



**HAL**  
open science

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

Martin Michaelis, Malte C. Kleinschmidt, Susanne Barth, Florian Rothweiler, Janina Geiler, Rainer Breitling, Bernd Mayer, Hedwig Deubzer, Olaf Witt, Jörg Kreuter, et al.

### ► To cite this version:

Martin Michaelis, Malte C. Kleinschmidt, Susanne Barth, Florian Rothweiler, Janina Geiler, et al.. Anti-cancer effects of artesunate in a panel of chemoresistant neuroblastoma cell lines. *Biochemical Pharmacology*, 2009, 79 (2), pp.130. 10.1016/j.bcp.2009.08.013 . hal-00535826

**HAL Id: hal-00535826**

**<https://hal.science/hal-00535826>**

Submitted on 13 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

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

Authors: Martin Michaelis, Malte C. Kleinschmidt, Susanne Barth, Florian Rothweiler, Janina Geiler, Rainer Breitling, Bernd Mayer, Hedwig Deubzer, Olaf Witt, Jörg Kreuter, Hans Wilhelm Doerr, Jaroslav Cinatl, Jindrich Cinatl Jr.



PII: S0006-2952(09)00693-5  
DOI: doi:10.1016/j.bcp.2009.08.013  
Reference: BCP 10297

To appear in: *BCP*

Received date: 2-7-2009  
Revised date: 10-8-2009  
Accepted date: 12-8-2009

Please cite this article as: Michaelis M, Kleinschmidt MC, Barth S, Rothweiler F, Geiler J, Breitling R, Mayer B, Deubzer H, Witt O, Kreuter J, Doerr HW, Cinatl J, Cinatl Jr. J, Anti-cancer effects of artesunate in a panel of chemoresistant neuroblastoma cell lines, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.08.013

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Anti-cancer effects of artesunate in a panel of chemoresistant**  
2  
3  
4 **neuroblastoma cell lines**  
5  
6  
7  
8

9 Martin Michaelis, PhD<sup>1</sup>, Malte C. Kleinschmidt, MD<sup>1</sup>, Susanne Barth, MSc<sup>2</sup>, Florian  
10 Rothweiler, MSc<sup>1</sup>, Janina Geiler MSc<sup>1</sup>, Rainer Breitling, PhD<sup>3</sup>, Bernd Mayer, PhD<sup>4</sup>,  
11 Hedwig Deubzer, MD<sup>5</sup>, Olaf Witt, MD<sup>5</sup>, Jörg Kreuter, PhD<sup>6</sup>, Hans Wilhelm Doerr,  
12 MD<sup>1</sup>, Jaroslav Cinatl, MD, PhD<sup>1</sup>, Jindrich Cinatl jr., PhD<sup>1\*</sup>  
13  
14  
15  
16  
17  
18  
19  
20

21 <sup>1</sup> Institut für Medizinische Virologie, Klinikum der J.W. Goethe-Universität, Paul  
22 Ehrlich-Str. 40, 60596 Frankfurt am Main, Germany  
23

24 <sup>2</sup> blue-drugs GmbH, Komturstr. 3A, 60528 Frankfurt am Main, Germany  
25

26 <sup>3</sup> Groningen Bioinformatics Centre, University of Groningen, Kerklaan 30, 9751 NN  
27 Haren, The Netherlands  
28

29 <sup>4</sup> emergentec biodevelopment GmbH, Rathausstr. 5/3, 1010 Vienna, Austria  
30

31 <sup>5</sup> Deutsches Krebsforschungszentrum (DKFZ), Klinische Kooperationseinheit  
32 Pädiatrische Onkologie (G340) and Pädiatrie III, Zentrum für Kinder- und  
33 Jugendmedizin, Im Neuenheimer Feld 280, 69120 Heidelberg  
34  
35

36 <sup>6</sup> Institut für Pharmazeutische Technologie, Johann Wolfgang Goethe-Universität,  
37 Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany  
38  
39  
40  
41  
42

43 **\*Corresponding author:** phone +49 69 6301 6409; fax +49 69 6301 4302  
44  
45

46 e-mail Cinatl@em.uni-frankfurt.de  
47  
48  
49  
50

51  
52  
53  
54  
55  
56  
57  
58 **Running title:** Artesunate in chemoresistant neuroblastoma cells  
59

60 **Classification:** (1) Antibiotics and Chemotherapeutics  
61  
62  
63  
64  
65

Accepted Manuscript

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 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<sup>r</sup>CDDP<sup>1000</sup> 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<sup>r</sup>CDDP<sup>1000</sup> from the other cell lines. L-Buthionine-S,R-sulfoximine, an inhibitor of GCL (glutamate-cysteine ligase), resensitised in part UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> 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.

**Key words:** neuroblastoma, artesunate, artemisinin, chemoresistance, cancer, chemotherapy

## 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.

## 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 (Manassass, 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<sup>VCR</sup><sup>10</sup> means UKF-NB-3 adapted to vincristine 10ng/ml, UKF-NB-3<sup>DOX</sup><sup>20</sup> means UKF-NB-3 adapted to doxorubicin 20ng/ml, UKF-NB-3<sup>CDDP</sup><sup>1000</sup> means UKF-NB-3 adapted to cisplatin 1000ng/ml. All cell lines were grown in IMDM supplemented with 10% FCS, 100IU/ml penicillin, and 100mg/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, 100IU/ml penicillin, and 100mg/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<sup>®</sup> 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 two hours 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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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<sup>r</sup>VCR<sup>10</sup>, UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, UKF-NB-6, UKF-NB-6<sup>r</sup>VCR<sup>10</sup>, or UKF-NB-6<sup>r</sup>CDDP<sup>1000</sup>. 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  $\geq 3$ , % 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](http://www.r-project.org/); Kendall's  $\tau$ -test, clustering and heatmaps were based on the package *stats*; correction for multiple testing was performed with *multtest*).

### 3. Results

#### 3.1. Sensitivity of neuroblastoma cells to artemisinin derivatives

The IC<sub>50</sub>-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 IC<sub>50</sub>-values for dihydroartemisinin and artesunate ranged from 1.43 to 11.95 µM whereas IC<sub>50</sub> values for most cell lines were below 5 µM. The only exception was the cell line UKF-NB-3<sup>rCDDP</sup><sup>1000</sup> for which the IC<sub>50</sub>-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). IC<sub>50</sub>-values for dihydroartemisinin and artesunate were below 10 µM and IC<sub>50</sub> values for artemisinin were > 50 µM.

