documented [18].

MATERIALS AND METHODS

Drugs

ARS was obtained from Sigma (Deisendorf, Germany). ART was obtained from Saokim Ltd. (Hanoi, Vietnam). Artesunate maltosyl-β-cyclodextrin (ART-MCD) was provided by Dr. Achille Benakis (Geneva, Switzerland). Each 1.24 g ART-MCD contained 100 mg ART. Artesunate and maltosyl-β-cyclodextrin (1:10) were dissolved in 100 ml water at 60°C and stirred for 12 h in a water bath, then cooled down and left at 3°C. The product was obtained by freeze-drying (patent of A. Benakis, No. CH 685391). DOX and transferrin were purchased from Sigma. Iron(II)–glycine sulfate (Ferrosanol) was obtained from Sanol (Monheim, Germany). Other drugs used in this study were arteether, artemether, artemisetene, arteanuine B, dihydroartemisinyl ester stereoisomers 1 and 2, daunorubicin (DNR), and cisplatin (DDP). While the IC_{50} values for ART in 60 cell lines of different origin of the National Cancer Institute (NCI, Bethesda, MD, USA), have been reported by us [13], the IC_{50} values for the other artemisinins as well as for DOX, DNR, and DDP have been deposited in the NCI's database (http://dtp.nci.nih.gov).

Cells

Human CCRF-CEM leukemia cells, human U373 astrocytoma cells, lymphoblastic TK6 cells with wild-type p53, and lymphoblastic WTK1 cells with a p53 Ile273 mutation [19] were used. All cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (Gibco-BRL) in a 5% CO_2 atmosphere at 37°C. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase.

Growth inhibition assay

The in vitro response to drugs was evaluated by means of a growth inhibition assay as described [20,21]. Aliquots of 5×10^4 cells/ml were seeded in 24-well plates, and drugs were added immediately at different concentrations. The concentrations of ART, ART-MCD, and ARS were in the dose range 0.001 to 30 μg/ml, and that of DOX, in the range 0.00001 to 0.3 μg/ml. For combination treatments, the same dose ranges of ART, ART-MCD, ARS, and DOX were used together with 1 mg/ml transferrin or 10 μg/ml iron(II)–glycine sulfate, respectively. Each dose of drug was applied in two independent wells. Cells were counted 7 days after treatment with the drugs. The cells in each well were counted twice. The resulting growth data represent the net outcome of cell proliferation and cell death. Vehicle controls were included for compounds dissolved in DMSO. Monoclonal antibody clone RVS10 against transferrin receptor (TfR; CD71), which competes with transferrin for binding to TfR and which blocks the TfR internalization pathway [22], was preincubated 2 h before application of ART plus iron(II)–glycine sulfate or transferrin. This antibody (Research Diagnostics, Flanders, NJ, USA) was generously provided by Dr. Kuk-Wha Lee and Dr. Pinchas Cohen (Division of Pediatric Endocrinology, Mattel Children's Hospital, David Geffen School of Medicine, University of California Los Angeles, CA, USA).

Clonogenic cell survival assay

Tumor cells (500 cells/well) were seeded in 60 mm dishes. ART and iron(II)–glycine sulfate were applied as described above. One week later, the colonies that grew from the surviving cells were fixed in methanol for 5 min and air-dried. Then, the colonies were stained with 1.25% Giemsa/0.125% crystal violet and counted.

Flow cytometry

Cell cycle and apoptosis analysis was done as described [23]. Briefly, after drug exposure CCRF-CEM cells were stained with propidium iodide (16.5 μg/ml) in PBS after RNase (0.03 μg/ml) digestion. Samples were analyzed on a FACS Calibur (Becton Dickinson). For each sample, 10,000 cells were analyzed. Cells within different cell cycle phases and the fraction of cells exhibiting a DNA content lower than G_1 (apoptotic cells) were quantified using the CellQuest software (Becton Dickinson).

Transferrin receptor (CD71) expression was determined with FITC-labeled mouse anti-human CD71 monoclonal IgG1 antibody Ber-T9 (DakoCytomation, Hamburg, Germany) and an EPICS XL-MCL flow cytometer (Beckman Coulter, Krefeld, Germany). As a negative control, FITC-labeled non-immunized mouse IgG1 was used (DAKO). All measurements were performed according to the manufacturer's instructions.

Statistical analysis

Baseline mRNA expression levels of 12 genes (aconitase 1 (soluble), aconitase 2 (mitochondrial), ceruloplasmin, ferredoxin 1, ferritin heavy and light polypeptides, ferrochelatase, iron-responsive element protein 2, lactoferrin, transferrin, transferrin receptor, zinc/iron regulated transporter-like RNA) involved in cellular iron uptake and metabolism in 60 cell lines of different origin were taken from the NCI's database (http://dtp.nci.nih.gov) to correlate them to the response of these cell lines to a panel of cytotoxic drugs. mRNA expression was determined by microarray analyses as reported [24].

Kendall's τ test was used to calculate significance (p values) and rank correlation coefficients (R values) as a relative measure of the linear dependency of two variables. This test was implemented in the WinSTAT Program (Kalmia). Kendall's τ test determines the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and is transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of Kendall's τ test. In addition to calculation of p and R values, the problem of multiple hypothesis testing was addressed. The probability of type I errors increases as the number of tests increases [25,26]. Therefore, a step-up resampling multicomparison procedure was applied to control the false discovery rate among the significant correlations at a significance level of .05. This program is available on the Internet (http://www.math.tau.ac.il). The false discovery rate (FDR) is the expected proportion α of erroneous rejections among all rejections of the null hypothesis [27].

RESULTS

As a starting point, growth of CCRF-CEM cells was determined after exposure to iron(II)–glycine sulfate. It was applied in the concentration range 0.001 to 1000 μg/ml. Within this concentration range, we observed no or only minimal inhibition of cell growth (<5%, data not

