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Anti-cancer effects of artesunate in a panel of chemoresistant neuroblastoma cell lines

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ABSTRACT

Artemisinin derivatives are well-tolerated anti-malaria drugs that also exert anti-cancer activity. Here, we investigated artemisinin and its derivatives dihydroartemisinin and artesunate in a panel of chemosensitive and chemoresistant human neuroblastoma cells as well as in primary neuroblastoma cultures. Only dihydroartemisinin and artesunate affected neuroblastoma cell viability with artesunate being more active. Artesunate-induced apoptosis and reactive oxygen species in neuroblastoma cells. Of 16 cell lines and two primary cultures, only UKF-NB-3⁺CDDP¹⁰⁰⁰ showed low sensitivity to artesunate. Characteristic gene expression signatures based on a previous analysis of artesunate resistance in the NCI60 cell line panel clearly separated UKF-NB-3⁺CDDP¹⁰⁰⁰ from the other cell lines. L-Buthionine-S,R-sulfoximine, an inhibitor of GCL (glutamate–cysteine ligase), resensitized in part UKF-NB-3⁺CDDP¹⁰⁰⁰ cells to artesunate. This finding together with bioinformatic analysis of expression of genes involved in glutathione metabolism showed that this pathway is involved in artesunate resistance. These data indicate that neuroblastoma represents an artesunate-sensitive cancer entity and that artesunate is also effective in chemoresistant neuroblastoma cells.

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1. Introduction

Neuroblastoma is the most frequent extracranial solid tumour of childhood. About half of all neuroblastoma patients are diagnosed with high-risk disease characterised by overall survival rates below 40% despite intensive multimodal treatment [1]. Therapy failure is basically caused by acquired chemoresistance [2].

Artemisinin derivatives including artemisinin, dihydroartemisinin, and artesunate are used as anti-malaria drugs, especially as constituents of drug combinations composed to counteract drug resistance in malaria [3]. Moreover, artemisinin derivatives were shown to exert anti-bacterial, antiviral, and anti-cancer effects [3]. Studies indicated that artemisinin derivatives are active against

cells from a broad spectrum of cancer entities [3–11]. Notably, artemisinin derivatives were described to be effective in many drug-resistant cancer cell lines [3]. Prominent resistance mechanisms like high expression of ATP binding cassette (ABC) transporters or p53 loss-of-function mutations did not substantially affect cancer cell sensitivity to artesunate [3,12,13].

Artemisinin derivatives have not been studied for anti-cancer activity in human neuroblastoma cells, yet. To investigate the effects of potential anti-cancer drugs on neuroblastoma cells, we established a panel of chemoresistant neuroblastoma cell lines by adaptation of chemosensitive neuroblastoma to increasing concentrations of cytotoxic drugs [14–20]. Here, we investigated the influence of artemisinin and its derivatives dihydroartemisinin and artesunate on chemosensitive or chemoresistant neuroblastoma cell lines and on primary neuroblastoma cultures. Moreover, gene expression signatures that correlate with artesunate sensitivity of neuroblastoma cells were established.

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2. Materials and methods

2.1. Cells

The cell lines UKF-NB-3 and UKF-NB-6 were isolated from bone marrow metastases of N-myc-amplified stage 4 neuroblastoma patients [15,20]. IMR-32 cells were obtained from ATCC (Manassas, VA, USA). The parental UKF-NB-3, UKF-NB-6 or IMR-32 cells are chemosensitive (no P-gp expression, wild-type p53) [15,17,20]. Cells were adapted to growth in the presence of vincristine (VCR, obtained from GRY Pharma GmbH, Kirchzarten, Germany), doxorubicin (DOX, obtained from medac Gesellschaft für klinische Spezialpräparate mbH, Hamburg, Germany), cisplatin (CDDP, obtained from GRY Pharma GmbH, Kirchzarten, Germany), melphalan (MEL, obtained from GlaxoSmithKline GmbH & Co. KG, Munich, Germany), etoposide (ETO, obtained from GRY Pharma GmbH, Kirchzarten, Germany), or topotecan (TOP, obtained from GlaxoSmithKline GmbH & Co. KG, Munich, Germany) by described methods and named following the published nomenclature [14,15], i.e., UKF-NB-3^{VCR}¹⁰ means UKF-NB-3 adapted to vincristine 10 ng/ml, UKF-NB-3^{DOX}²⁰ means UKF-NB-3 adapted to doxorubicin 20 ng/ml, UKF-NB-3^{CDDP}¹⁰⁰⁰ means UKF-NB-3 adapted to cisplatin 1000 ng/ml. All cell lines were grown in IMDM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C. All cell culture materials were purchased from PromoCell GmbH (Heidelberg, Germany).

Fresh neuroblastoma cells (MYCN amplified) were isolated from the bone marrow aspirate of two patients with metastasised INSS stage 4 neuroblastoma following informed consent. Primary cells were cultivated in IMDM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C.