Since dihydroartemisinin and artesunate exerted similar anti-neuroblastoma effects and the IC<sub>50</sub>-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

1 activated caspase-3 (Fig. 1A). Fourty eight hour-treatment of neuroblastoma cells  
2 with artesunate 10  $\mu$ M significantly enhanced the number of cells in sub-G1 phase  
3  
4 (Fig. 1B).  
5  
6  
7  
8  
9

### 10 **3.3. Determination of cross-resistance profiles in the cell line UKF-NB-3 and its** 11 **chemoresistant sub-lines** 12 13

14  
15 The cytotoxic mechanism of action of artesunate was suggested to differ from  
16 the mechanisms of established anti-cancer drugs [3,9,10]. Cross-resistance profiles  
17 were determined in a cell line panel consisting of the parental chemosensitive  
18 neuroblastoma cell line UKF-NB-3 and its sub-lines adapted to the cytotoxic drugs  
19 vincristine (UKF-NB-3<sup>r</sup>VCR<sup>10</sup>), doxorubicin (UKF-NB-3<sup>r</sup>DOX<sup>20</sup>), cisplatin (UKF-NB-  
20 3<sup>r</sup>CDDP<sup>1000</sup>), topotecan (UKF-NB-3<sup>r</sup>TOP<sup>15</sup>), melphalan (UKF-NB-3<sup>r</sup>MEL<sup>2000</sup>), or  
21 etoposide (UKF-NB-3<sup>r</sup>ETO<sup>100</sup>). IC<sub>50</sub>-values were determined for each drug used for  
22 adaptation (Table 3). Moreover, relative cellular resistances (IC<sub>50</sub> chemoresistant  
23 UKF-NB-3 sub-line / IC<sub>50</sub> UKF-NB-3) indicating resistance/sensitivity of  
24  
25 chemoresistant sub-lines to each drug relative to parental chemosensitive UKF-NB-3  
26 cells were calculated for each cytotoxic drug and artesunate (Table 3). In general,  
27 adaptation of UKF-NB-3 cells to one cytotoxic drug resulted in decreased sensitivity  
28 to the whole panel of anti-cancer drugs. In contrast, drug resistant UKF-NB-3 sub-  
29 lines showed comparable or increased sensitivity to artesunate (with the exception of  
30 UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>).  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

### 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  $\tau$ -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 (Suppl. Table 1). Cluster analysis of the cell lines UKF-NB-3, UKF-NB-3<sup>r</sup>VCR<sup>10</sup>, UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, UKF-NB-6, UKF-NB-6<sup>r</sup>VCR<sup>10</sup>, and UKF-NB-6<sup>r</sup>CDDP<sup>1000</sup> for expression of these genes could clearly differentiate the artesunate-resistant UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cell line from the other cell lines showing substantially higher artesunate sensitivity (Figure 2).

For 8 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 (Suppl. Table 1). For 5 of these 8 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 3 genes was correlated to the artesunate  $IC_{50}$  in the opposite direction (Suppl. Table 1).

### 3.5. Sensitisation of UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> 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  $\gamma$ -glutamylcysteine synthetase), which has been shown to sensitise artesunate-resistant cells to artesunate [22]. Moreover, GCL transfection decreased the cancer cell sensitivity to

1 artesunate [10,22]. GCL catalyses the first and rate-limiting step in the cellular  
2 synthesis of glutathione. The enzyme is encoded by two different genes. *GCLC*  
3 encodes for the catalytic subunit, *GCLM* for a modulatory subunit. Only the gene  
4 encodes for the catalytic subunit, *GCLM* for a modulatory subunit. Only the gene  
5 product of *GCLC* is regarded to be essential for GCL function [24-29].  
6  
7

8  
9 In UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells *GCLC* expression was found to be significantly  
10 higher than in UKF-NB-3 cells while *GCLM* expression did not differ between these  
11 cell lines (Suppl. Table 2). Treatment of UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells with a fixed BSO  
12 concentration (50  $\mu$ M) that did not affect cell viability ( $92.61 \pm 6.77$  % relative to non-  
13 treated control) resulted in a 4.6-fold decrease of the IC<sub>50</sub> for artesunate (IC<sub>50</sub> with  
14 BSO:  $6.48 \pm 2.18$   $\mu$ M; IC<sub>50</sub> without BSO  $29.70 \pm 0.35$   $\mu$ M). However, the IC<sub>50</sub>-value  
15 remained clearly (2.41-fold) higher than that detected in artesunate-treated UKF-NB-  
16 3 cells ( $2.69 \pm 0.10$   $\mu$ M)  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27

28  
29 Different glutathione-related enzymes had been shown to contribute to  
30 resistance to artesunate [22]. Hierarchical clustering of the expression of glutathione-  
31 related enzymes that had been suggested before to be correlated to artesunate  
32 sensitivity/resistance [22] (Table S2) distinguished between low artesunate-sensitive  
33 UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells and the more sensitive cell lines (Figure 3).  
34  
35  
36  
37  
38  
39  
40  
41

42 The anti-cancer mechanism of action of artesunate is suspected to include  
43 induction of oxidative stress by cleavage of its endoperoxide moiety catalysed by Fe<sup>2+</sup>  
44 ions [3,30]. Since BSO partly sensitised UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells to artesunate and  
45 the glutathione metabolism is known to be involved in the cellular response to  
46 oxidative stress [31,32], UKF-NB-3 or UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> were treated for 24 h with  
47 different concentrations of artesunate and the cellular formation of reactive oxygen  
48 species (ROS) was measured. Artesunate induced in both cell lines oxidative stress  
49 in a concentration-dependent manner, however, ROS production was much more  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

pronounced in UKF-NB-3 cells, especially in lower artesunate concentrations (Figure 4).