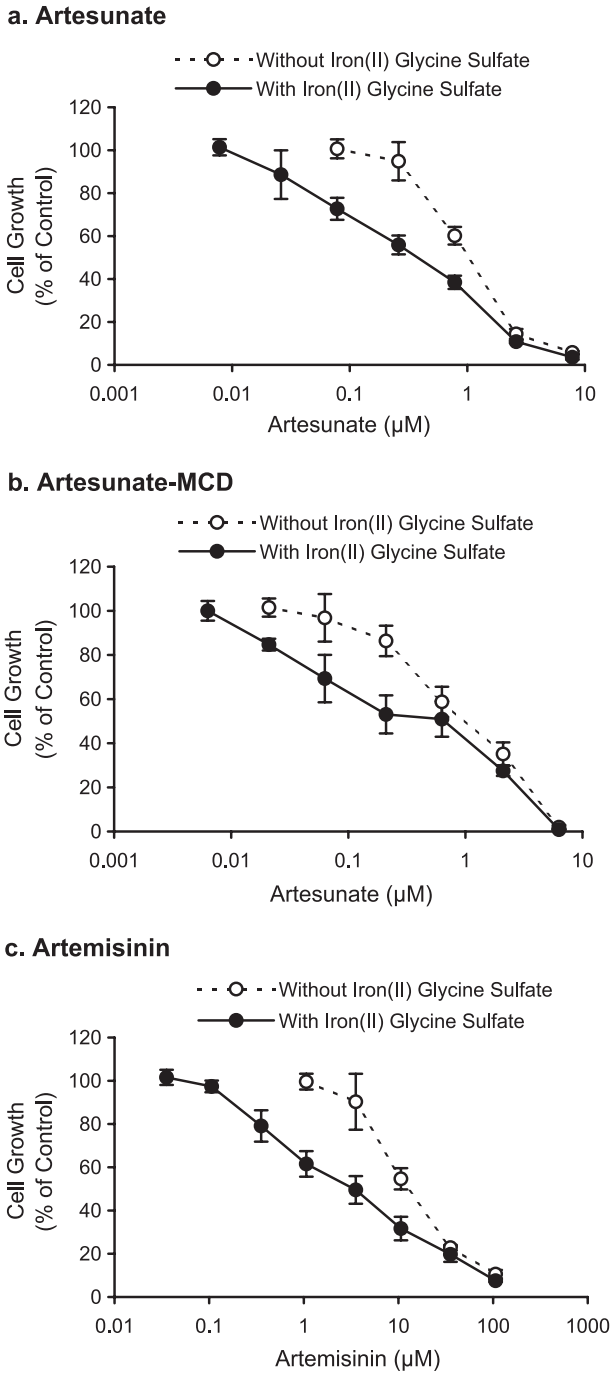

Fig. 1. Growth inhibition of human CCRF-CEM leukemia cells by (a) ART, (b) ART-MCD, and (c) ARS with or without addition of 10 μg/ml iron(II)–glycine sulfate (Ferrosanol).

shown). For further combination treatments, we used a concentration of 10 μg/ml iron(II)–glycine sulfate. CCRF-CEM leukemia cells were treated with different concentrations of ART, ART-MCD, or ARS in combination with or without iron(II)–glycine sulfate (10 μg/ml). As shown in Fig. 1, each of the artemisinins inhibited cell growth in a dose-dependent manner. The dose–response curves were used to calculate the 50% inhibition concentration (IC_{50}) (Table 1). While ART and ART-MCD were active in a similar molar range (IC_{50}: 1.1 and 1.2 μM for CCRF-CEM, respectively), ARS was 10-fold less active (IC_{50}: 11.5 μM). The addition of iron(II)–glycine sulfate increased the growth inhibitory activity of ART, ART-MCD, and ARS (2.9-, 1.9-, and 3.5-fold, respectively (Fig. 1, Table 1).

To compare this result obtained from leukemia cells with that from a cell line of a solid tumor, we used U373 astrocytoma cells. U373 cells were treated with ART, ART-MCD, or ARS with or without addition of iron(II)–glycine sulfate. The growth of U373 cells was not inhibited by iron(II)–glycine sulfate alone. Again, we observed dose-dependent inhibition of cell growth by the three artemisinins. Based on the IC_{50} values for ART and ART-MCD (3.5 and 1.0 μM for U373, respectively), the latter was 3.5-fold more active than free ART (Table 1). ARS was similar in activity to free ART (IC_{50}: 3.3 μM). The cytotoxicity of the artemisinins was increased by iron(II)–glycine sulfate (Fig. 2). Combination of iron(II)–glycine sulfate with ART, ART-MCD, and ARS resulted in 8.3-, 7.9-, and 4.5-fold increased cytotoxicity compared with the artemisinins alone (Table 1).

As a next step, we analyzed the effect of transferrin on the growth inhibitory activity of artemisinins. Transferrin was first applied alone to CCRF-CEM cells in the

Table 1. 50% Inhibition Concentrations (IC_{50}) of Human CCRF-CEM Leukemia and Human U373 Astrocytoma Cells after Treatment with Artemisinins and Iron(II)–Glycine Sulfate or Transferrin in Growth Inhibition Assays

	IC_{50} (μM)	
	CCRF-CEM	U373
Iron(II)–glycine sulfate		
ART	1.1	3.5
ART+ iron(II)glycine sulfate	0.38	0.42
Modulation index[a]	2.9	8.3
ART-MCD	1.2	1.0
ART-MCD+ iron(II)glycine sulfate	0.65	0.13
Modulation index	1.9	7.9
ARS	11.5	3.3
ARS+ iron(II)glycine sulfate	3.3	0.73
Modulation Index	3.5	4.5
Transferrin		
ART	0.73	3.8
ART+ transferrin	0.49	0.37
Modulation index	1.5	10.3
ART-MCD	1.4	0.58
ART-MCD+ transferrin	0.40	0.13
Modulation index	3.5	4.5
ARS	6.4	3.8
ARS+ transferrin	0.71	0.7
Modulation index	9	5.4

[a] IC_{50} for ART, ART-MCD, or ARS alone divided by IC_{50} for artemisinins+ iron(II)–glycine sulfate or transferrin.