2.2. Viability assay

Viability of cells from cancer cell lines was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described before [16].

Viability of primary neuroblastoma cells was examined by the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Mannheim, Germany) following the manufacturer's instructions.

2.3. Apoptosis

Activated caspase-3 was detected by immune staining. The primary antibody was: caspase-3 (active) (R&D Systems, Wiesbaden, Germany). Biotin-conjugated secondary monoclonal antibodies were used and visualisation was performed with streptavidin–peroxidase complex with AEC (3-amino-9-ethylcarbazole, purchased from Sigma–Aldrich Chemie GmbH, Munich, Germany) as a substrate.

In addition to staining for activated caspase-3, apoptotic cells were detected as the cells with fractional DNA content (“sub-G1” cell subpopulation). Cells were fixed with 70% ethanol for 2 h at –20 °C. The cellular DNA was stained using propidium iodide (20 µg/ml, purchased from Sigma–Aldrich Chemie GmbH, Munich, Germany) and analysed by flow cytometry (FacsCalibur, BD Biosciences, Heidelberg, Germany).

2.4. Determination of cellular oxidative stress

Reactive oxygen species (ROS) were detected in artesunate-treated cells using the Image-iT LIVE Green Reactive Oxygen Species Kit (Molecular Probes, distributed by Invitrogen, Karlsruhe, Germany).

2.5. Gene expression analysis

Gene expression analysis using AB1700 Human Genome Survey Microarray V2.0 chips (Applied Biosystems, Darmstadt, Germany) was performed by IMGM laboratories (Martinsried, Germany) for the cell lines UKF-NB-3, UKF-NB-3^{VCR}¹⁰, UKF-NB-3^{CDDP}¹⁰⁰⁰, UKF-NB-6, UKF-NB-6^{VCR}¹⁰, or UKF-NB-6^{CDDP}¹⁰⁰⁰. mRNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Experiments were performed in triplicates.

Expression data were processed using the R/bioconductor package ‘ABarray’ (<http://www.r-project.org/>; <http://www.bioconductor.org/>) with default parameters (SN threshold ≥ 3 , %detect samples: 0.5). This included quality control, quantile normalisation [21], and filtering of unspecific hybridisation.

For every microarray experiment, the expression pattern of 50 randomly chosen genes was verified by quantitative real-time PCR resulting in confirmation of expression of >80% of investigated genes (data not shown).

Cluster analysis was performed using Euclidean distance and complete linkage and was visualised as heatmap. All steps were processed in R (www.r-project.org; Kendall's τ -test, clustering and heatmaps were based on the package stats; correction for multiple testing was performed with multitest).

3. Results

3.1. Sensitivity of neuroblastoma cells to artemisinin derivatives

The IC₅₀-values for the effects of artemisinin, dihydroartemisinin, or artesunate on the viability of the investigated neuroblastoma cell lines are shown in Table 1. Artemisinin concentrations of 50 µM failed to reduce the viability of neuroblastoma cells by 50%. The IC₅₀-values for dihydroartemisinin and artesunate ranged from 1.43 to 11.95 µM whereas IC₅₀-values for most cell lines were below 5 µM. The only exception was the cell line UKF-NB-3^{CDDP}¹⁰⁰⁰ for which the IC₅₀-values were 24.9 ± 0.28 µM for dihydroartemisinin and 29.7 ± 0.35 µM for artesunate.

In addition to testing in neuroblastoma cell lines, artemisinin, dihydroartemisinin, and artesunate were investigated in primary neuroblastoma cells isolated from the bone marrow of two patients with metastasised INSS stage 4 neuroblastoma (Table 2). IC₅₀-values for dihydroartemisinin and artesunate were below 10 µM and IC₅₀-values for artemisinin were >50 µM.

Since dihydroartemisinin and artesunate exerted similar anti-neuroblastoma effects and the IC₅₀-values for artesunate were in general lower, subsequent experiments were performed with artesunate.

3.2. Artesunate induces apoptosis in neuroblastoma cell lines

To investigate if artesunate induces apoptosis in neuroblastoma cells, the parental chemosensitive UKF-NB-3 cell line and its chemoresistant sub-lines were investigated for caspase-3 activation and cells in sub-G1 phase. Artesunate 10 µM treatment for 24 h resulted in a significant increase of cells positively stained for activated caspase-3 (Fig. 1A). Forty-eight hour-treatment of neuroblastoma cells with artesunate 10 µM significantly enhanced the number of cells in sub-G1 phase (Fig. 1B).

