Artesunate induces oxidative DNA damage, sustained DNA double-strand breaks and the ATM/ATR damage response in cancer cells

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Artesunate-induced oxidative DNA damage
Abstract

Artesunate, the active agent from *Artemisia annua* L. used in the traditional Chinese medicine, is being applied as first-line drug for malaria treatment, and trials are ongoing that include this drug in cancer therapy. Despite increasing interest in its therapeutic application, the mode of cell kill provoked by artesunate in human cells is unknown. Here, we show that artesunate is a powerful inducer of oxidative DNA damage, giving rise to FPG-sensitive sites and the formation of 8-oxo-guanine and 1,N6-ethenoadenine. Oxidative DNA damage was induced in LN-229 human glioblastoma cells dose dependently and was paralleled by cell death executed by apoptosis and necrosis, which could be attenuated by radical scavengers such as N-acetyl cysteine. Oxidative DNA damage resulted in DNA double-strand breaks (DSB) as determined by γH2AX foci that co-localized with 53BP1. Upon chronic treatment with artesunate, the level of DSB continuously increased over the treatment period up to a steady-state level, which is in contrast to ionizing radiation that induced a burst of DSB followed by a decline due to their repair. Knockdown of Rad51 by siRNA and inactivation of DNA-PK strongly sensitized glioma cells to artesunate. The data indicate that both homologous recombination and non-homologous end-joining are involved in the repair of artesunate-induced DSB. Artesunate provoked a DNA damage response (DDR) with phosphorylation of ATM, ATR, Chk1 and Chk2. Overall, the data revealed that artesunate induces oxidative DNA lesions and DSB that continuously increase during the treatment period and accumulate until they trigger DDR and finally tumor cell death.
Introduction

The natural compound artemisinin, derived from *Artemisia annua* L., which has been used for centuries in the traditional Chinese medicine (TCM), and its semi-synthetic derivatives artemunate (Fig. 1) and artemether are anti-malaria drugs that also exert remarkable anti-cancer activity *in vitro* and *in vivo* [1-3]. The active chemical moiety of artemisinin-type compounds is an endoperoxide bridge, which is cleaved in the presence of ferrous iron by a Fenton-type reaction. This leads, in plasmodia, to the generation of reactive oxygen species (ROS), e.g. hydroxyl radicals and superoxide anions [4] as well as carbon-centered radical molecules [5]. In plasmodia, these reactive species were shown to alkylate proteins [5-7], which is thought to be the underlying reason of the bacteriostatic effect. Since ferrous iron is present in high amounts in erythrocytes bearing plasmodium parasites and in cancer cells, the activity towards malaria infections and cancer cells might be explained by the same biochemical mechanism. Indeed, exogenous application of iron to cancer cells in the form of transferrin or the drug Ferrosanol® increased the activity of artemunate [8, 9]. Furthermore, oxidative stress response genes are determinants for artemunate resistance [8, 9].

While the present model claims that protein alkylation is the cause for artemunate induced toxicity [10], we have recently shown that artemunate is able to induce DNA damage, which is repaired by specific repair pathways [11]. The molecular nature of the DNA damage induced by artemunate has not yet been identified. Since artemunate gives rise in a Fenton-type reaction to ROS, it is tempting to hypothesize that artemunate-induced ROS formation may lead to oxidative DNA damage, which ultimately accounts for the cytotoxic activity observed in cancer cells. To prove this hypothesis, we measured the formation of 8-oxoguanine (8-oxoG) and 1,N6-etheno adenine (ethenoA), which are main oxidative DNA lesions, following treatment of LN-229 human glioblastoma cells with artemunate. Oxidative DNA damage, induction of DNA double-strand breaks (DSB), DNA damage signaling and apoptosis/necrosis were observed to be induced, indicating that artemunate acts as a potential genotoxic anticancer drug. The role of oxidative DNA damage was confirmed by ROS scavengers, e.g. N-acetyl cysteine, which reversed the detrimental effects of artemunate on LN-229 cells. Hence, we were able to identify for the first time critical oxidative DNA lesions induced by artemunate in human cells that activate cell death.
mechanisms and thus cause cancer cell death. We also show that artesunate induces during the treatment period a sustained level of oxidative DNA lesions and DSB, which is different from ionizing radiation, and that DSB repair strongly contributes to resistance of glioma cells to artesunate.