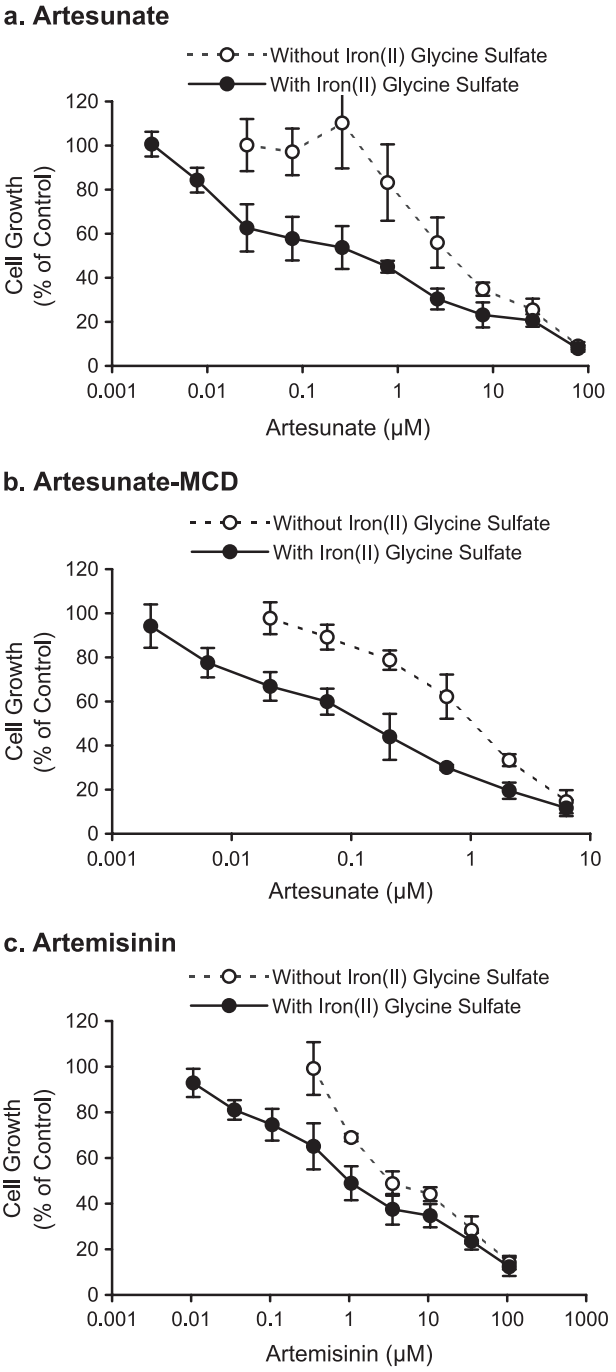

Fig. 2. Growth inhibition of human U373 astrocytoma cells by (a) ART, (b) ART-MCD, and (c) ARS with or without addition of 10 μg/ml iron(II)–glycine sulfate (Ferrosanol).

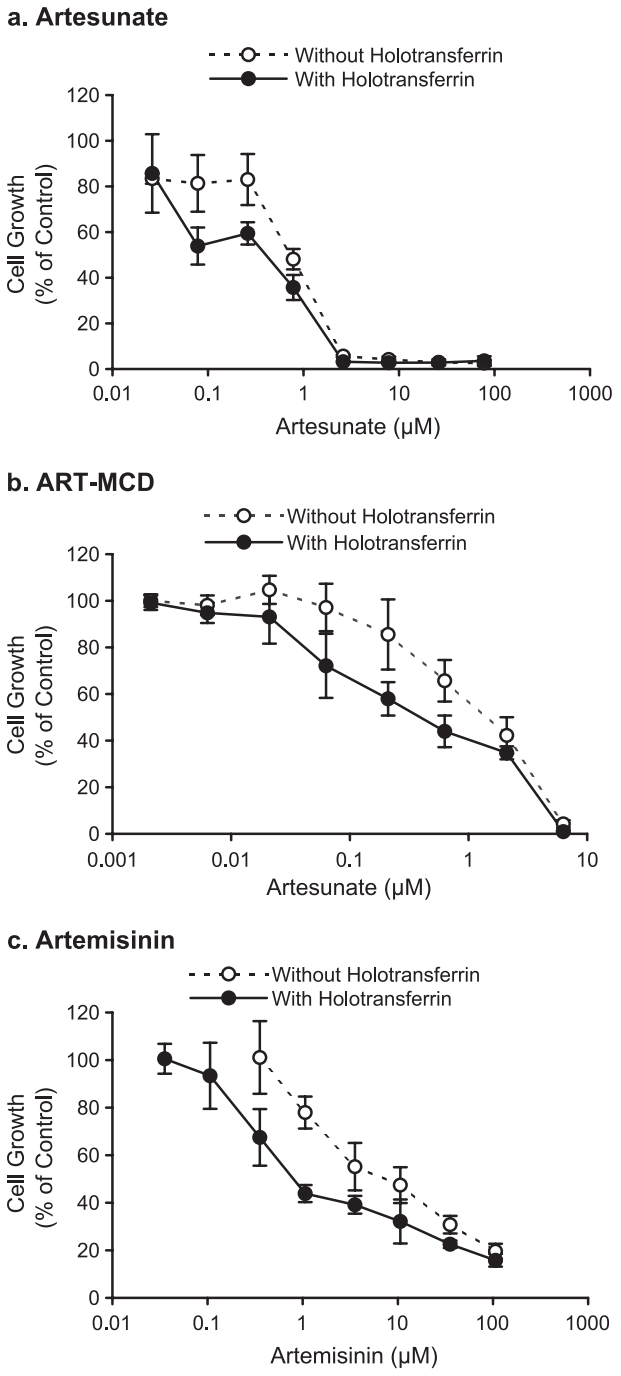

Fig. 3. Growth inhibition of human CCRF-CEM leukemia cells by (a) ART, (b) ART-MCD, and (c) ARS with or without addition of 1 mg/ml transferrin.

concentration range 0.1 to 2 mg/ml. Within this concentration range, we observed no or only minimal inhibition of cell growth (<5%, data not shown). For further combination treatments, we used a concentration of 1 mg/ml transferrin. CCRF-CEM cells were then treated with ART, ART-MCD, or ARS at various concentrations with or without 1 mg/ml transferrin. The growth inhibitory activity of all three artemisinins was increased in combination with transferrin 1.5-, 3.5-, and 9-fold, respectively, compared with that of the three artemisinins alone (Fig. 3, Table 1).

Under identical experimental conditions, transferrin was then investigated in U373 cells. No inhibition of cell growth was observed when transferrin was applied alone. Again, the dose-dependent inhibition of ART, ART-