Accepted Manuscript

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

#### 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  $\mu$ M, dihydroartemesinin and artesunate exerted anti-cancer effects in concentrations between 1.43 and 11.95  $\mu$ M (with the exception of UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>: IC<sub>50</sub> artesunate 29.7  $\mu$ M; IC<sub>50</sub> dihydroartemesinin 24.9  $\mu$ M). 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  $6.88 \pm 4.69$  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

1 (inhibits topoisomerase I), melphalan (alkylating agent), etoposide (inhibits  
2 topoisomerase II). In clear contrast, only one of the chemoresistant sub-lines of UKF-  
3 NB-3 (UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>) showed a profound decreased sensitivity to artesunate  
4 relative to the sensitivity of the parental chemosensitive cell line. This supports  
5 previous findings suggesting that artesunate differs in its anti-cancer mechanism  
6 from that of other cytotoxic drugs [3,9,10]. Most recently, artesunate was found to  
7 target topoisomerase II $\alpha$  with a similar potency like etoposide [11]. Notably,  
8 artesunate was similar effective in parental UKF-NB-3 cells ( $IC_{50} = 2.69 \pm 0.10$ ) and  
9 in the sub-line resistant to the topoisomerase II $\alpha$  inhibitor etoposide UKF-NB-  
10 3<sup>r</sup>ETO<sup>100</sup> ( $IC_{50} = 2.53 \pm 0.50$ ). Moreover, different chemoresistance mechanisms  
11 including overexpression of the ABC transporter P-glycoprotein and p53 inactivation  
12 did not attenuate cancer cell sensitivity to artesunate in previous studies [3,12,13]. In  
13 accordance, neuroblastoma cells that overexpress P-glycoprotein (e.g. UKF-NB-  
14 3<sup>r</sup>DOX<sup>20</sup>, UKF-NB-6<sup>r</sup>VCR<sup>10</sup>, UKF-NB-6<sup>r</sup>DOX<sup>20</sup>) [15,20] and those that harbour a loss-  
15 of-function p53 mutation in addition to P-glycoprotein expression (UKF-NB-3<sup>r</sup>VCR<sup>10</sup>)  
16 [15,19] were similar sensitive to artesunate like chemosensitive parental  
17 neuroblastoma cells.

18  
19 The anti-cancer mechanism of artesunate is suspected to include induction of  
20 oxidative stress by cleavage of its endoperoxide moiety catalysed by Fe<sup>2+</sup> ions  
21 [3,30,34]. In concordance, increased expression of genes encoding for anti-oxidative  
22 gene products including *DHHDH* (encoding for dihydrodiol dehydrogenase), *GCLC* /  
23 *GCLM* (encoding for GCL), *GSTM4*, *GSTT2*, *GSTZ1* (all encoding for glutathione S-  
24 transferases), and *MGST3* (encoding for microsomal glutathione S-transferase 3)  
25 have been found to correlate with cancer cell resistance to artesunate [9,10] and the  
26 GCL inhibitor BSO that depletes cellular glutathione pools sensitised cancer cells to  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1 artesunate [22]. Moreover, GCL transfection decreased the cellular sensitivity to  
2 artesunate [10,22].  
3

4 Anti-oxidative gene expression has also been reported to contribute to  
5 cisplatin resistance [35-37]. Intracellularly, the cis-chloro ligands of cisplatin are  
6 displaced by water molecules resulting in a complex that reacts with nucleophilic  
7 centres of biomolecules including DNA, RNA, proteins, and membrane  
8 phospholipids. Thereby, the formation of inter- and intra-strand DNA cross-links is  
9 regarded to be critical for the anti-cancer and pro-apoptotic activity of cisplatin  
10 [35,38]. Moreover, cisplatin-induced ROS induction has been found to contribute to  
11 the pro-apoptotic activity of cisplatin [39-42] and anti-oxidative gene expression  
12 contributes to cancer cell resistance to cisplatin by interfering with DNA binding as  
13 well as by antagonising ROS formation [35-37,39-44].  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28

29 Artesunate-induced ROS formation was strongly decreased in the artesunate-  
30 resistant UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cell line in comparison to the sensitive parental cell line  
31 UKF-NB-3. GCL catalyses the first and rate-limiting step in the cellular synthesis of  
32 the tripeptide glutathione ( $\gamma$ -glutamylcysteinylglycine). The enzyme is encoded by two  
33 different genes. *GCLC* encodes for the catalytic subunit. *GCLM* encodes for a  
34 modulatory subunit. The gene product of *GCLC* is regarded to be essential for GCL  
35 function and homozygous *GCLC* knock-out mice were not viable. The gene product  
36 of *GCLM* increases the affinity of the catalytic subunit for glutamate and decreases  
37 the sensitivity to feedback inhibition by glutathione but appears not to be essential for  
38 enzyme function since homozygous *GCLM* knock-out mice are viable and fertile [24-  
39 29]. The artesunate-resistant UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cell line showed increased  
40 expression of *GCLC* but not of *GCLM*. BSO increased the artesunate sensitivity of  
41 UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells but the artesunate IC<sub>50</sub> value remained clearly higher than  
42 that determined in the parental chemosensitive UKF-NB-3 cell line. Notably, a  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

signature of genes encoding for glutathione-related enzymes that were suggested before to be involved in artesunate resistance [22] distinguished between UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> and the more artesunate-sensitive cell lines (Figure 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 IC<sub>50</sub>-values in these cells [9,10,22,23] separated UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> from the more artesunate-sensitive cell lines (Figure 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 are 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<sup>r</sup>CDDP<sup>1000</sup> 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.

Accepted Manuscript

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Acknowledgements

The authors thank the friendly society “Hilfe für krebskranke Kinder Frankfurt e.V.” and its foundation “Frankfurter Stiftung für krebskranke Kinder” for support. The work was in part supported by the European Commission (project acronym: SYNLET, contract no. 043312).