**Material and Methods**

**Cell culture and drug treatment**

For the experiments we used the cell line LN-229, which is a human glioblastoma line wild-type for p53 [12]. The line was kindly provided by Dr. Weller (Tübingen, Germany) and used and described in our previous work [13, 14]. LN-229 cells were stably transfected with shRNA targeting Rad51 or with the empty vector. Selection occurred with G418 as previously described [15]. We also used the cell lines MO59K and MO59J, which are isogenic human glioblastoma lines wild-type for p53, but proficient and deficient in DNA-PKcs respectively [16]. The lines were a kind gift of Dr. M. Rave-Fränk (Göttingen, Germany) and described previously [15]. Cells were cultured in DMEM/high glucose medium supplemented with 5 % FCS at 7 % CO2 and 37° C. They were cultured for a maximal period of three month and then replaced by a new batch. They were checked routinely for mycoplasma contamination. Artesunate (Dafra Pharma, Turnhout, Belgium) was prepared by dissolving the drug in dimethyl sulphoxide (DMSO) and diluting it in sterile phosphate buffered saline (PBS) to a final concentration of 2 mg/ml. Stock solution was aliquoted and stored at -20° C until use. Iron(II)–glycine sulfate (Ferrosanol) was obtained from Sanol (Monheim, Germany) and used at a concentration of 10 µg/ml. T-butyl hydroperoxide (BOOH) was from Sigma (Germany) and diluted in PBS.

**Determination of apoptosis and necrosis**

The apoptotic frequency was determined by SubG1 fraction or annexin V/propidium iodide double-staining quantified by flow cytometry using a FACS Calibur as previously described [17]. The number of apoptotic cells and the cell populations were calculated using the CellQuestPro program of Beckdon Dickinson (BD, Heidelberg, Germany).
Determination of ROS formation
To evaluate ROS production following artesunate treatment, the membrane permeable indicator H$_2$DCFDA was employed. Cells were pre-incubated with 10 µM H$_2$DCFDA in serum-free medium at 37 °C for 30 min and thereafter treated with artesunate. After different times, cells were washed twice with PBS and monitored with a FACS Calibur (BD, Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The ROS level was determined by comparing the changes in fluorescence intensity with that of the control.

Single cell gel electrophoresis (comet assay)
Exponentially growing cells were exposed to artesunate and after different time periods trypsinized and washed with ice-cold PBS. Alkaline cell lysis and electrophoresis was essentially performed as described previously [18]. Formamidopyrimidine-DNA glycosylase (FPG) (generous gift from Bernd Epe, Mainz) was added to the agarose gel on the slide at a dilution of 1 mg/ml. The slides were incubated for 45 min at 37°C. Electrophoresis (23 V) was performed at 4°C for 15 min in 90 mmol/L Tris, 90 mmol/L boric acid, and 2 mmol/L EDTA (pH 7.5). Propidium iodide stained slides were evaluated using a fluorescence microscope and the Komet 4.0.2 software from Kinetic Imaging Ltd. (Liverpool, UK). Data were expressed as Tail Moment (TM), which represents the percentage of DNA in the tail multiplied by the length between the centre of the head and tail [19].