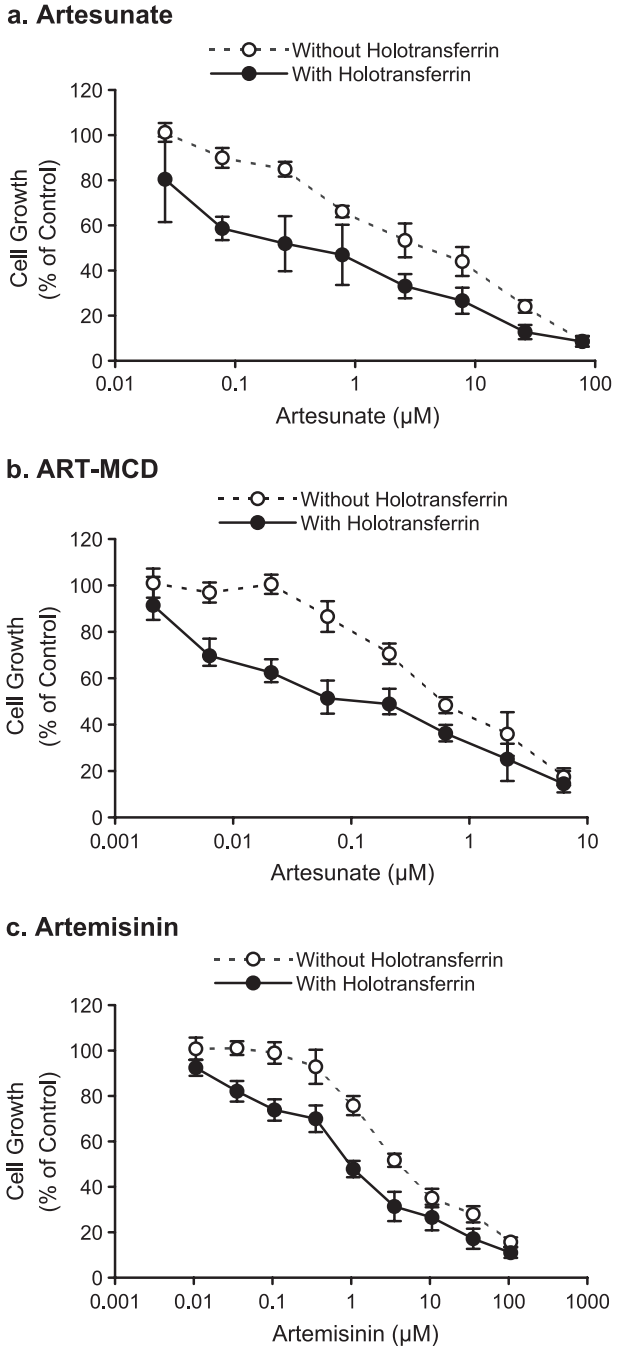

Fig. 4. Growth inhibition of human U373 astrocytoma cells by (a) ART, (b) ART-MCD, and (c) ARS with or without addition of 1 mg/ml transferrin.

MCD, or ARS was increased by the addition of 1 mg/ml transferrin (Fig. 4). The increase in cytotoxicity was 10.3-, 4.5-, and 5.4-fold, respectively (Table 1). In this set of experiments, ART-MCD had an IC_{50} value of 0.58 μM and was 6.6-fold more cytotoxic than free ART (IC_{50}: 3.8 μM), whereas the cytotoxicities of ARS and ART were identical.

We proved the results obtained by the growth inhibition assay with the clonogenic cell survival assay, as this method is frequently looked on as a gold standard. U373 cells were exposed to ART with or without iron(II)–glycine sulfate. As shown in Fig. 5, the combination of ART plus iron(II)–glycine sulfate inhibited clonogenic cell growth more than ART alone. This result is in accordance with the observations obtained by the growth inhibition assay.

In a further control experiment, we attempted to demonstrate the high degree of iron(II) sensitivity of the cells used in this study and to allow quantitative comparison of growth-inhibitory effects between artemisinins and a reference drug. We used DOX as a reference drug for our experiments, as iron is known to increase the cytotoxic activity of DOX [28]. Treatment of CCRF-CEM cells with DOX plus iron(II)–glycine sulfate resulted in a 1.4-fold reduced cell growth compared with treatment with DOX alone (Fig. 6a). Comparable results were obtained for DOX plus transferrin, which was 2.4-fold more cytotoxic (based on the IC_{50} values) than DOX alone (Fig. 6b).

In the next set of experiments, we investigated the cellular and molecular mechanisms by which ferrous iron increases the cytotoxicity of artemisinins. As cytotoxic compounds frequently induce cell cycle perturbations and apoptosis, we measured cellular DNA content using

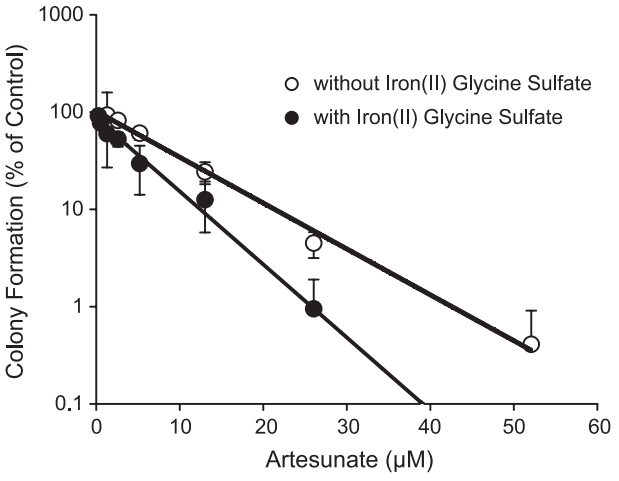

Fig. 5. Inhibition of colony formation of human U373 astrocytoma cells by ART with or without addition of 10 μg/ml iron(II)–glycine sulfate (Ferrosanol) as measured in clonogenic cell survival assays. Mean values of three independent experiments are shown.

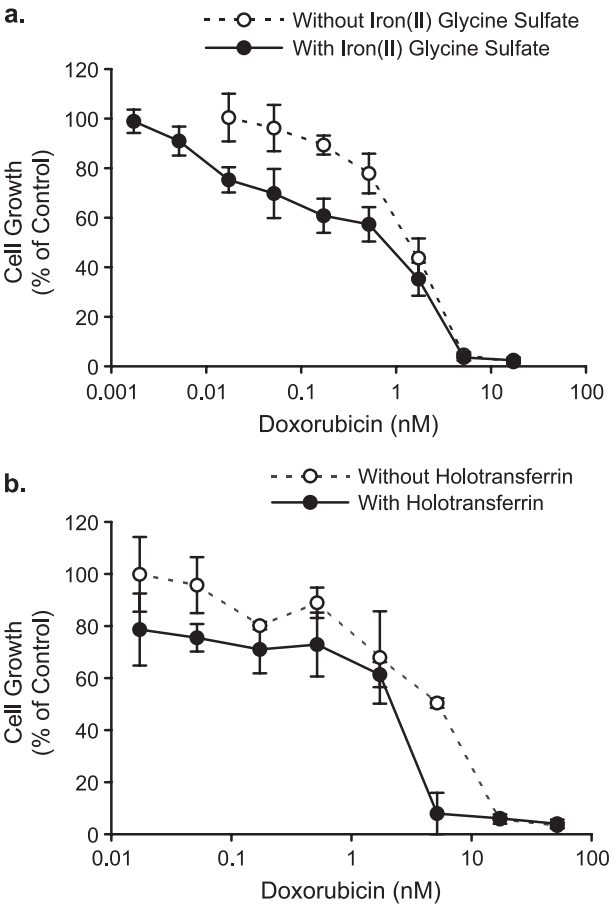

Fig. 6. Growth inhibition of leukemic CCRF-CEM cells by doxorubicin with or without addition of (a)10 μg/ml iron(II)–glycine sulfate (Ferrosanol) or (b) 1 mg/ml transferrin.