3.3. Determination of cross-resistance profiles in the cell line UKF-NB-3 and its chemoresistant sub-lines

The cytotoxic mechanism of action of artesunate was suggested to differ from the mechanisms of established anti-cancer drugs

Table 1

Anti-neuroblastoma effects of the artemisinin derivatives artemisinin, dihydroartemisinin, and artesunate in a panel of parental chemosensitive cell lines and their chemoresistant sub-lines as indicated by the concentrations that cause 50% reduction in cell viability (IC_{50}).

Cell line	IC_{50} (μ M)		
	Artemisinin	Dihydroartemisinin	Artesunate
UKF-NB-3	>50	4.50 \pm 0.30	2.69 \pm 0.10
UKF-NB-3 ^{VCR} ¹⁰	>50	2.09 \pm 0.20	1.43 \pm 0.12
UKF-NB-3 ^{DOX} ²⁰	>50	3.18 \pm 0.50	2.24 \pm 0.06
UKF-NB-3 ^{CDDP} ¹⁰⁰⁰	>50	24.9 \pm 0.28	29.70 \pm 0.35
UKF-NB-3 ^{TOP} ¹⁵	>50	2.24 \pm 0.08	1.93 \pm 0.20
UKF-NB-3 ^{MEL} ²⁰⁰⁰	>50	2.85 \pm 0.08	1.62 \pm 0.40
UKF-NB-3 ^{ETO} ¹⁰⁰	>50	3.90 \pm 0.13	2.53 \pm 0.50
UKF-NB-6	>50	6.24 \pm 0.19	3.54 \pm 0.42
UKF-NB-6 ^{VCR} ¹⁰	>50	11.95 \pm 0.20	7.13 \pm 0.17
UKF-NB-6 ^{DOX} ²⁰	>50	7.30 \pm 0.14	9.90 \pm 1.20
UKF-NB-6 ^{CDDP} ¹⁰⁰⁰	>50	6.67 \pm 0.10	2.27 \pm 0.06
UKF-NB-6 ^{TOP} ¹⁵	>50	5.35 \pm 0.48	3.72 \pm 0.20
IMR-32	>50	3.15 \pm 0.16	2.69 \pm 0.18
IMR-32 ^{DOX} ²⁰	>50	3.69 \pm 0.13	3.80 \pm 0.90
IMR-32 ^{CDDP} ¹⁰⁰⁰	>50	4.03 \pm 0.23	3.05 \pm 0.37
IMR-32 ^{MEL} ²⁰⁰⁰	>50	2.46 \pm 0.15	2.40 \pm 0.23

[3,9,10]. Cross-resistance profiles were determined in a cell line panel consisting of the parental chemosensitive neuroblastoma cell line UKF-NB-3 and its sub-lines adapted to the cytotoxic drugs vincristine (UKF-NB-3^{VCR}¹⁰), doxorubicin (UKF-NB-3^{DOX}²⁰), cisplatin (UKF-NB-3^{CDDP}¹⁰⁰⁰), topotecan (UKF-NB-3^{TOP}¹⁵), melphalan (UKF-NB-3^{MEL}²⁰⁰⁰), or etoposide (UKF-NB-3^{ETO}¹⁰⁰). IC_{50} -values were determined for each drug used for adaptation (Table 3). Moreover, relative cellular resistances (IC_{50} chemoresistant UKF-NB-3 sub-line/ IC_{50} UKF-NB-3) indicating resistance/sensitivity of chemoresistant sub-lines to each drug relative to parental chemosensitive UKF-NB-3 cells were calculated for each cytotoxic drug and artesunate (Table 3). In general, adaptation of UKF-NB-3 cells to one cytotoxic drug resulted in decreased sensitivity to the whole panel of anti-cancer drugs. In contrast, drug-resistant UKF-NB-3 sub-lines showed comparable or increased sensitivity to artesunate (with the exception of UKF-NB-3^{CDDP}¹⁰⁰⁰).

3.4. Investigation of neuroblastoma cells for gene expression signatures that predict cancer cell response to artesunate

Gene expression signatures that correlated with cancer cell sensitivity to artesunate had been established before by correlation of gene expression with IC_{50} levels of artesunate in 55 cell lines from the NCI60 panel using Kendall's τ -test (9, 10, 21, 22). From these signatures, we created a list of 94 genes for which expression has been shown to directly or inversely correlate to artesunate resistance in the cells from the NCI60 panel (Appendix Supplementary Table 1). Cluster analysis of the cell lines UKF-NB-3, UKF-NB-3^{VCR}¹⁰, UKF-NB-3^{CDDP}¹⁰⁰⁰, UKF-NB-6, UKF-NB-6^{VCR}¹⁰, and UKF-NB-6^{CDDP}¹⁰⁰⁰ for expression of these genes could clearly differentiate the artesunate-resistant UKF-NB-3^{CDDP}¹⁰⁰⁰ cell line from the other cell lines showing substantially higher artesunate sensitivity (Fig. 2).