Phospho-H2AX and 53BP1 immunofluorescence staining
LN-229 cells were seeded on cover slips. Following treatment with artesunate or with ionising radiation (2 Gy), cells were fixed with 4 % formaldehyde at different time points. Cover slips were then blocked using 5 % BSA in PBS containing 0.3 % Triton-X100. The antibodies used were monoclonal anti-γH2AX (Millipore) and/or anti-53BP1 (Millipore GmbH) and Alexa Fluor 488 (Invitrogen) or Cy3 (Abcam). Before mounting, DNA was stained with 1 µM ToPro3 for 15 min. Between all staining steps cells on the cover slips were washed three times in PBS containing 0.3 % Triton-X100 for 5 min. Slides were mounted in anti-fade medium (Glycerol:PBS 1:1, 2.5 % DABCO, pH 8.6 with HCl) and scored using a laser scanning microscope (LSM 710) and the ZEN Software from Carl Zeiss (Jena, Germany).
8-oxoG and ethenoA immunofluorescence staining

LN-229 cells were seeded on cover slips. Following treatment with artesunate, cells were fixed with methanol:acetone (7:3) for 7 min at -20° C and rehydrated in PBS. After treatment with RNase A (200 µg/ml in PBS) and RNase T1 (50 units/ml in PBS) for 1 h at 37° C, cover slips were washed in PBS. DNA was denatured by incubation in a solution containing 70 mM NaOH, 0.14 M NaCl and 40 % methanol for 5 min at 0° C. For proteolysis cells were incubated with PBS containing 0.1 % trypsin for 30 sec. followed by incubation with proteinase K (2 mg/ml in 20 mM Tris/HCl and 2 mM CaCl₂) for 10 min at 37° C. To avoid nonspecific antibody binding, cells were first incubated with PBS containing 1 % caseine for 30 min at room temperature (RT), and then with a rabbit anti–8-oxoG antibody, or a mouse anti-ethenoA antibody (0.5 mg antibody/ml PBS containing 1 % caseine (Squarix Biotechnology, Marl, Germany) for 16 h at 4° C. Unbound antibodies were removed by extensive washing with PBS containing 0.05 % Tween 20. Cy3 goat anti-rabbit or anti mouse (2 mg/ml in PBS containing 1 % BSA) (Jackson ImmunoResearch Europe Ltd., UK) were added for 1 h at 37° C. After washing with PBS, nuclear DNA was stained with ToPro3 (Invitrogen, Oregon, USA) (1 µM in PBS). Samples were then mounted in an anti-fade medium (Glycerol:PBS 1:1, 2.5 % DABCO, pH 8.6 with HCl) and kept in the dark until microscopic analysis.

Image Analysis

Fluorescence images were recorded and quantified using a laser scanning microscope (LSM 710) and the ZEN Software from Carl Zeiss AG (Jena, Germany). Each value represents the average fluorescence intensity of at least 50 nuclei.

Preparation of protein extracts and western blot analysis

Whole cell extracts were prepared by lysis of the cells on the plates in 95° C preheated 1 x loading buffer (Roti®-Load 1, Roth, Karlsruhe, Germany). For preparing whole-cell extracts for the other western blot analyses, cells were washed in ice-cold PBS, harvested and re-suspended in whole-cell extract buffer (20 mM Tris-HCl; pH 8.5, 1 mM EDTA, 1 µM β-mercaptoethanol, 5 % glycerol, 10 mM DDT, 0.5 mM PMSF, 1 mM Na₃VO₄, proteinase inhibitor). After sonication on ice (two cycles of 10 pulses) with a Branson sonifier (Cell Disruptor B15; output control=4, 40
% duty cycle) homogenates were stored at -20°C. Western blot analysis was performed as previously described [20]. Protein-antibody complexes were visualized by ECL (Amersham). Antibodies were purchased from Amersham Biosciences AB, Millipore GmbH, Calbiochem, Merck Chemicals Ltd., and Cell Signalling Technology.

Statistics

Experiments were conducted at least three times. Values were compared statistically using the unpaired T test. Significance levels: * p<0.05; ** p<0.005; *** p<0.001.