Accepted Manuscript

## References

- 1  
2  
3  
4 [1] Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet*  
5  
6 2007;369:2106-20.  
7  
8 [2] Maris JM, Matthay KK. Molecular biology of neuroblastoma. *J Clin Oncol* 1999;  
9  
10 17: 2264-79.  
11  
12 [3] Efferth T. Willmar Schwabe Award 2006: antiplasmodial and antitumor activity of  
13  
14 artemisinin--from bench to bedside. *Planta Med* 2007;73:299-309.  
15  
16  
17 [4] Woerdenbag HJ, Moskal TA, Pras N, Malingré TM, el-Feraly FS, Kampinga HH et  
18  
19 al. Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. *J*  
20  
21 *Nat Prod* 1993;56:849-56.  
22  
23 [5] Zheng GQ. Cytotoxic terpenoids and flavonoids from *Artemisia annua*. *Planta*  
24  
25 *Med* 1994;60:54-7.  
26  
27  
28  
29  
30   
31 [6] Beekman AC, Barentsen AR, Woerdenbag HJ, Van Uden W, Pras N, Konings AW  
32  
33 et al. Stereochemistry-dependent cytotoxicity of some artemisinin derivatives. *J Nat*  
34  
35 *Prod* 1997;60:325-30.  
36  
37 [7] Beekman AC, Woerdenbag HJ, Van Uden W, Pras N, Konings AW, Wikström HV.  
38  
39 Stability of artemisinin in aqueous environments: impact on its cytotoxic action to  
40  
41 Ehrlich ascites tumour cells. *J Pharm Pharmacol* 1997;49:1254-8.  
42  
43 [8] Beekman AC, Wierenga PK, Woerdenbag HJ, Van Uden W, Pras N, Konings AW  
44  
45 et al. Artemisinin-derived sesquiterpene lactones as potential antitumour compounds:  
46  
47 cytotoxic action against bone marrow and tumour cells. *Planta Med* 1998;64:615-9.  
48  
49 [9] Efferth T, Olbrich A, Bauer R. mRNA expression profiles for the response of  
50  
51 human tumor cell lines to the antimalarial drugs artesunate, arteether, and  
52  
53 artemether. *Biochem Pharmacol* 2002;64:617-23.  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [10] Efferth T, Sauerbrey A, Olbrich A, Gebhart E, Rauch P, Weber HO et al. Molecular modes of action of artesunate in tumor cell lines. *Mol Pharmacol* 2003;64:382-94.
- [11] Youns M, Efferth T, Reichling J, Fellenberg K, Bauer A, Hoheisel JD. Gene expression profiling identifies novel key players involved in the cytotoxic effect of Artesunate on pancreatic cancer cells. *Biochem Pharmacol* 2009;78:273-83.
- [12] Efferth T, Davey M, Olbrich A, Rücker G, Gebhart E, Davey R. Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. *Blood Cells Mol Dis* 2002;28:160-8.
- [13] Hou J, Wang D, Zhang R, Wang H. Experimental therapy of hepatoma with artemisinin and its derivatives: in vitro and in vivo activity, chemosensitization, and mechanisms of action. *Clin Cancer Res* 2008;14:5519-30.
- [14] Kotchetkov R, Cinatl J, Blaheta R, Vogel JU, Karaskova J, Squire J et al. Development of resistance to vincristine and doxorubicin in neuroblastoma alters malignant properties and induces additional karyotype changes: a preclinical model. *Int J Cancer* 2003;104:36-43.
- [15] Kotchetkov R, Driever PH, Cinatl J, Michaelis M, Karaskova J, Blaheta R et al. Increased malignant behavior in neuroblastoma cells with acquired multi-drug resistance does not depend on P-gp expression. *Int J Oncol* 2005;27:1029-37.
- [16] Michaelis M, Fichtner I, Behrens D, Haider W, Rothweiler F, Mack A et al. Anti-cancer effects of bortezomib against chemoresistant neuroblastoma cell lines in vitro and in vivo. *Int J Oncol* 2006;28:439-46.
- [17] Michaelis M, Cinatl J, Anand P, Rothweiler F, Kotchetkov R, von Deimling A et al. Onconase induces caspase-independent cell death in chemoresistant neuroblastoma cells. *Cancer Lett* 2007;250:107-16.

1 [18] Michaelis M, Bliss J, Arnold SC, Hinsch N, Rothweiler F, Deubzer HE et al.

2 Cisplatin-resistant neuroblastoma cells express enhanced levels of epidermal growth  
3 factor receptor (EGFR) and are sensitive to treatment with EGFR-specific toxins. Clin  
4 Cancer Res 2008;14:6531-7.  
5  
6  
7

8 [19] Michaelis M, Rothweiler F, Klassert D, von Deimling A, Weber K, Fehse B et al.  
9 Reversal of P-glycoprotein-mediated multidrug resistance by the murine double  
10 minute 2 antagonist nutlin-3. Cancer Res 2009;69:416-21.  
11  
12  
13  
14

15 [20] Blaheta RA, Michaelis M, Natsheh I, Hasenberg C, Weich E, Relja B et al.  
16 Valproic acid inhibits adhesion of vincristine- and cisplatin-resistant neuroblastoma  
17 tumour cells to endothelium. Br J Cancer 2007;96:1699-706.  
18  
19  
20  
21

22 [21] Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization  
23 methods for high density oligonucleotide array data based on variance and bias.  
24 Bioinformatics 2003;19:185-93.  
25  
26  
27  
28

29 [22] Efferth T, Volm M. Glutathione-related enzymes contribute to resistance of tumor  
30 cells and low toxicity in normal organs to artesunate. In Vivo 2005;19:225-32.  
31  
32  
33  
34

35 [23] Anfosso L, Efferth T, Albini A, Pfeffer U. Microarray expression profiles of  
36 angiogenesis-related genes predict tumor cell response to artemisinin.  
37 Pharmacogenomics J 2006;6:269-78.  
38  
39  
40  
41

42 [24] Huang CS, Chang LS, Anderson ME, Meister A. Catalytic and regulatory  
43 properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. J  
44 Biol Chem 1993;268:19675-80.  
45  
46  
47  
48

49 [25] Huang CS, Anderson ME, Meister A. Amino acid sequence and function of the  
50 light subunit of rat kidney gamma-glutamylcysteine synthetase. J Biol Chem  
51 1993;268:20578-83.  
52  
53  
54  
55

56 [26] Huang CS, He W, Meister A, Anderson ME. Amino acid sequence of rat kidney  
57 glutathione synthetase. Proc Natl Acad Sci U S A 1995;92:1232-6.  
58  
59  
60  
61

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

[27] Dalton TP, Dieter MZ, Yang Y, Shertzer HG, Nebert DW. Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. *Biochem Biophys Res Commun* 2000;279:324-9.