Table 2

Anti-neuroblastoma effects of the artemisinin derivatives artemisinin, dihydroartemisinin, and artesunate in primary neuroblastoma cell cultures as indicated by the concentrations that cause 50% reduction in cell viability (IC_{50}).

	IC_{50} (μ M)	
	Patient 1	Patient 2
Artemisinin	>50	>50
Dihydroartemisinin	8.30 \pm 2.95	6.58 \pm 2.72
Artesunate	6.69 \pm 2.28	5.11 \pm 2.61

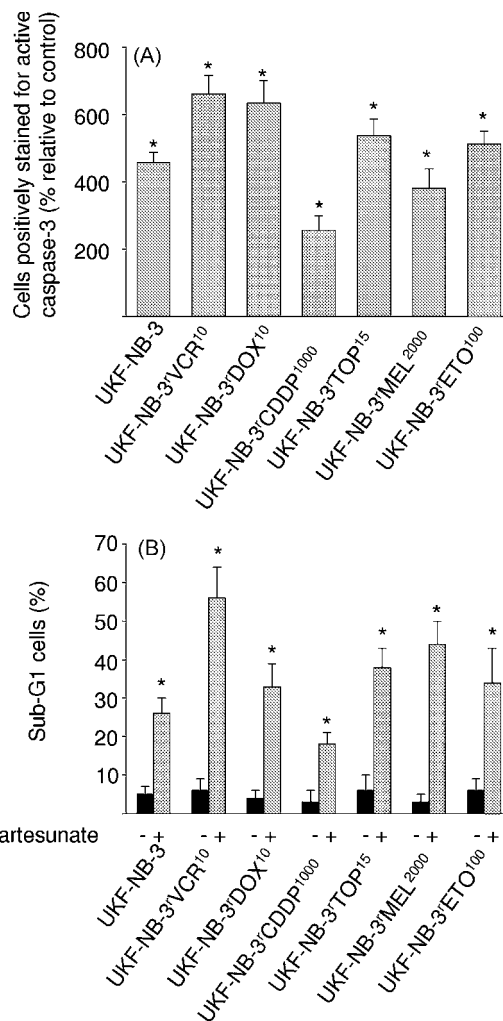


Fig. 1. Apoptosis induction by artesunate in neuroblastoma cells. (A) Activated caspase-3 positive cells after artesunate 10 μ M treatment for 24 h relative to untreated control. The artesunate-induced increase in active caspase-3 positive cells was statistically significant ($p < 0.05$) in all cell lines (determined by t -test). (B) Sub-G1 cell fraction in artesunate-treated (10 μ M, 48 h) and non-treated control cells. The artesunate-induced increase in sub-G1 cells was statistically significant ($p < 0.05$) in all cell lines (determined by t -test).

For eight genes of the 94 gene list, a statistically significant correlation between gene expression and the IC_{50} for artesunate was detected in our cell lines (Appendix Supplementary Table 1). For five of these eight genes, the correlation was found to be of the same type (direct or inverse) as determined in the previous works [9,10,22,23]. The expression of the remaining three genes was correlated to the artesunate IC_{50} in the opposite direction (Appendix Supplementary Table 1).

3.5. Sensitisation of UKF-NB-3^{CDDP}¹⁰⁰⁰ cells to artesunate by L-buthionine-S,R-sulfoximine (BSO)

L-Buthionine-S,R-sulfoximine (BSO) is a glutathione-depleting agent that inhibits GCL (glutamate-cysteine ligase, also known as γ -glutamylcysteine synthetase), which has been shown to sensitise artesunate-resistant cells to artesunate [22]. Moreover, GCL transfection decreased the cancer cell sensitivity to artesunate [10,22]. GCL catalyses the first and rate-limiting step in the cellular synthesis of glutathione. The enzyme is encoded by two different genes. *GCLC* encodes for the catalytic subunit, *GCLM* for a modulatory subunit. Only the gene product of *GCLC* is regarded to be essential for GCL function [24–29].

Table 3Drug sensitivity (IC_{50}) and relative resistances of the neuroblastoma cell line UKF-NB-3 and its chemoresistant sub-lines.