propidium iodide staining and flow cytometry. The results in Fig. 7a show that exposure to 10 μg/ml iron(II)–glycine sulfate neither affected the distribution of CCRF-CEM cells in the different cell cycle phases (G_0/G_1, S, G_2/M) nor induced apoptosis (sub-G_0/G_1) within the period investigated (24–96 h). After application of 3 μg/ml ART in combination with iron(II)–glycine sulfate, we did not observe cell cycle perturbations (Fig. 7b). However, the fraction of apoptotic cells increased constantly up to 33% after 96 h (Fig. 7b). Simultaneously, the percentage of G_0/G_1 cells decreased, indicating that the major fraction of apoptotic cells was recruited from this cell cycle phase. Representative DNA histograms of untreated contol CCRF-CEM cells and of cells treated with iron(II)–glycine sulfate alone, ART alone, or a combination of both drugs are shown in Fig. 7c. Cells treated with 50% DMSO, which served as the solvent for ART, did not show an increased rate of apoptotic cells compared with untreated controls (data not shown).

Furthermore, we asked whether the tumor suppressor p53 might play a role in the cytotoxic action of ART. As CCRF-CEM and U373 cells used in the experiments

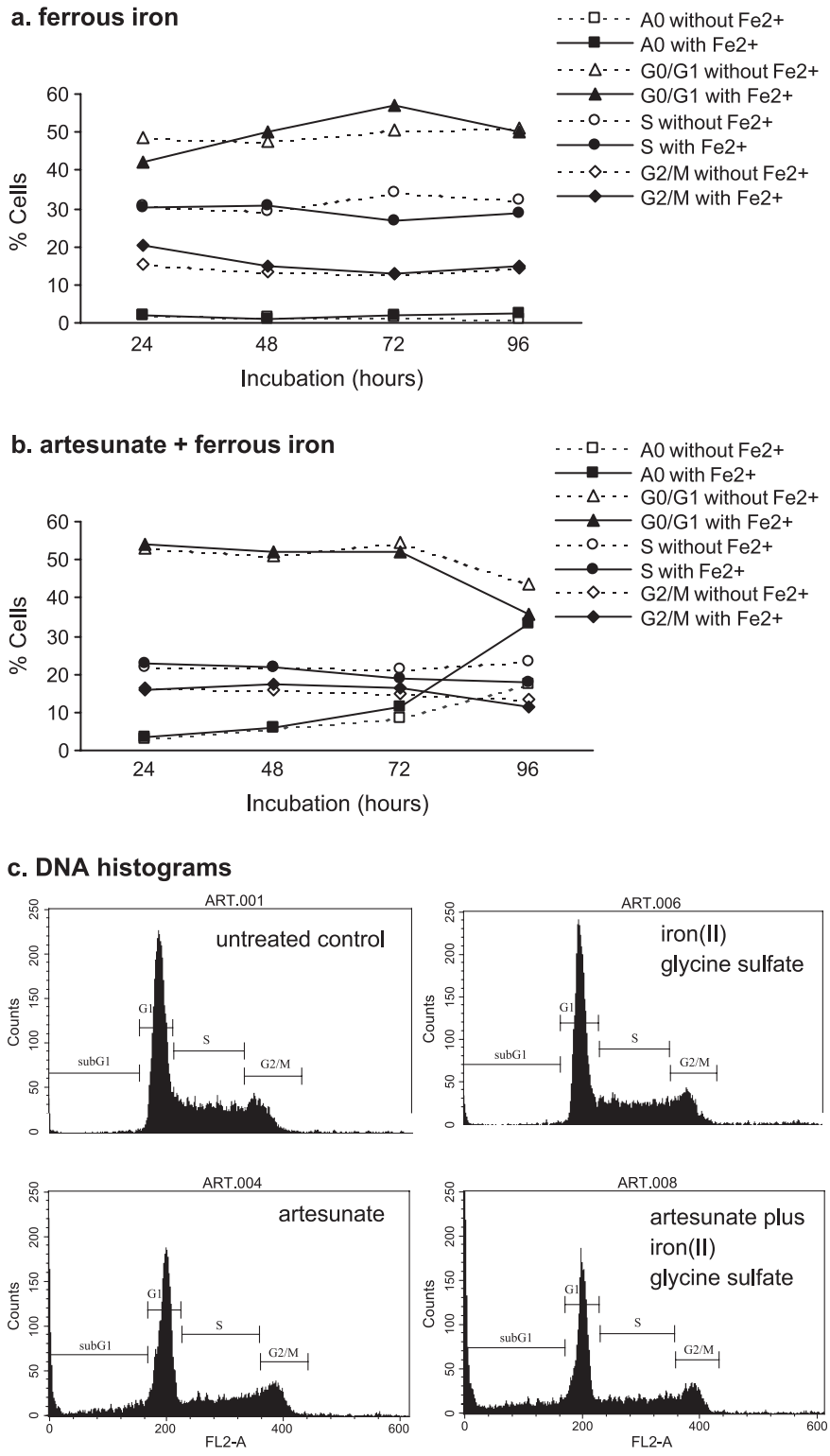

Fig. 7. Influence of ART and ferrous iron on cell cycle distribution and induction of apoptosis in CCRF-CEM cells. Percentages of cells in the different cycle phases and of apoptotic cells after treatment (a) with 10 μg/ml iron(II)–glycine sulfate (Ferrosanol) alone and (b) with 3 μg/ml ART plus iron(II)–glycine sulfate (Ferrosanol). (c) Representative histograms. Cells were harvested and analyzed by flow cytometry at the indicated time points.

above both carry mutated p53 genes [29,30], they are not suited to address this question. Therefore, we analyzed p53 wild-type TK6 and p53 mutated WTK1 cells. The growth inhibition curves in Fig. 8 show that both cell lines responded similarly to ART, indicating that p53 mutational status does not influence cellular response to ART.