Results

Artesunate induces apoptosis and necrosis in human glioma cells

Experiments were performed with LN-229 human glioblastoma cells, which are wild-type for p53 [14]. In previous studies we showed that these cells are repair competent and undergo apoptosis upon genotoxic anticancer drug treatment [15]. Following artesunate exposure, LN-229 cells undergo apoptosis dose-dependently. In the range of 5-50 µg/ml (chronic exposure) artesunate induces apoptosis, as determined by subG1 fraction (Fig. 1B). Using annexin V/PI double-staining, we quantified the level of apoptosis and necrosis and observed that artesunate also induces a considerable amount of necrosis (Fig. 1C). Cell death by apoptosis is a late response that starts two days after the onset of treatment and remains at a high level for at least 5 days (Fig. 1D). The addition of ferrosanol to the medium significantly ameliorated the frequency of apoptosis and necrosis (Fig. 1E), supporting the paradigm that a Fenton-type reaction that gives rise to ROS formation is involved in artesunate activation in cancer cells.

Artesunate induces oxidative DNA damage

To elucidate the role of ROS in artesunate-induced cell death we determined the intracellular ROS level by CH2-staining (Fig. 2A for a representative example). Intracellular ROS clearly increased with artesunate exposure time, reaching a level after 24 h exposure (15 µg/ml) that even exceeded the level induced by t-butyl hydroperoxide (BOOH), which is a powerful oxidative genotoxin (Fig. 2B).
Intracellular ROS is thought to damage the nuclear DNA. Therefore, we determined the level of 8-oxoguanine (8-oxoG), which is the major DNA oxidation product. We utilized a modified alkaline comet assay, which is based on treatment of lysed cells with formamidopyrimidine-DNA glycosylase (FPG) protein prior to electrophoresis. FPG is a bacterial damage-specific DNA glycosylase that recognizes 8-oxoG, and by removing the base, FPG generates apurinic sites that are converted to single-strand breaks (SSB) under the alkaline conditions of the assay. As shown in Fig. 2C, without FPG no SSB were induced whereas in the presence of FPG SSB were induced that increased significantly as a function of artesunate dose. These breaks were not immediately induced at significant amounts. They were first detected 6 h after the addition of artesunate to the medium and later on remained at a high level (Fig. 2D), which indicates that DNA base damage formation by artesunate is a slow process, not comparable to direct oxidizing agents such as BOOH, for which 8-oxoG formation can be observed immediately after treatment (Fig. 3A).

To confirm this data, we determined the 8-oxoG levels by immunocytochemistry. As shown in Fig. 3A (for representative cytochemistry) and Fig. 3B (for quantification), artesunate clearly induces 8-oxoG immunoreactive cells. Similar to the data obtained with the FPG-comet assay, 8-oxoG formation was not significantly enhanced in the first hours after addition of artesunate to the medium. It started to increase significantly only 6 h following treatment. Interestingly, the 8-oxoG level determined after an exposure period of 24 h was not higher than after 6 and 8 h, indicating that an equilibrium is reached between the formation of 8-oxoG and its removal by DNA repair. Removal of 8-oxoG from DNA in the post-exposure period does occur as demonstrated in Fig. 3C. It shows that in LN-229 cells treated for 24 h with artesunate 8-oxoG is removed from the DNA in a time dependent manner reaching control levels 6 h after exposure. Overall, the data support the view that artesunate in the exposure period produces a dose-dependent level of ROS that continuously attack DNA, reaching an equilibrium level that is likely determined by the concentration of artesunate in the medium and the 8-oxoG repair capacity of the cell.

Another biologically relevant DNA oxidation product is 1,N6-ethenoadenine (ethenoA). Since ethenoA is supposed to be a replication blocking DNA adduct [21] and, therefore, a presumptive cytotoxic DNA lesion [22], we wondered whether
Artesunate is able to induce this type of DNA damage as well. LN-229 cells treated with artesunate (15 μg/ml) clearly showed the formation of ethenoA, as demonstrated by immunostaining that increased with treatment time (Fig. 4A). Quantification revealed that after 6 h the level was significantly enhanced and 24 h following treatment an ethenoA level similar to a toxic dose (400 mM) of BOOH was reached (Fig. 4B). The finding substantiates that artesunate generates a sustained ROS level that continuously induces oxidative DNA damage and that this damage accumulates over the treatment period.

