

# Artesunate exerts specific cytotoxicity in retinoblastoma cells via CD71

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**Abstract.** Retinoblastoma (RB) is an intraocular cancer that affects young children. There is an ongoing effort to find new agents for RB management that are effective, specific and with few side-effects. In the present study, we tested artesunate (ART), a synthetic derivative from the herbal drug artemisinin, used in the clinic for the treatment of malaria. We analyzed ART cytotoxicity in an RB cell line (RB-Y79) and in a retinal epithelial cell line (hTERT-RPE1) by flow cytometric analysis (FCM). We related the effect of ART to the expression of transferrin receptor 1 (TfR-1, also known as CD71) by knocking down CD71 with RNAi and analyzing cell cycle variables by FCM. We found that the cytotoxic action of ART is specific for RB cells in a dose-dependent manner, with low toxicity in normal retina cells. ART is more effective in RB than carboplatin with a markedly strong cytotoxic effect on carboplatin-resistant RB cells. RB had higher CD71 levels at the membrane compared to normal retinal cells. We showed that ART internalization in RB cells is dependent upon the expression of the CD71. In addition, ART blocked the cell cycle progression at the G1 phase, even at low doses, and decreased the proportion of RB cells in the S phase. In conclusion, we showed that ART is a promising drug exhibiting high selective cytotoxicity even against multidrug-resistant RB cells. Thus, we suggest that ART could be used in the treatment of RB.

## Introduction

Retinoblastoma (RB) is the most common intraocular malignancy that affects young children (1-3). It is a rare disease with a 1:15,000/1:20,000 incidence worldwide (4). The mortality rate ranges from 30 to 60% in developing countries, in opposition to 10% in other countries such as the USA (4-9). The low survival is due mainly to its late diagnosis (3,10).

Before the 1990's, RB treatment consisted of the removal of the eye and radiation, but the loss of eyes and facial deformity as a consequence of the treatment resulted in severe morbidity for the young patients (11). After the 1990's, chemotherapy became an effective treatment for cancer (12,13). Extensive research has been carried out since then in RB management to save the life and vision of the child. The most common chemotherapy protocol currently in use consists of carboplatin, vincristine and etoposide (14-16). In general, carboplatin is given in 1 day doses (17-19) and is then stopped for several weeks. During this interval, the other anti-neoplastic drugs can be administered. The application of these anti-neoplastic drugs aims to reduce the tumor size (chemoreduction) but a second focal treatment such as cryotherapy or brachytherapy is normally used giving this combined therapy a positive prognosis. Adequate tumor reduction requires 2-6 cycles of chemotherapy (20).

However, the systemic treatment of RB is effective at the beginning of therapy but the long term use of chemotherapy may be limited as these drugs cause serious adverse side-effects, including myelosuppression, ototoxicity, nephrotoxicity and anemia (21-24). In addition, tumor size is reduced by only 3% after the third chemotherapeutic cycle, compared with a 30% reduction after the first drug application (25). This suggests that chemotherapy effectiveness in RB patients drops over time, increasing the risk of developing multidrug-resistant RB cells, which increases the chances of tumor re-growth and secondary metastases, thereby limiting the application of chemotherapy in the treatment for RB (26-28). As a result, there is a need for alternative drugs that are effective, selective and with fewer adverse side-effects for RB treatment to overcome the limitations of the current chemotherapeutic drugs.

Artemisinin is a promising drug to test anti-neoplastic activity against RB. Artemisinin is an herbal drug that has been used in traditional Chinese medicine for thousands

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of years (29) and it is clinically used as an anti-malarial drug (30). Artemisinin has low solubility in water or oil, poor bioavailability, and a short half-life *in vivo* (30,31). However, some semi-synthetic derivatives have been developed, such as artesunate, dihydroartemisinin, artemether and arteether, that overcome the problems associated with the natural product (30,31). In recent years, artemisinin and its derivatives (Arts) have been shown to inhibit cell growth in various types of cancer and cancer cells, such as leukemia, fibrosarcoma, ovarian, breast cancer and cervical cancer cells (32-35). In RB, the cytotoxicity and specificity of these compounds has not been studied. It is a promising drug to test anti-neoplastic activity against RB as Arts induce practically no side-effects and are therefore suitable for a long-term use (36).