Then we analyzed, whether the ART effects measured under iron(II) co-treatment resulted from the presence of a specific transferrin–transferrin receptor (TfR) pathway in these cells. As transferrin and free iron (after binding to transferrin) enter the cell via TfR, we treated U373 cells with anti-TfR monoclonal antibody RVS10, which competes with transferrin for binding to TfR (CD71) and which blocks the TfR internalization pathway [22]. Pretreatment of U373 cells for 2 h with this antibody reduced the growth-inhibitory effect of ART plus iron(II)–glycine sulfate or ART plus transferrin to the level of ART treatment alone (Fig. 9b). For this experiment, antibody RVS10 was used at a concentration of 30 ng/ml. This concentration caused only minimal cytotoxicity in U373 cells, while higher antibody concentrations (100–300 ng/ml) inhibited cell proliferation (Fig. 9a).

Next, TfR expression in U373 and CCRF-CEM tumor cells was compared with that of peripheral mononuclear blood cells (PMBC) of four healthy donors. As can be seen in Figs. 10a and b, 95% of CCRF-CEM cells and 48% of U373 cells expressed TfR. In contrast, the TfR expression in PMBC was 1.3% (Fig. 10c), 0.4%, 0.6%, and 1.1%, respectively. In another experiment, we determined the expression of TfR mRNA by RT-PCR as described elsewhere (primer receptor sense 5′ -TAT CTG CTA TGG GAC TAT TGC-3′ , primer receptor antisense 5′ -CGC CAC ATA ACC CCC AGG ATT-3′ [31]). We clearly detected specific signals for the expression of TfR in total RNA prepared from CCRF-CEM and U373 cells. In this semiquantitative procedure,

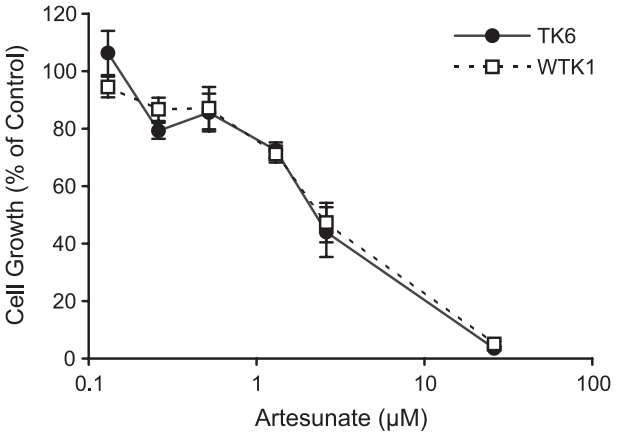

Fig. 8. Growth inhibition of human TK6 (wild-type p53) and WTK1 (mutated p53) cells by ART. Means and SEM of two independent experiments with each fourfold determination.

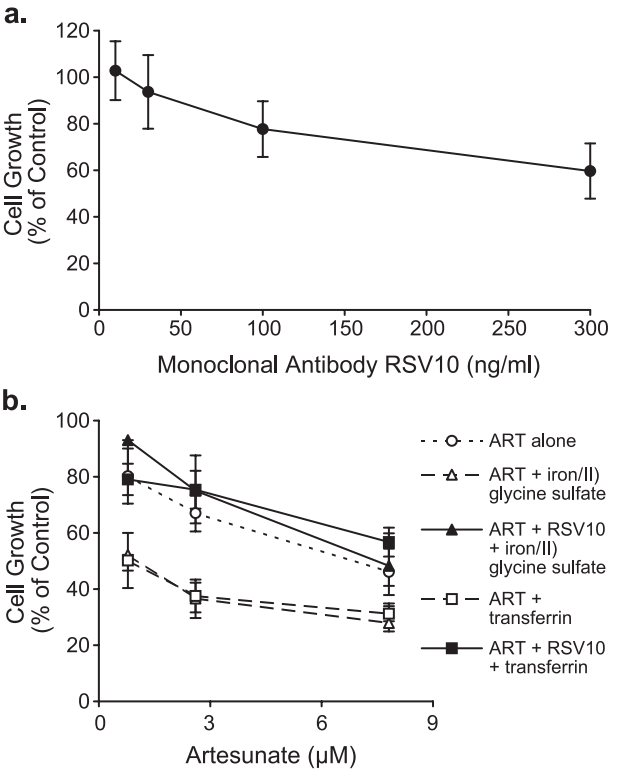

Fig. 9. Effect of anti-TfR antibody RVS10 on iron-mediated growth inhibition of U373 astrocytoma cells by ART. (a) Growth inhibition by RVS10 alone. (b) Growth inhibition by ART with and without 10 μg/ml iron(II)–glycine sulfate or 1 mg/ml transferrin after pretreatment with 30 ng/ml RVS10 for 2 h.

we could easily distinguish between the relatively high level of expression of TfR in these cells and the much lower level of signals in samples from PMBC tested as a control (data not shown).

Although the elevated levels of TfR in tumor cells point to the strengthened role of this pathway compared with that of normal cells, there remains the question of why the distinct levels of TfR expression differing between CCRF-CEM and U373 cells (Fig. 10) did not correlate quantitatively with the sensitivity of these cells to co-treatment with iron(II) (Table 1). To search for additional factors involved in the synergism between iron(II) and ART, e.g., iron(II)-responsive genes, we investigated the relationships between the baseline mRNA expression levels of 12 genes involved in cellular iron uptake and metabolism in 60 cell lines of different origin (NCI) and IC_{50} values for ART and ARS. These genes were aconitase 1 (soluble), aconitase 2 (mitochondrial), ceruloplasmin, ferredoxin 1, ferritin heavy and light polypeptides, ferrochelatase, iron-responsive element protein 2, lactoferrin, transferrin, TfR, and zinc/iron regulated transporter-like RNA. For comparison, we also correlated mRNA expression to the IC_{50} values for other artemisinins (arteether, artemether, artemisetene, artea-

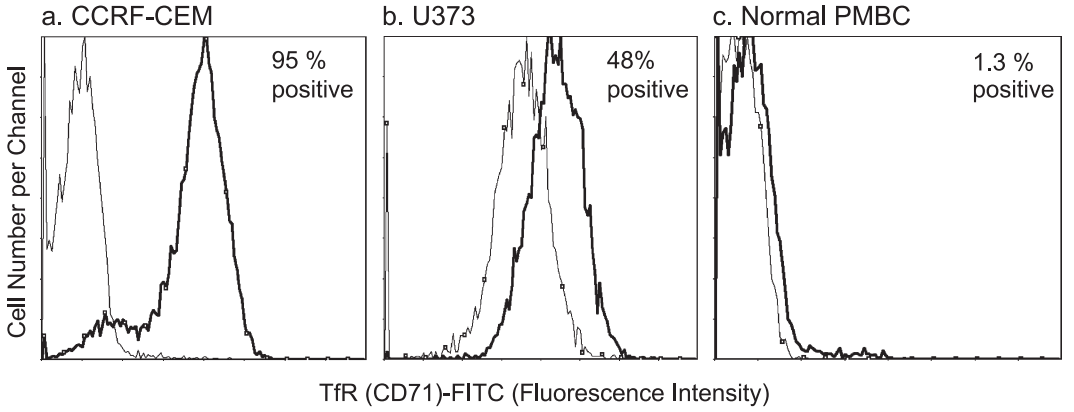

Fig. 10. Determination of transferrin receptor (CD71) expression by flow cytometry using FITC-labeled antibody Ber-T9 (bold line) and corresponding negative control (thin line) in (a) human CCRF-CEM leukemia cells, (b) human U373 astrocytoma cells, and (c) human peripheral blood mononuclear cells of a healthy donor.