[28] Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM et al. Glutathione synthesis is essential for mouse development but not for cell growth in culture. *Proc Natl Acad Sci U S A* 2000;97:5101-6.

[29] Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP. Initial characterization of the glutamate-cysteine ligase modifier subunit Gclm(-/-) knockout mouse. Novel model system for a severely compromised oxidative stress response. *J Biol Chem* 2002;277:49446-52.

[30] Efferth T, Giaisi M, Merling A, Krammer PH, Li-Weber M. Artesunate induces ROS-mediated apoptosis in doxorubicin-resistant T leukemia cells. *PLoS ONE* 2007;2:e693.

[31] Circu ML, Aw TY. Glutathione and apoptosis. *Free Radic Res* 2008;42:689-706.

[32] Tan SH, Lee SC, Goh BC, Wong J. Pharmacogenetics in breast cancer therapy. *Clin Cancer Res* 2008;14:8027-41.

[33] Batty KT, Davis TM, Thu LT, Binh TQ, Anh TK, Ilett KF. Selective high-performance liquid chromatographic determination of artesunate and alpha- and beta-dihydroartemisinin in patients with falciparum malaria. *J Chromatogr B Biomed Appl* 1996;677:345-50.

[34] Li PC, Lam E, Roos WP, Zdzienicka MZ, Kaina B, Efferth T. Artesunate derived from traditional Chinese medicine induces DNA damage and repair. *Cancer Res* 2008;68:4347-51.

[35] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003;22:7265-79.



- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [36] Biaglow JE, Miller RA. The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer Biol Ther* 2005;4:6-13.
- [37] Stewart DJ. Mechanisms of resistance to cisplatin and carboplatin. *Crit Rev Oncol Hematol* 2007;63:12-31.
- [38] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov* 2005;4:307-20.
- [39] Spitz DR, Phillips JW, Adams DT, Sherman CM, Deen DF, Li GC. Cellular resistance to oxidative stress is accompanied by resistance to cisplatin: the significance of increased catalase activity and total glutathione in hydrogen peroxide-resistant fibroblasts. *J Cell Physiol* 1993;156:72-9.
- [40] Sasada T, Iwata S, Sato N, Kitaoka Y, Hirota K, Nakamura K et al. Redox control of resistance to cis-diamminedichloroplatinum (II) (CDDP): protective effect of human thioredoxin against CDDP-induced cytotoxicity. *J Clin Invest* 1996;97:2268-76.
- [41] Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H, Murai M. Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. *Br J Cancer* 1997;76:206-10.
- [42] Hellberg V, Wallin I, Eriksson S, Hernlund E, Jerremalm E, Berndtsson M et al. Cisplatin and oxaliplatin toxicity: importance of cochlear kinetics as a determinant for ototoxicity. *J Natl Cancer Inst.* 2009;101:37-47.
- [43] Jiang M, Wei Q, Pabla N, Dong G, Wang CY, Yang T et al. Effects of hydroxyl radical scavenging on cisplatin-induced p53 activation, tubular cell apoptosis and nephrotoxicity. *Biochem Pharmacol* 2007;73:1499-510.
- [44] Jing XB, Cai XB, Hu H, Chen SZ, Chen BM, Cai JY. Reactive oxygen species and mitochondrial membrane potential are modulated during CDDP-induced apoptosis in EC-109 cells. *Biochem Cell Biol* 2007;85:265-71.

1 [45] Sjöblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD et al. The  
2 consensus coding sequences of human breast and colorectal cancers. *Science*  
3  
4 2006;314:268-74.  
5

6 [46] Cardoso J, Boer J, Morreau H, Fodde R. Expression and genomic profiling of  
7 colorectal cancer. *Biochim Biophys Acta* 2007;1775:103-37.  
8  
9

10 [47] Karunajeewa HA, Mueller I, Senn M, Lin E, Law I, Gomorrai PS et al. A trial of  
11 combination antimalarial therapies in children from Papua New Guinea. *N Engl J*  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

[48] Kobbe R, Klein P, Adjei S, Amemasor S, Thompson WN, Heidemann H et al. A  
randomized trial on effectiveness of artemether-lumefantrine versus artesunate plus  
amodiaquine for unsupervised treatment of uncomplicated *Plasmodium falciparum*  
malaria in Ghanaian children. *Malar J* 2008;7:261.

[49] Koram K, Quaye L, Abuaku B. Efficacy of amodiaquine/artesunate combination  
therapy for uncomplicated malaria in children under five years in Ghana. *Ghana Med*  
*J* 2008;42:55-60.

**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	$IC_{50}$ ( $\mu$ M)		
	artemisinin	dihydroartemisinin	artesunate
UKF-NB-3	> 50	4.50 $\pm$ 0.30	2.69 $\pm$ 0.10
UKF-NB-3 <sup>r</sup> VCR <sup>10</sup>	> 50	2.09 $\pm$ 0.20	1.43 $\pm$ 0.12
UKF-NB-3 <sup>r</sup> DOX <sup>20</sup>	> 50	3.18 $\pm$ 0.50	2.24 $\pm$ 0.06
UKF-NB-3 <sup>r</sup> CDDP <sup>1000</sup>	> 50	24.9 $\pm$ 0.28	29.70 $\pm$ 0.35
UKF-NB-3 <sup>r</sup> TOP <sup>15</sup>	> 50	2.24 $\pm$ 0.08	1.93 $\pm$ 0.20
UKF-NB-3 <sup>r</sup> MEL <sup>2000</sup>	> 50	2.85 $\pm$ 0.08	1.62 $\pm$ 0.40
UKF-NB-3 <sup>r</sup> ETO <sup>100</sup>	> 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 <sup>r</sup> VCR <sup>10</sup>	> 50	11.95 $\pm$ 0.20	7.13 $\pm$ 0.17
UKF-NB-6 <sup>r</sup> DOX <sup>20</sup>	> 50	7.30 $\pm$ 0.14	9.90 $\pm$ 1.20
UKF-NB-6 <sup>r</sup> CDDP <sup>1000</sup>	> 50	6.67 $\pm$ 0.10	2.27 $\pm$ 0.06
UKF-NB-6 <sup>r</sup> TOP <sup>15</sup>	> 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 <sup>r</sup> DOX <sup>20</sup>	> 50	3.69 $\pm$ 0.13	3.80 $\pm$ 0.90
IMR-32 <sup>r</sup> CDDP <sup>1000</sup>	> 50	4.03 $\pm$ 0.23	3.05 $\pm$ 0.37
IMR-32 <sup>r</sup> MEL <sup>2000</sup>	> 50	2.46 $\pm$ 0.15	2.40 $\pm$ 0.23

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**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 ( $IC_{50}$ ).