If artesunate generates DNA damaging ROS in mammalian cells, it is anticipated that ROS scavengers are able to reduce the level of DNA damage. This was indeed the case as curcumin, resveratrol and N-acetyl cysteine, all of which have radical scavenging properties [23-26], completely prevented artesunate mediated 8-oxoG formation (Fig. 4C). Furthermore, the ROS scavenger N-acetyl cysteine greatly reduced the killing effect of artesunate in LN-229 cells (Fig. 4D), indicating that artesunate-induced oxidative DNA damage is responsible for the killing effect of the drug.

**Artesunate induces a sustained-increasing level of DNA double-strand breaks, which is different from ionizing radiation**

The most severe lethal lesions produced by ROS are DSB [27], which are powerful inducer of apoptosis [28]. To elucidate whether artesunate is able to induce DSB, we determined the phosphorylation level of histone 2AX (γH2AX). As shown in Fig. 5A, H2AX phosphorylation at ser139 occurred already 2 h after the onset of artesunate treatment, increased with time 6 h later and remained at this high level up to 24 h following treatment. We also determined γH2AX foci by immunocytochemistry (Fig. 5B for a representative example and 5C for quantification), which is a widely accepted marker for DSB [29, 30]. As shown in a dose-dependent experiment in Fig. 5D, artesunate induces γH2AX foci in LN-229 cells that increased nearly linearly with exposure time, which is in stark contrast to ionizing radiation (IR) for which γH2AX foci reached a maximum 1 h after treatment and thereafter declined (Fig. 5D) as a result of DSB repair [31]. In artesunate exposed cells, a maximum level was induced at 10 h that did not increase further when the exposure time was extended to 24 h (Fig. 5D), which is conform with the hypothesis that DSB are formed and repaired.
leading finally to an equilibrium level. γH2AX foci co-localized with 53BP1 (Fig. 5E for a representative example and Fig. 5F for quantification), which is a strong additional indication for the ability of artesunate to induce “true” DSB.

Both HR and NHEJ protect against artesunate-induced cell death

Previously, we reported that mutant Chinese hamster cells impaired in the homologous recombination (HR) pathway of DSB repair are hypersensitive to artesunate [11]. This finding prompted us to translate the finding to cancer cells and determined whether down-regulation of HR in glioma LN-229 cells impacts their killing response following artesunate treatment. To this end we stably transfected LN-229 cells with a Rad51 shRNA vector, which clearly down-regulates Rad51 protein expression (Fig. 6A, insert). Treatment of these cells with artesunate led to a dramatic increase in the level of apoptosis, as compared to empty vector transfected cells (Fig. 6A). The data substantiate the finding that the HR repair pathway is involved in protection against artesunate-induced genotoxicity.

Is non-homologous end-joining (NHEJ) involved in DSB repair following artesunate treatment as well? To this end we assessed the killing response of a pair of glioma cells wild-type (MO59K) and mutated for DNA-PKcs (MO59J). MO59K cells were highly resistant to artesunate and underwent cell death (executed mostly by necrosis) only at the high dose level of 50 µg/ml (Fig. 6B). In contrast, MO59J cells were more sensitive, undergoing death already at 10 µg/ml artesunate (Fig. 6C). In these cells death was executed mostly by apoptosis and less by necrosis (compare Fig. 6B and C). To substantiate that NHEJ is involved in protection against artesunate, we treated p53wt glioma cells (LN-229) with a DNA-PKcs inhibitor (NU7026). Inhibition of DNA-PKcs, which is a main component of NHEJ, greatly sensitized glioma cells to artesunate (Fig. 6D, compare with Fig. 1C). Similar to MO59K cells, a large amount of necrosis was induced (Fig. 6D). This necrosis induction is related to ATP depletion, which is currently under study (data not shown). The data suggests that NHEJ, together with HR, is involved in the defense against artesunate-induced DNA damage.
Artesunate induces the DNA damage response