nuine B, and dihydroartemisinyl ester stereoisomers 1 and 2) as well as to the IC_{50} values for DOX and DNR, the activity of which is influenced by iron, and for cisplatin (DDP) whose action is independent of the presence of iron. The mRNA expression as determined by microarray analyses has been reported [24] and deposited in the N.C.I.'s database (http://dtp.nci.nih.gov). The IC_{50} values for ART in 60 cell lines of the N.C.I. have been reported by us [13], while the IC_{50} values for the other ARS derivatives as well as for DOX, DNR, and DDP have also been deposited in the N.C.I.'s database.

Table 2. Correlation of Constitutive mRNA Expression of Genes Involved in Cellular Iron Metabolism and Response with IC_{50} Values for Artemisinin Derivatives and Established Cytostatic Drugs of 60 NCI Cell Lines by Kendall's τ Test and False Discovery Rate (FDR) Calculation

	CP	FDX1	ZIRTL	ACO2
ART[a]	0.00224	0.03106*	n.s.[b]	0.00373
ARS	0.02437*	n.s.	0.01447*	0.00082
ARE	n.s.	0.01727*	0.01152	0.00014
ARM	n.s.	n.s.	0.00334	0.00013
ARAB	n.s.	n.s.	n.s.	0.02266*
ARTEMIS	0.0077	n.s.	0.02680*	0.02162*
ARTEST 1	0.00534	0.02482*	0.04462*	0.00004
ARTEST 2	0.00165	n.s.	0.02902*	0.00011
DOX	n.s.	0.03228*	0.03856*	0.08221*
DNR	n.s.	0.01204	0.02786*	0.02969*
DDP	n.s.	0.02637*	n.s.	n.s.

[a] ART, artesunate; ARS, artemisinin; ARE, arteether; ARM, artemether; ARAB; arteanuine B; ARTEMIS, artemisetene; ARTEST 1, dihydroartemisinyl ester stereoisomer 1; ARTEST 2, dihydroartemisinyl ester stereoisomer 2; DOX, doxorubicin, DNR, daunorubicin; DDP, cisplatin; CP, ceruloplasmin (ferroxidase; GenBank Accession No. H86554); FDX1, ferredoxin 1 (GenBank Accession No. N89718); ZIRTL, zinc/iron transporter-like (GenBank Accession No. AA044290); ACO2, aconitase 2, mitochondrial (GenBank Accession No. W44340).

[b] n.s., not significant ($p > .05$).

* Correlations with $p > .01447$ were identified as having an error probability of ≥5% by FDR calculations.

mRNA expression of ACO2, CP, FDX1, and ZIRTL correlated with the IC_{50} values for at least five drugs at $p < .05$ (Table 2). These correlations obtained with Kendall's τ test were then subjected to false discovery rate (FDR) calculations. p values obtained by correlation tests such as Kendall's τ test may be errorprone if large data sets are analyzed. This is due to the well-known fact that the probability of type I errors increases as the number of correlation tests increases [25,26]. If mathematical correction systems for the determination of FDR are applied, the rate of p values that reflect false-positive correlations (e.g., significant correlations by chance) can be diminished. Therefore, we applied FDR calculations to our data set and adjusted the significance level to .05. We revealed an α value of .01447, which means that all correlations with $p > .01447$ have an error probability of 5% or above. According to this FDR calculation, the mRNA expression of ACO2, CP, and ZIRTL genes correlated with the IC_{50} values for six, four, and two of the eight artemisinins assayed, respectively, with error rates below this threshold. The FDR calculations revealed that the correlations between FDX1 and the IC_{50} values of the different drugs are errorprone with a probability of ≥5%. Application of the FDR approach to DOX, DNR, and DDP as control drugs showed that only the correlation between FDX1 mRNA expression and IC_{50} valued for DNR fulfilled the criterion of having an error probability of <5%.

DISCUSSION

In the present investigation, we analyzed the role of free and transferrin-bound ferrous iron on the growth-inhibitory activity of free artesunate (ART), artesunate microencapsulated in maltosyl-β-cyclodextrin (ART-MCD), and artemisinin (ARS) on human CCRF-CEM leukemia

and human U373 astrocytoma cells. We observed that cell growth was inhibited in a dose-dependent manner by these artemisinins. It has been shown here for the first time that ART-MCD is also cytotoxic to cancer cells. While ART-MCD did not reveal increased cytotoxicity compared with free ART toward CCRF-CEM leukemia cells, its activity was 3.5- to 6.6-fold more active in U373 astrocytoma cells. Different cyclodextrins including MCD have recently been used to improve the water solubility of artemisinins and, thereby, their bioavailability in malaria patients [32–34]. Our results are a first clue that ART-MCD may be more active against adherently growing cells of solid tumors than free ART. Whether the improved features of ART-MCD are also advantageous for in vivo applications still has to be demonstrated. ARS was less active against CCRF-CEM leukemia cells as compared with ART and ART-MCD. This is in accord with our earlier observations [21]. In U373 astrocytoma cells, the activity of ART was similar to that of ARS, pointing to cell type-specific differences in the activity of the various artemisinins.

In contrast to these findings with CCRF-CEM and U373 cells, it seems important to stress that exposure of nontumor cells to ART produces no or only minimal cytotoxicity. In a previous study, we showed that growth of primary human fibroblasts is almost unaffected by ART concentrations up to 100 μM [16]. Even more important in this respect is the recent finding that normal human breast cells do not or merely respond to treatment with transferrin plus dihydroartemisinin (the active metabolite of ART), whereas growth of breast cancer cells is strictly inhibited [14].