	$IC_{50}$ ( $\mu M$ )	
	Patient 1	Patient 2
artemisinin	> 50	> 50
dihydroartemisinin	$8.30 \pm 2.95$	$6.58 \pm 2.72$
artesunate	$6.69 \pm 2.28$	$5.11 \pm 2.61$

**Table 3.** Drug sensitivity (IC<sub>50</sub>) and relative resistances of the neuroblastoma cell line UKF-NB-3 and its chemoresistant sub-lines.

Cell line	drug sensitivity (IC <sub>50</sub> (ng/ml))/ relative resistance (IC <sub>50</sub> UKF-NB-3 sub-line / IC <sub>50</sub> 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 <sup>i</sup> VCR <sup>10</sup>	114.2 ± 11.0/ 475.8	99.3 ± 2.5 15.2	587.6 ± 46.5 6.3	2.84 ± 0.45 2.1	887.3 ± 14.8 6.1	392.3 ± 16.8/ 13.6	1.43 ± 0.12/ 0.5
UKF-NB-3 <sup>i</sup> DOX <sup>20</sup>	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 ± 0.06 0.8
UKF-NB-3 <sup>i</sup> CDDP <sup>1000</sup>	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 <sup>i</sup> TOP <sup>15</sup>	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 <sup>i</sup> MEL <sup>2000</sup>	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 <sup>i</sup> ETO <sup>100</sup>	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

## Figure legends

**Figure 1.** Apoptosis induction by artesunate in neuroblastoma cells. A) Activated caspase-3 positive cells after artesunate 10  $\mu$ 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  $\mu$ 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).

**Figure 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\* corresponds to gene expression profiles determined together with UKF-NB-3<sup>r</sup>VCR<sup>10</sup>, UKF-NB-3 to gene expression profiles determined together with UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> cells), UKF-NB-3<sup>r</sup>VCR<sup>10</sup>, UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, UKF-NB-6 (UKF-NB-6\* corresponds to gene expression profiles determined together with UKF-NB-6<sup>r</sup>VCR<sup>10</sup>, UKF-NB-6 to gene expression profiles determined together with UKF-NB-6<sup>r</sup>CDDP<sup>1000</sup> cells), UKF-NB-6<sup>r</sup>VCR<sup>10</sup>, or UKF-NB-6<sup>r</sup>CDDP<sup>1000</sup>.

**Figure 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\* corresponds to gene expression profiles determined together with UKF-NB-3<sup>r</sup>VCR<sup>10</sup>,

1 UKF-NB-3 to gene expression profiles determined together with UKF-NB-3<sup>rCDDP</sup><sup>1000</sup>  
2 cells), UKF-NB-3<sup>rVCR</sup><sup>10</sup>, UKF-NB-3<sup>rCDDP</sup><sup>1000</sup>, UKF-NB-6 (UKF-NB-6\* corresponds  
3 to gene expression profiles determined together with UKF-NB-6<sup>rVCR</sup><sup>10</sup>, UKF-NB-6 to  
4 gene expression profiles determined together with UKF-NB-3<sup>rCDDP</sup><sup>1000</sup> cells), UKF-  
5 NB-6<sup>rVCR</sup><sup>10</sup>, or UKF-NB-6<sup>rCDDP</sup><sup>1000</sup>.  
6  
7  
8  
9  
10  
11  
12  
13

14 **Figure 4.** Influence of artesunate on formation of reactive oxygen species (ROS) in  
15 neuroblastoma cell lines after 24 h. Nuclei are DAPI-stained in blue. Cellular ROS  
16 formation is indicated in green.  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Figure 1