As shown in Fig. 6E, artesunate treatment of LN-229 glioma cells gives rise to a dose-dependent increase in the level of phosphorylated ATM, ATR, Chk2 and Chk1, which was determined 8 h after the onset of treatment. The data are conform with the notion that artesunate generates oxidative DNA damage, yielding DSB that trigger activation of the ATM/ATR/Chk1/Chk2 damage response pathway.

Discussion

This study was conducted to provide evidence that artesunate, which is a widely used drug for the treatment of malaria, is able to induce oxidative DNA damage in mammalian cells. We utilized a glioma cell line (LN-229) for the study because the mechanism of death in glioma cells following DNA damage has been well established in our previous work [13-15]. Here we show for the first time that artesunate is able to induce oxidative DNA damage, i.e. 8-oxoG and ethenoA, which are two main oxidative DNA base adducts. We should note that 8-oxoG is a mispairing lesion that is considered to be responsible for the induction of point mutations following ROS [32-34]. This points to the possibility that artesunate bears a mutagenic potential, which should be considered a side effect of its use as an anti-malaria drug. EthenoA is likely a replication-blocking lesion [21, 22] that, if not repaired in time, may cause stalled DNA replication that ends up with DSB formation and activation of the DNA damage response. This was indeed observed in LN-229 glioma cells. Our observation that down-regulation of HR (by siRad51) greatly ameliorates the killing effect of artesunate is compatible with the model that stalled and finally collapsed replication forks yield the formation of DSB that finally trigger apoptosis [35]. Artesunate-induced ROS may also produce DSB directly. This minor fraction is, similar to ionizing radiation [36], likely to be subject to NHEJ, which explains why both HR and NHEJ are significantly involved in the defense against artesunate-induced DNA damage. The finding that artesunate activates ATM/Chk2 and ATR/Chk1 supports the hypothesis that directly induced and replication mediated DSB contribute to the genotoxic and killing responses observed.

It is striking that following treatment with artesunate, oxidative lesions and DSB are not formed immediately at high amount, but slowly accumulate up to a saturation
level is reached. This is in contrast to the well-known effect of ionizing radiation that induces a burst of DSB immediately after irradiation (Fig. 5D and ref. [37]). Interestingly, the frequency of DSB (as determined by phosphorylation of ser139 of H2AX) following artesunate increased with time after exposure reaching a plateau, which is explained by the equilibrium between the formation and repair of DNA lesions. Thus, although artesunate is a ROS producer, it differs significantly from ionizing radiation and chemical oxidative agents in their ability to produce a sustained level of oxidative DNA lesions, including DSB. Chronic and sustained formation of low-level DNA adducts and DSB that accumulate in the treatment period is not achieved by acute radiation and chemical direct oxidizing agents. We hypothesize that the sustained formation of DNA oxidation products, notably DSB, following artesunate may differ in their biological consequences from lesions that are formed in a pulse-wise manner being, if generated above a particular threshold, an activator of death signaling, which is tolerated in normal but not tumor cells.

Another reasonable supposition for tumor specificity of the killing effect of artesunate rests on artesunate activation by ferrous iron, which gained support by our experiments with ferrosanol that strongly enhanced the killing effect of artesunate in LN-229 glioma cells. Since tumors frequently over-express iron transporter, a tumor-specificity of artesunate-induced cell kill is anticipated. The finding that the cytotoxic effect of artesunate in human cells is due to ROS-induced DSBs, which are repaired by HR and NHEJ, may open up strategies for the inclusion of artesunate in cancer therapy protocols.