Cell line	Drug sensitivity (IC_{50} (ng/ml))/relative resistance (IC_{50} UKF-NB-3 sub-line/ IC_{50} UKF-NB-3)						
	Vincristin	Doxorubicin	Cisplatin	Topotecan	Melphalan	Etoposide	Artesunate
UKF-NB-3	0.24 ± 0.02/1	6.53 ± 1.20/1	93.7 ± 1.2/1	1.36 ± 0.56/1	144.5 ± 45.2/1	28.8 ± 3.3/1	2.69 ± 0.10/1
UKF-NB-3 ^r VCR ¹⁰	114.2 ± 11.0/475.8	99.3 ± 2.515.2	587.6 ± 46.56.3	2.84 ± 0.452.1	887.3 ± 14.86.1	392.3 ± 16.8/13.6	1.43 ± 0.12/0.5
UKF-NB-3 ^r DOX ²⁰	37.1 ± 0.18/154.6	105.2 ± 10.4/16.1	239.4 ± 32.5/2.6	2.08 ± 0.21/1.5	231.0 ± 16.4/1.6	241.0 ± 21.2/8.4	2.24 ± 0.060.8
UKF-NB-3 ^r CDDP ¹⁰⁰⁰	0.71 ± 0.02/3.0	21.2 ± 1.09/3.2	900.1 ± 29.7/9.6	3.47 ± 0.41/2.6	969.0 ± 17.4/6.7	67.9 ± 12.5/2.4	29.70 ± 0.35/11.0
UKF-NB-3 ^r TOP ¹⁵	0.92 ± 0.11/3.8	34.6 ± 0.82/5.3	507.0 ± 12.9/5.4	5.68 ± 1.36/4.2	606.7 ± 48.7/4.2	136.5 ± 36.6/4.7	1.93 ± 0.20/0.7
UKF-NB-3 ^r MEL ²⁰⁰⁰	0.69 ± 0.07/2.9	29.3 ± 0.54/4.5	596.5 ± 11.9/6.4	2.63 ± 0.61/1.9	628.6 ± 25.5/4.4	106.8 ± 14.4/3.7	1.62 ± 0.40/0.6
UKF-NB-3 ^r ETO ¹⁰⁰	0.57 ± 0.02/2.4	24.5 ± 1.24/3.8	123.8 ± 4.0/1.3	1.98 ± 0.28/1.5	224.1 ± 24.8/1.6	138.1 ± 15.4/4.8	2.53 ± 0.50/0.9

In UKF-NB-3^rCDDP¹⁰⁰⁰ cells *GCLC* expression was found to be significantly higher than in UKF-NB-3 cells while *GCLM* expression did not differ between these cell lines (Supplementary Table 2). Treatment of UKF-NB-3^rCDDP¹⁰⁰⁰ cells with a fixed BSO concentration (50 μ M) that did not affect cell viability (92.61 ± 6.77% relative to non-treated control) resulted in a 4.6-fold decrease of the IC_{50} for artesunate (IC_{50} with BSO: 6.48 ± 2.18 μ M; IC_{50} without BSO 29.70 ± 0.35 μ M). However, the IC_{50} -value remained clearly

(2.41-fold) higher than that detected in artesunate-treated UKF-NB-3 cells (2.69 ± 0.10 μ M).

Different glutathione-related enzymes had been shown to contribute to resistance to artesunate [22]. Hierarchical clustering of the expression of glutathione-related enzymes that had been suggested before to be correlated to artesunate sensitivity/resistance [22] (Table S2) distinguished between low artesunate-sensitive UKF-NB-3^rCDDP¹⁰⁰⁰ cells and the more sensitive cell lines (Fig. 3).

The anti-cancer mechanism of action of artesunate is suspected to include induction of oxidative stress by cleavage of its endoperoxide moiety catalysed by Fe^{2+} ions [3,30]. Since BSO partly sensitised UKF-NB-3^rCDDP¹⁰⁰⁰ cells to artesunate and the