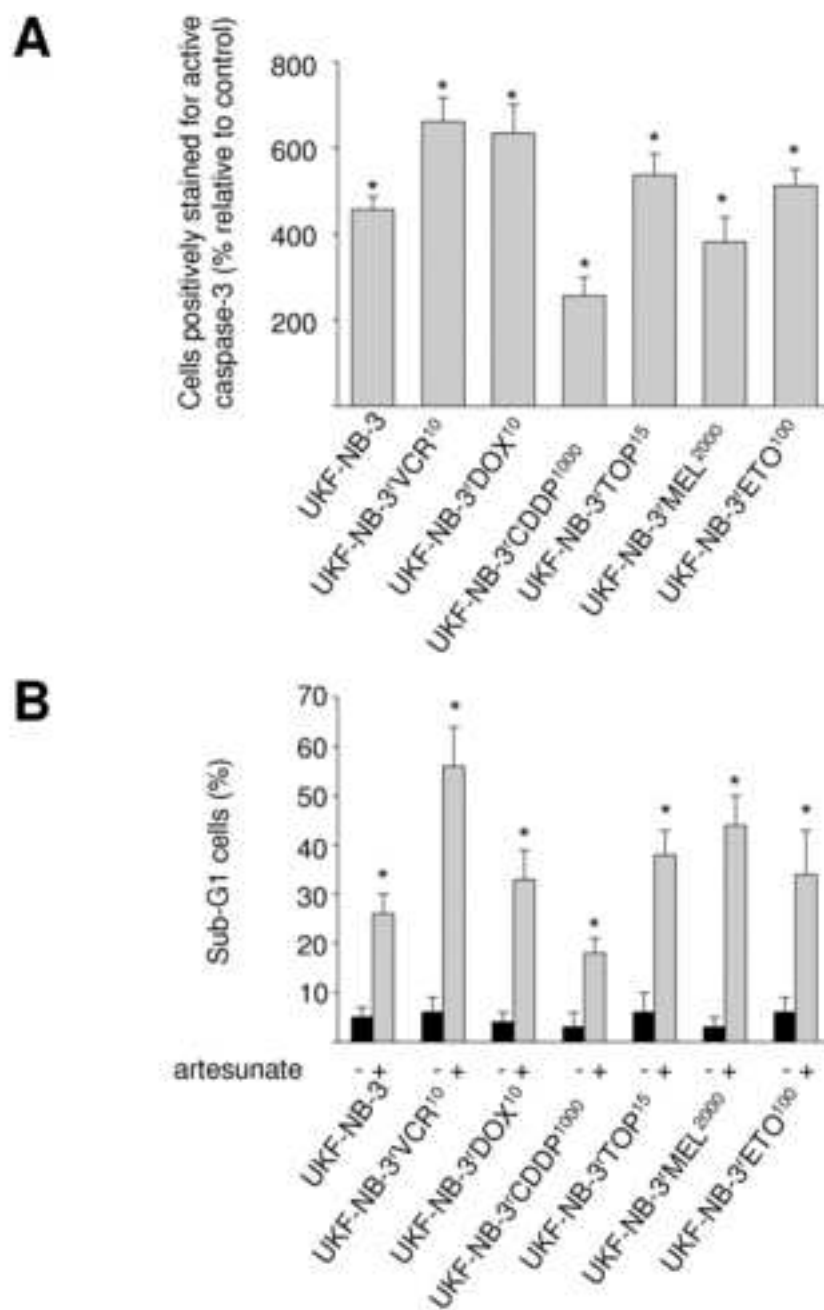




Figure 2

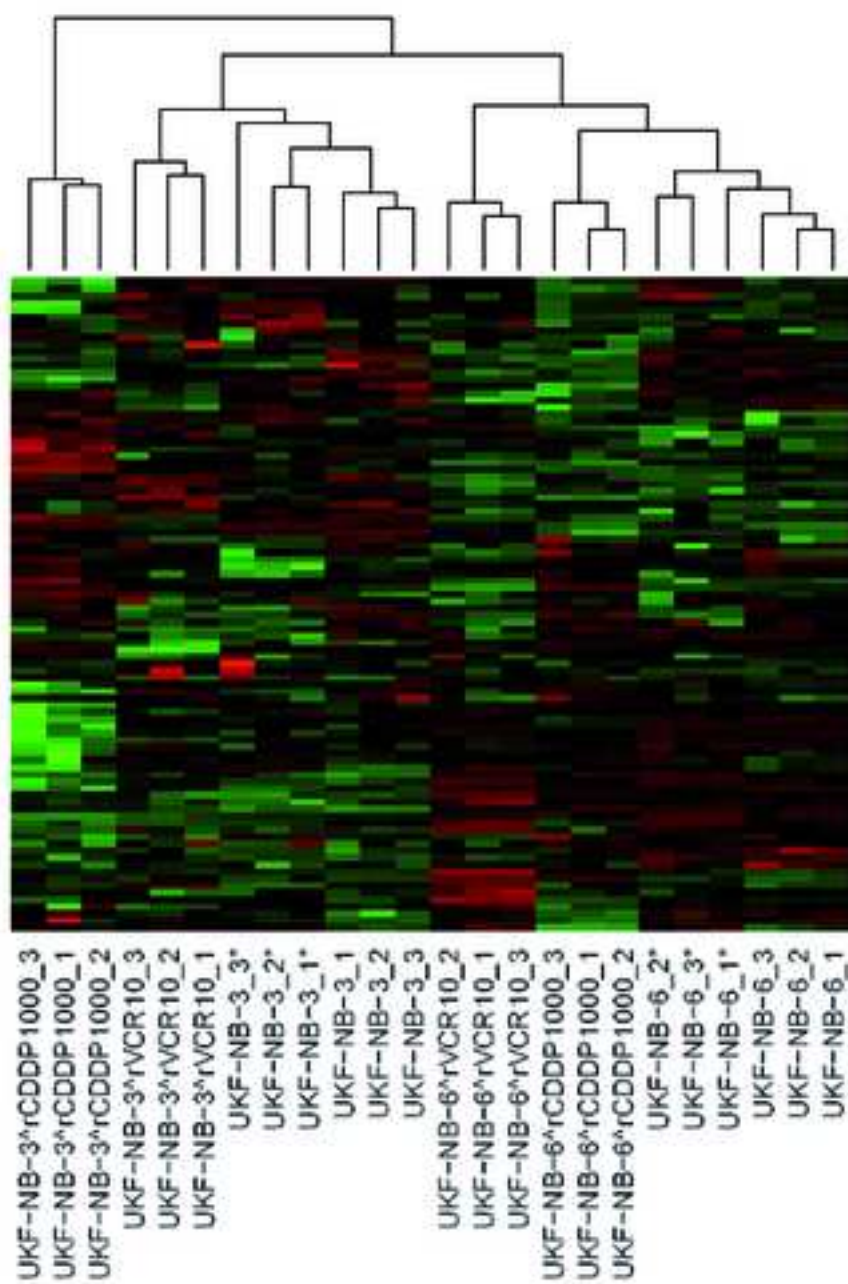


Figure 2