In our experiments, we observed DSB formation and activation of DDR within the artesunate treatment period much earlier than the onset of apoptosis. This lag phase between the induction of critical lesions and apoptosis execution is also known from other experimental systems and genotoxic exposures [15, 28]. It is tempting to speculate that critical components of the cell death pathway need to become accumulated until the pathway can be fully activated. We should also note that compared to other genotoxic anticancer drugs such as temozolomide artesunate induces a comparatively high level of necrosis. This could be the consequence of extensive base damage that activates base excision repair, which in turn leads to
ATP depletion. Since apoptosis requires ATP [33], the necrotic pathway will be activated. Work is in progress to clarify this question.

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References


Legends

Figure 1
Cell death induced by artesunate in LN-229 glioblastoma cells. (A) Structural formula of artesunate. (B) Quantification of the Sub-G1 fraction in the cell population that was treated for 48 h with different doses of artesunate. Cells were seeded 2 days before the onset of artesunate treatment. (C) Cell death (apoptosis plus necrosis) induced in LN-229 cells as measured 48 h after artesunate treatment by annexin V/PI double-staining and flow cytometry. (D) Time course of induction of apoptosis, determined by Sub-G1 measurements. (E) Cell death (apoptosis plus necrosis) induced in LN-229 as measured by annexin V/PI staining 48 h after treatment with artesunate alone and artesunate together with Iron(II)–glycine sulfate (10 µg/ml). All data are the mean of at least three independent experiments +/- SD. Significance levels were compared to the non-treated control.

Figure 2
Formation of reactive oxygen species (ROS) and induction of FPG sensitive sites by artesunate. (A) Chloromethyl-H\textsubscript{2}DCFDA staining, an indicator for ROS formation, determined by FACS at different time points after treatment with 15 µg/ml ART; 1 h-treatment with BOOH was used as a positive control. (B) Relative ROS production measured at different time points after treatment with 15 µg/ml ART, BOOH was used as a positive control. (C) FPG sensitive sites induced by artesunate. LN-229 cells were exposed to artesunate with different doses for 24 h, harvested, embedded in agarose, lysed, incubated in the presence and absence of FPG protein and subjected to alkaline single-cell gel electrophoresis. TM, tail moment. (D) Cells were treated with a concentration of 25 µg/ml artesunate and harvested at the indicated time points. Cells were subjected to alkaline single-cell gel electrophoresis as described above in the presence or absence of FPG protein. Data are the mean of at least three independent experiments +/- SD.

Figure 3
Formation of 8-oxoguanine following artesunate treatment of LN-229 glioblastoma cells. (A) Immunofluorescence staining of 8-oxoG after artesunate (15 µg/ml) at different times of exposure; BOOH (400 mM, 1 h treatment) was used as a positive
control. The negative control was only treated with the secondary antibody. ToPro3, nuclear staining. (B) The 8-oxoG level was measured by quantifying the fluorescence intensity using LSM and the appropriate Zeiss software. (C) 8-oxo-G level as a function of time following artesunate treatment. Exponentially growing LN-229 cells were treated with artesunate for 24 h. The medium was replaced and cells were harvested for 8-oxoG staining at indicated times post-exposure. The fluorescence intensity was determined using LSM software. At least 50 cells were counted per experiment and measure point. Data are the mean of three independent experiments +/- SD.

Figure 4
Induction by artesunate of 1,N6-ethenoadenine (EthenoA) in LN-229 glioblastoma cells and effect of radical scavengers. (A) Immunofluorescence staining of EthenoA after artesunate (15 µg/ml) at different times following treatment; BOOH (400 mM, 1 h treatment) was used as positive control. (B) The EthenoA level was measured by quantifying the fluorescence intensity using LSM and the ZEN software. (C) Effects of radical scavenger on artesunate-induced death in LN-229 cells. Cells were incubated with different radical scavengers, N-acetyl cystein (NAC, 10 mM), curcumin (CUC, 5 mM) and resveratrol (RES, 5 mM) in the presence and absence of artesunate (15 µg/ml). The intensity of 8-oxoG was measured by quantifying the fluorescence intensity using LSM. (D) Effect of NAC on artesunate-induced cell death. Cells were incubated with either the radical scavenger N-acetyl cysteine (NAC, 10 mM), artesunate (ART, 15 µg/ml) or with the combination of both agents (ART 15 µg/ml + NAC 10 mM) and cell death was measured 48 h after treatment by annexin V/PI staining and FACS. Experiments were performed three times. Data in Fig. 4B show a representative experiment (50 cells counted +/- SD).