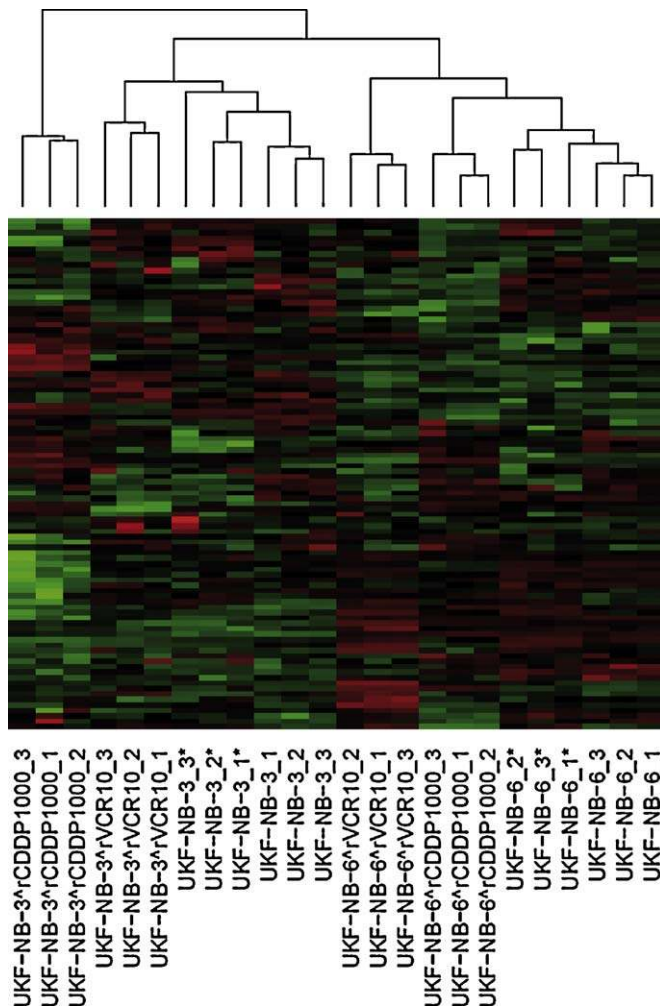


Fig. 2. Hierarchical cluster analysis and heatmap of expression of genes reported to be involved in cancer cell sensitivity to artesunate [9,10,21,22] in the cell lines UKF-NB-3 (two independent experiments were performed for the comparison of parental and chemoresistant cells; UKF-NB-3^r corresponds to gene expression profiles determined together with UKF-NB-3^rVCR¹⁰, UKF-NB-3 to gene expression profiles determined together with UKF-NB-3^rCDDP¹⁰⁰⁰ cells), UKF-NB-3^rVCR¹⁰, UKF-NB-3^rCDDP¹⁰⁰⁰, UKF-NB-6 (UKF-NB-6^r corresponds to gene expression profiles determined together with UKF-NB-6^rVCR¹⁰, UKF-NB-6 to gene expression profiles determined together with UKF-NB-6^rCDDP¹⁰⁰⁰ cells), UKF-NB-6^rVCR¹⁰, or UKF-NB-6^rCDDP¹⁰⁰⁰.

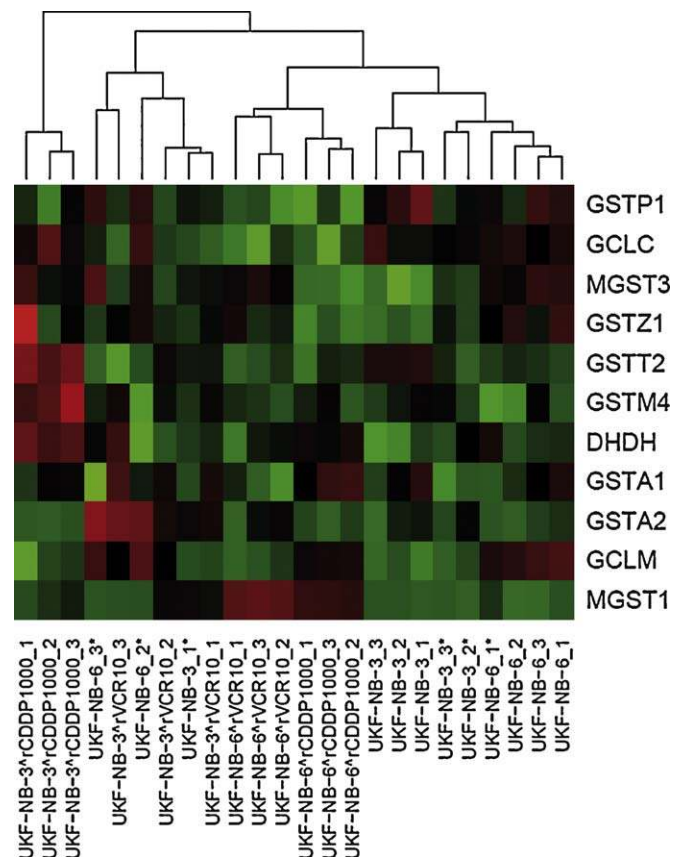


Fig. 3. Hierarchical cluster analysis and heatmap of expression of genes encoding for glutathione-related enzymes reported to be involved in cancer cell sensitivity to artesunate [21] in the cell lines UKF-NB-3 (two independent experiments were performed for the comparison of parental and chemoresistant cells; UKF-NB-3^r corresponds to gene expression profiles determined together with UKF-NB-3^rVCR¹⁰, UKF-NB-3 to gene expression profiles determined together with UKF-NB-3^rCDDP¹⁰⁰⁰ cells), UKF-NB-3^rVCR¹⁰, UKF-NB-3^rCDDP¹⁰⁰⁰, UKF-NB-6 (UKF-NB-6^r corresponds to gene expression profiles determined together with UKF-NB-6^rVCR¹⁰, UKF-NB-6 to gene expression profiles determined together with UKF-NB-6^rCDDP¹⁰⁰⁰ cells), UKF-NB-6^rVCR¹⁰, or UKF-NB-6^rCDDP¹⁰⁰⁰.