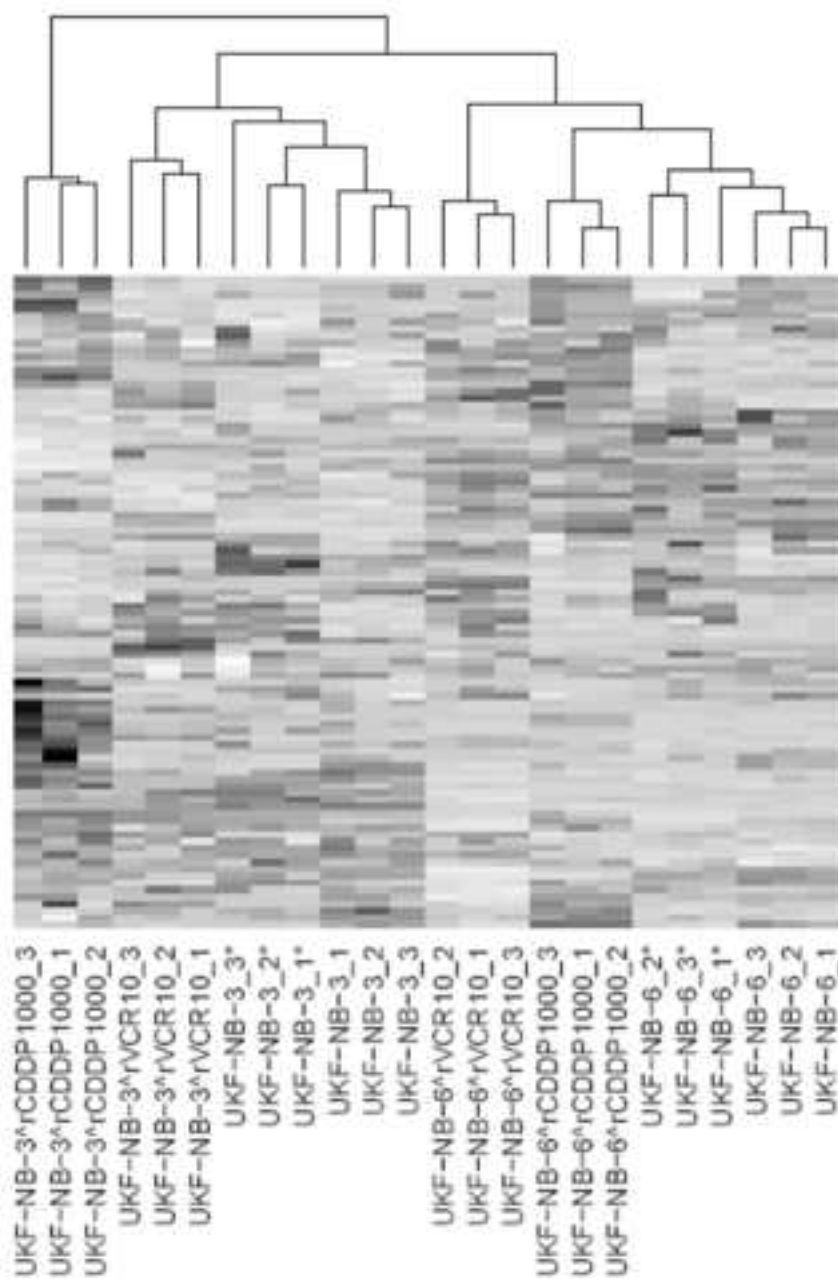


Figure 3

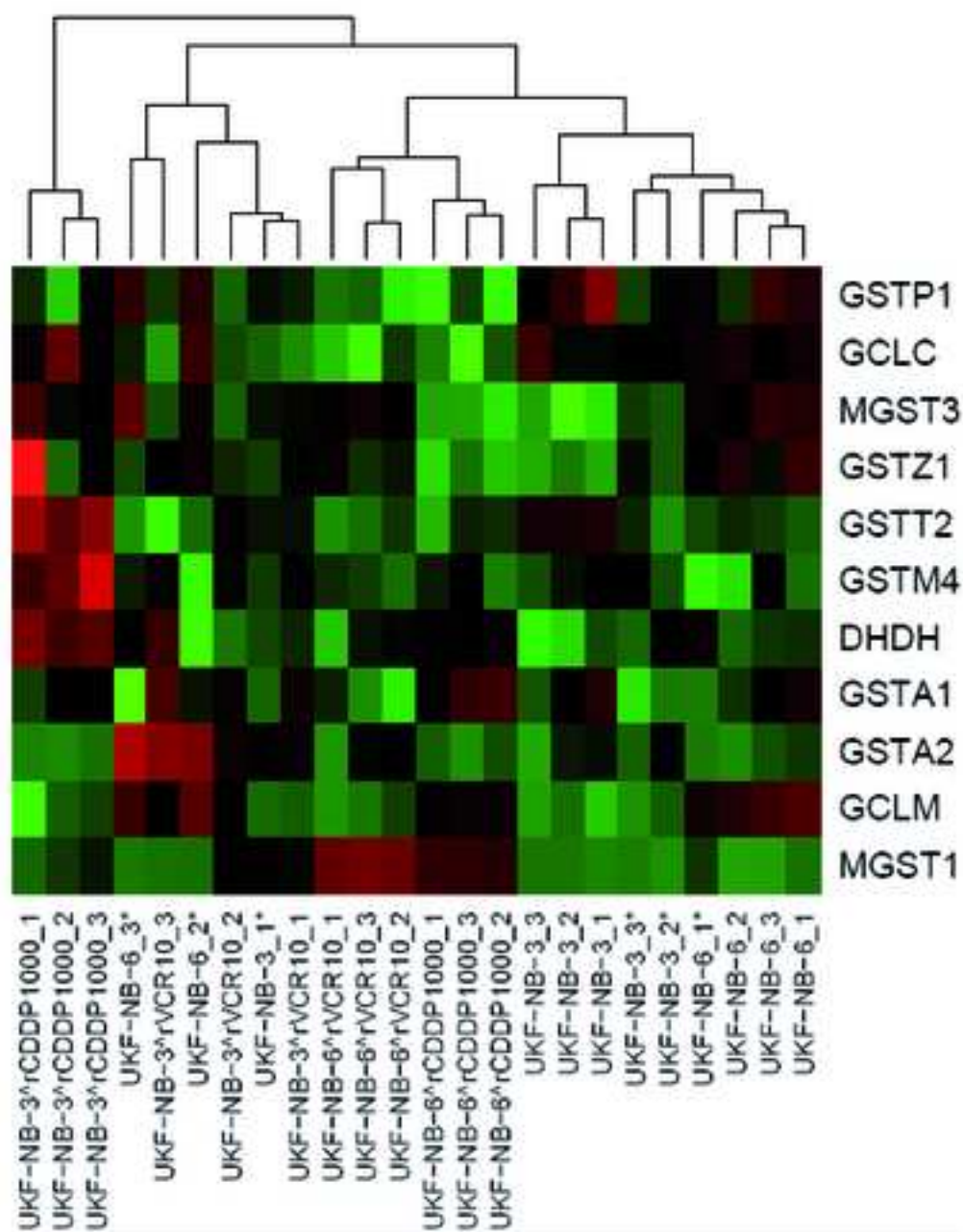


Figure 3