Figure 5
Level of γH2AX and 53BP1 following artesunate treatment. (A) Exponentially growing LN-229 cells were exposed to artesunate up to 24 h, harvested and subjected to western blot analysis. As loading control, the filter was re-incubated with extracellular signal-regulated kinase 2 (ERK2). IF, induction factor (γH2AX/ERK2 in relation to the control). (B) Representative example of γH2AX nuclear foci and (C) the average number of foci per cell after ART treatment (15 µg/ml) of LN-229 cells. (D) Number of
γH2AX foci per LN-229 cell after treatment with γ-rays (2 Gy) and with 15 µg/ml artesunate. Cells were in the exponentially growth phase and irradiated at zero time. Artesunate was added to the medium at zero time and left there until cells were harvested at the indicated times. Data are the mean of three independent experiments +/- SD. (E) Co-localization of γH2AX and 53BP1, showing a representative example, and (F) the average number of foci per cell after artesunate treatment (15 µg/ml).

**Figure 6**
Artesunate-induced glioma cell death in Rad51 downregulated and DNA-PK<sub>CS</sub> mutated cells as well as phosphorylation of players of the DDR. (A) LN-229 cells were stably transfected with shRNA targeting Rad51, or with empty vector, and induced cell death (apoptosis plus necrosis) was determined 48 h after treatment with different doses of artesunate by annexin V/PI flow cytometry. Insert: The efficiency of Rad51 knockdown was tested by western blot analysis. As loading control, the filter was re-incubated with ERK2. (B) Artesunate-induced death (the sum of apoptosis and necrosis determined by annexin V/PI flow cytometry) in DNA-PK<sub>CS</sub> wildtype glioma MO59K cells and (C) DNA-PK<sub>CS</sub> MO59J mutant cells. (D) Effect of the DNA-PK inhibitor NU7026 on death of artesunate treated LN-229 cells. Cells were treated with the inhibitor (10 µM) 2 h prior to the addition of artesunate to the medium. (E) Phosphorylation of ATM, ATR, Chk2 and Chk1 in LN-229 cells after artesunate treatment, which was determined 8 h after the onset of treatment. To verify equal protein levels, the total amount of non-phosphorylated proteins and β-Actin was determined.
**Fig. 1**

(A) Chemical structure of Artesunate.

(B) Cell death (Sub-G1 fraction %) vs. Artesunate (µg/ml).

(C) Apoptosis/necrosis (%) vs. Artesunate (µg/ml).

(D) Cell death (Sub-G1 fraction %) vs. Time of exposure (h).

(E) Apoptosis/necrosis (%) vs. Artesunate (µg/ml).
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Fig. 2

**DNA breaks (TM)**

**Artesunate (µg/ml)**

**Time (h)**
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Fig. 4
Fig. 5
A

![Graph showing cell death (Sub-G1 fraction %) versus Artesunate (µg/ml) for LN229 cells with Empty vector and Rad51 knockdown.]

B

![Bar graph showing cell death (%) for MO59K cells.]

C

![Bar graph showing cell death (%) for MO59J cells.]

D

![Bar graph showing cell death (%) for LN-229 cells with and without DNA-PK inhibition.]

E

![Western blot analysis showing changes in phosphorylated forms of ATM, ATR, CHK2, and CHK1 with increasing ART concentrations.]

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Fig. 6
Artesunate induces oxidative DNA damage, sustained DNA double-strand breaks and the ATM/ATR damage response in cancer cells

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