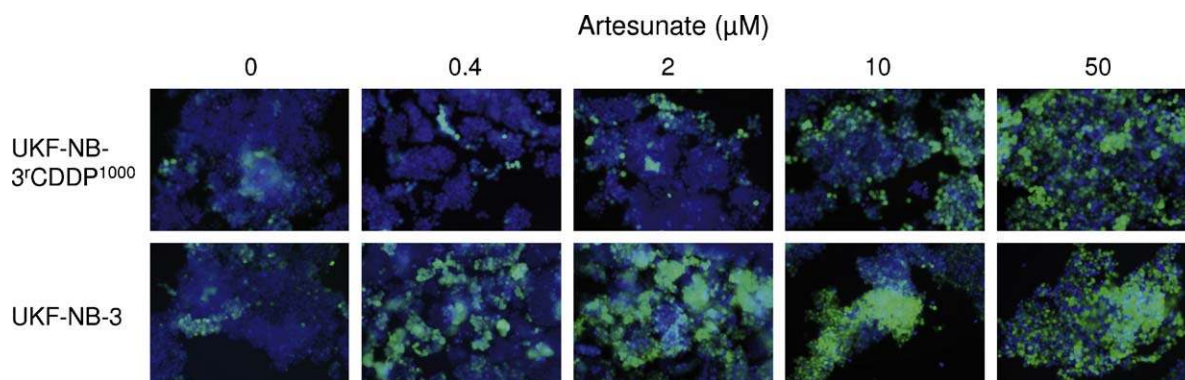


Fig. 4. Influence of artesunate on formation of reactive oxygen species (ROS) in neuroblastoma cell lines after 24 h. Nuclei are DAPI-stained in blue. Cellular ROS formation is indicated in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

glutathione metabolism is known to be involved in the cellular response to oxidative stress [31,32], UKF-NB-3 or UKF-NB-3^{CDDP}¹⁰⁰⁰ were treated for 24 h with different concentrations of artesunate and the cellular formation of reactive oxygen species (ROS) was measured. Artesunate-induced in both cell lines oxidative stress in a concentration-dependent manner, however, ROS production was much more pronounced in UKF-NB-3 cells, especially in lower artesunate concentrations (Fig. 4).

4. Discussion

Artemisinin and its derivatives dihydroartemesinin and artesunate were investigated for anti-neuroblastoma activity in a panel of chemosensitive and chemoresistant neuroblastoma cell lines. While artemisinin was not effective in concentrations up to 50 μM , dihydroartemesinin and artesunate exerted anti-cancer effects in concentrations between 1.43 and 11.95 μM (with the exception of UKF-NB-3^{CDDP}¹⁰⁰⁰: IC₅₀ artesunate 29.7 μM ; IC₅₀ dihydroartemesinin 24.9 μM). In the UKF-NB-3 cell line and its chemoresistant sub-lines, the artesunate-induced decrease in neuroblastoma cell viability was paralleled by increased numbers of cells stained positively for activated caspase-3 or enhanced amounts of cells in sub-G1 phase indicating apoptosis induction. In addition to examination of cell lines, the substances were tested in primary cultures obtained from two patients with metastasised INSS stage 4 neuroblastoma with similar results. The investigated artesunate concentrations are in the range of those found effective in melanoma cell lines [9] and about 1000-fold lower than peak plasma levels determined in malaria patients that were reported to be $6.88 \pm 4.69 \text{ mM}$ after i.v. administration of artesunate 2 mg/kg [33]. Notably, two melanoma patients showed favourable response to artesunate treatment [3].

As shown by the determination of cross-resistance profiles established for the parental chemosensitive cell line UKF-NB-3 and its sub-lines adapted to various cytotoxic drugs, adaptation of UKF-NB-3 cells to one cytotoxic drug usually decreased its sensitivity to the whole panel of structurally different anti-cancer including vincristine (interferes with formation of the spindle apparatus), doxorubicin (intercalating agent, inhibits topoisomerase II), cisplatin (DNA crosslinker), topotecan (inhibits topoisomerase I), melphalan (alkylating agent), etoposide (inhibits topoisomerase II). In clear contrast, only one of the chemoresistant sub-lines of UKF-NB-3 (UKF-NB-3^{CDDP}¹⁰⁰⁰) showed a profound decreased sensitivity to artesunate relative to the sensitivity of the parental chemosensitive cell line. This supports previous findings suggesting that artesunate differs in its anti-cancer mechanism from that of other cytotoxic drugs [3,9,10]. Most recently, artesunate was found to target topoisomerase II α with a similar potency like etoposide [11]. Notably, artesunate was similar effective in

parental UKF-NB-3 cells (IC₅₀ = 2.69 ± 0.10) and in the sub-line resistant to the topoisomerase II α inhibitor etoposide UKF-NB-3^{ETO}¹⁰⁰ (IC₅₀ = 2.53 ± 0.50). Moreover, different chemoresistance mechanisms including overexpression of the ABC transporter P-glycoprotein and p53 inactivation did not attenuate cancer cell sensitivity to artesunate in previous studies [3,12,13]. In accordance, neuroblastoma cells that overexpress P-glycoprotein (e.g., UKF-NB-3^{DOX}²⁰, UKF-NB-6^{VCR}¹⁰, UKF-NB-6^{DOX}²⁰) [15,20] and those that harbour a loss-of-function p53 mutation in addition to P-glycoprotein expression (UKF-NB-3^{VCR}¹⁰) [15,19] were similarly sensitive to artesunate like chemosensitive parental neuroblastoma cells.