Interestingly, the redox properties of iron may also explain why high cellular iron content makes tumor cells sensitive to oxidative stress induced by H_2O_2, whereas H_2O_2-resistant cells have lower amounts of iron [35]. This is in accord with both the results of the present study showing that iron sensitized tumor cells to artemisinins and with previous results showing that H_2O_2-resistant cells exhibit cross-resistance to ART [36], as both H_2O_2 and ART produce reactive oxygen species.

The addition of iron(II)–glycine sulfate or transferrin increased growth inhibition induced by the artemisinins alone in the range 1.5- to 10.3-fold. In previous investigations, ferrous iron has been described as a determinant of the activity of antimalarial artemisinins [10]. The growth inhibitory effect of DOX was also increased by ferrous iron. It is noteworthy that ART, ART-MCD, and ARS were all active even without exogenously supplied iron and that the growth inhibition of CCRF-CEM cells induced by ART, ART-MCD, or ARS is not strictly dependent on the availability of exogenous iron. DOX is a standard anticancer agent, and was used as control drug in this study. The hydroxynaphthoquinone moiety of DOX represents an iron-chelating structure [28]. The DOX–iron complex catalyzes the generation of reactive oxygen species and hydroxyl radicals. This result well fits the data obtained with ART, ART-MCD, and ARS. Our finding that iron(II)–glycine sulfate increased the action of artemisinins is interesting, as we used a clinically approved formulation, Ferrosanol. Hence, artemisinins might be easily applied in combination with Ferrosanol in a clinical setting.

A recent investigation reported that transferrin increased the growth-inhibitory effect of ARS on HTB125 breast cancer cells, while ARS alone did not inhibit the growth of these cells [14]. Our results are in accord with respect to the increase in growth inhibition produced by transferrin. On the other hand, we found that ARS alone had growth-inhibitory activity against CCRF-CEM leukemia and U373 astrocytoma cells. Again, these differences may be cell type-specific. The fact that transferrin increased the action of artemisinins raised our interest, as it may be used to develop new treatment approaches. The absorption of iron in growing cells and tissues is increased, and there is a relationship between the uptake of transferrin and the rate of tumor proliferation [37]. Cellular iron uptake and internalization are mediated by binding of transferrin–iron complexes to TfR (CD71) expressed on the cell surface membrane and subsequent endocytosis. TfR expression in normal tissues is limited, e.g., to basal epidermis, endocrine pancreas, hepatocytes, Kupffer cells, testis, and pituitary, whereas most other tissues are TfR-negative [38]. In contrast, TfR is expressed in much larger amounts in proliferating and malignant cells [39–41] and it is widely distributed among clinical tumors [38]. In the present analysis, we showed that TfR expression was much higher in CCRF-CEM and U373 tumor cells than in peripheral mononuclear blood cells of four healthy donors. The fact that transferrin in combination with ART, ART-MCD, or ARS decreased cell growth of CCRF-CEM cells raises the attractive possibility that tumors that express more TfR than normal cells are preferentially affected by the ART/transferrin combination treatment. Further detailed investigations are required to explore the full potential of this hypothesis.

ART and ART plus ferrous iron induced apoptosis in CCRF-CEM cells. This confirms earlier results in KG-1a cells [12] and WEHI7.2 cells stably transfected with BCL-2 which were more resistant to ART than mock vector-transfected control cells [36]. The finding that ART alone and in combination with ferrous iron did not provoke cell cycle perturbations is also in accord with earlier results with KG-1a cells [12]. p53 wild-type TK6 and p53 mutated WTK1 cells were similarly sensitive to ART. This is a clue that p53-independent mechanisms are operative in response to ART. This may explain the lack

of cell cycle arrest after ART treatment. Our results are in accord with p53−/− knockout and p53+/+ knock-in HCT-116 human colon carcinoma cells that were similarly sensitive to ART [15].

Based on the results with the anti-TfR antibody RVS10, which neutralizes transferrin entry into cells, we hypothesize that TfR causes the iron-mediated increase in cytotoxicity of artemisinins. In a recent investigation, this antibody was shown to block apoptosis induced by insulin-like growth factor binding protein 3 [22]. This protein is a known apoptosis-inducing molecule that binds to transferrin to enter cells. The results of that investigation and the data from the present study speak for an inhibitory function of this antibody to compete for transferrin binding an internalization via TfR.

The observation that the TfR expression of CCRF-CEM and U373 cells did not correlate with the degree of increase in cytotoxicity caused by iron(II)–glycine sulfate or transferrin was surprising. To prove this result in another set of cell lines, we correlated the IC_{50} values for different artemisinin derivatives and the mRNA expression values determined by microarray analyses for TfR that are deposited in the database of the Developmental Therapeutics Program of the National Cancer Institute (http://dtp.nci.nih.gov). In addition, expression levels of other genes involved in iron uptake, storage, and metabolism were analyzed. TfR expression did not correlate with the IC_{50} values of the artemisinins, indicating that other iron-related genes may also be involved in the cellular response to artemisinins. Instead, our analysis pointed to the ACO2 and CP genes, the expression levels of which correlated with the IC_{50} values for several artemisinins, DOX, and DNR, but not DDP. Aconitases catalyze the conversion of citrate to isocitrate in the tricarboxylic acid cycle either in the cytoplasm (ACO1) or in the mitochondria (ACO2) [42]. Iron activates ACO1 and ACO2 by iron–sulfur complexes [43] and regulates the expression of ACO2, making aconitases important for iron homeostasis and energy production. The ACO2 gene is likely to be an essential enzyme [42]. It has been hypothesized that citrate triggers expression of the transferrin receptor in the presence of low iron stores in the mitochondria to increase cellular iron uptake [44]. CP (also known as ferroxidase or iron(II):oxygen oxidoreductase) is important for iron homeostasis. The enzyme is involved in the peroxidation of Fe(II) transferrin to form Fe(III) transferrin. CP, together with transferrin and its receptor, plays a role in the internalization of iron and cellular proliferation and also in carcinogenesis [45,46].

These results point to mitochondrial aconitase and ceruloplasmin (ferroxidase) as important targets of the cellular response to artemisinins when administered with ferrous iron. The validation of whether these candidate genes are mechanistically relevant for cellular response to artemisinins and ferrous iron deserves thorough investigation.

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ABBREVIATIONS

ACO2 — mitochondrial aconitase
ARS — artemisinin
ART — artesunate
ART-MCD — artesunate maltosyl-β-cyclodextrin
CP — ceruloplasmin (ferroxidase)
DDP — cisplatin
DNR — daunorubicin
DOX — doxorubicin
FDR — false discovery rate
FDX1 — ferredoxin 1
TfR — transferrin receptor (CD71)
ZIRTL — zinc/iron regulated transporter-like RNA