The anti-cancer mechanism of artesunate is suspected to include induction of oxidative stress by cleavage of its endoperoxide moiety catalysed by Fe²⁺ ions [3,30,34]. In concordance, increased expression of genes encoding for anti-oxidative gene products including *DHAP* (encoding for dihydrodiol dehydrogenase), *GCLC/GCLM* (encoding for GCL), *GSTM4*, *GSTT2*, *GSTZ1* (all encoding for glutathione S-transferases), and *MGST3* (encoding for microsomal glutathione S-transferase 3) have been found to correlate with cancer cell resistance to artesunate [9,10] and the GCL inhibitor BSO that depletes cellular glutathione pools sensitised cancer cells to artesunate [22]. Moreover, GCL transfection decreased the cellular sensitivity to artesunate [10,22].

Anti-oxidative gene expression has also been reported to contribute to cisplatin resistance [35–37]. Intracellularly, the cis-chloro ligands of cisplatin are displaced by water molecules resulting in a complex that reacts with nucleophilic centres of biomolecules including DNA, RNA, proteins, and membrane phospholipids. Thereby, the formation of inter- and intra-strand DNA cross-links is regarded to be critical for the anti-cancer and pro-apoptotic activity of cisplatin [35,38]. Moreover, cisplatin-induced ROS induction has been found to contribute to the pro-apoptotic activity of cisplatin [39–42] and anti-oxidative gene expression contributes to cancer cell resistance to cisplatin by interfering with DNA binding as well as by antagonising ROS formation [35–37,39–44].

Artesunate-induced ROS formation was strongly decreased in the artesunate-resistant UKF-NB-3^{CDDP}¹⁰⁰⁰ cell line in comparison to the sensitive parental cell line UKF-NB-3. GCL catalyses the first and rate-limiting step in the cellular synthesis of the tripeptide glutathione (γ -glutamylcysteinylglycine). The enzyme is encoded by two different genes. *GCLC* encodes for the catalytic subunit. *GCLM* encodes for a modulatory subunit. The gene product of *GCLC* is regarded to be essential for GCL function and homozygous *GCLC* knock-out mice were not viable. The gene product of *GCLM* increases the affinity of the catalytic subunit for glutamate and decreases the sensitivity to feedback inhibition by glutathione but appears not to be essential for enzyme function since homozygous *GCLM* knock-out mice are

viable and fertile [24–29]. The artesunate-resistant UKF-NB-3^{CDDP}1000 cell line showed increased expression of *GCLC* but not of *GCLM*. BSO increased the artesunate sensitivity of UKF-NB-3^{CDDP}1000 cells but the artesunate IC₅₀-value remained clearly higher than that determined in the parental chemosensitive UKF-NB-3 cell line. Notably, a signature of genes encoding for glutathione-related enzymes that were suggested before to be involved in artesunate resistance [22] distinguished between UKF-NB-3^{CDDP}1000 and the more artesunate-sensitive cell lines (Fig. 3).

Moreover, a 94-gene list assembled from different previous studies that reported on the correlation of gene expression data from the NCI60 panel to the artesunate IC₅₀-values in these cells [9,10,22,23] separated UKF-NB-3^{CDDP}1000 from the more artesunate-sensitive cell lines (Fig. 2). This agreement between our data and the previous expression signature is striking, given the tremendous heterogeneity even of cancers from related entities [45,46] and the fact that no neuroblastoma cell lines are included in the NCI60 cell line panel. The finding that gene expression signatures derived from the NCI60 panel is suited to differentiate between artesunate-sensitive and resistant neuroblastoma cell lines illustrates the general applicability of pharmacogenomic approaches.

In conclusion, our data show that artesunate is effective in the majority of the investigated chemosensitive and chemoresistant neuroblastoma cell lines and in primary neuroblastoma cell cultures. The anti-cancer mechanism of artesunate differs from that of established anti-cancer drugs and involves induction of oxidative stress. Gene expression signatures derived from previous investigations using cells from other entities than neuroblastoma were able to discriminate between the artesunate-resistant UKF-NB-3^{CDDP}1000 cell line and the more sensitive neuroblastoma cell lines investigated. Since clinical studies investigating artesunate for malaria treatment revealed that the drug is very well-tolerated in children and that side-effects are low [47–49] and neuroblastoma is a neoplasm of early childhood (96% of cases occur before the age of 10 years [2]), our findings warrant the further investigation of artesunate as anti-neuroblastoma drug.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.013.

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