Although the underlying mechanism is not clearly known, it is likely that Arts work by a multiple mechanisms dependent on iron (37). Several studies suggested that the antitumor and anti-malarial activities of Arts appear to be exerted through oxidative damage (38), by blocking the cell cycle progression (35,39), by induction of cell apoptosis (32), and others (40). Iron is a key player in the anticancer activity of Arts as it mediates the production of oxidative radicals and, also, as iron metabolism promotes cell growth (34,36). Transferrin receptor 1 (TfR-1, also known as CD71), a type II transmembrane protein, plays important roles in the cellular iron uptake and iron metabolism (41). The expression of TfR-1 in cancer cells is elevated compared to normal tissues, which helps absorb more iron and keep the proliferative profile of the cancer cells (42,43). However, it is not clear whether there is any functional relationship between CD71 and Arts cytotoxicity.

Therefore, Arts may be a good candidate to treat RB. Nevertheless, the ability of these drugs to kill cancer cells is variable and dependent on the tumor cell lines (33,44). It is unknown whether these drugs could have a cytotoxic action on RB cell lines. Therefore, in this study, we analyzed the cytotoxic activity and specificity of artesunate (ART) in an RB cell line, in comparison with its normal counterpart, the epithelial retina cell line. We explored the possible relationship between CD71 and its connection with ART cytotoxicity. In addition, we explored the effect of ART on cell cycle progression in RB cells. We found that the cytotoxic action of ART is specific to RB cells in a dose-dependent manner, with low toxicity in normal retina cells. Markedly, ART exerted high cytotoxicity in carboplatin-resistant RB (RB-R) cells. Also, RB had higher CD71 levels at the membrane than normal retina cells. ART internalization and ART cytotoxic action was dependent, in part, on the CD71 receptor. In addition, ART blocked the cell cycle progression at the G1 phase, even at low doses. In summary, we showed that ART is a promising drug to be used for RB treatment, highly cytotoxic against RB cells and multidrug-resistant cells with limited function in normal retina cells.

## Materials and methods

**Antibodies and reagents.** ART was purchased from Guilin Pharmaceutical Co., Ltd. (Guilin, Guangxi, China; H10930195). Carboplatin was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, Shandong, China; H10920028). ART and carboplatin were freshly prepared and diluted in 5% NaHCO<sub>3</sub> to

the required concentrations. CD71-FITC and IgG1-FITC antibodies were purchased from BD Biosciences, USA. Propidium iodide (PI) was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). shRNAs were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Aurum Total RNA Mini kit, iScript cDNA Synthesis kit, iQ SYBR Green SuperMix kit were all purchased from Bio-Rad Laboratories, Inc.

**Cell lines.** Human RB cell line RB-Y79 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), human retinal pigment epithelium cell line (hTERT-RPE1) was purchased from JENNIO Biological Company (Guangzhou, China). The cells were cultured and passaged advisably in Complete Media (RPMI-1640 medium; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Australia), 1% Penicillin-Streptomycin Solution (Gibco, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Carboplatin-resistant RB-Y79 cells.** RB-Y79 cells in logarithmic phase were incubated with 40 µg/ml at 37°C in a humidified 5% CO<sub>2</sub> incubator for 2 h. After centrifuging and washing, the medium containing the drug was discarded. Cells were then cultured in complete culture medium. Once the culture growth was in the logarithmic phase again the carboplatin treatment was repeated. The same procedure was reiterated for several months until a stable resistant cell line at 40 µg/ml carboplatin was generated. To test RB cell resistance to carboplatin, RB-Y79 and RB-R cells were cultured in the presence of 10, 20, 40, 50, 60, 70, 80, 90 or 100 µg/ml carboplatin respectively. After 24 h, cell numbers were counted in the culture using Counter Star with the Automated Cell Counter software (unpublished data).

**Cytotoxicity assay.** To assess the potential inhibitory capacity of ART, 3x10<sup>4</sup> RB-Y79 and hTERT-RPE1 cells were seeded in triplicate in 96-well plates at a density of 1x10<sup>6</sup> cells/ml. Four hours later, cells were cultured with different concentrations of ART (0, 12.5, 25, 50, 100 and 200 µg/ml) and carboplatin (50 µg/ml) for 24 and 48 h, respectively. Following collection, cells were washed twice with PBS and stained with PI (final concentration at 1 µg/ml) for 10 min in the dark, at room temperature. Cytotoxicity analysis was carried out using the FACSCalibur flow cytometer. Data were analyzed with CellQuest-Pro software, and the percentage of dead cells was calculated. In all experiments the cytotoxic activity was defined as the percentage of dead cells after treatment minus the natural death percentage of the respective cell type. To test the carboplatin cytotoxicity on carboplatin-resistant RB cells, 3x10<sup>4</sup> cells were seeded in triplicate in 96-well plates at a density of 1x10<sup>6</sup> cells/ml and cytotoxicity assay was performed by flow cytometry, as explained above.

**Membrane CD71 expression assay.** To determine the CD71 expression level on cell membrane, RB-Y79 and hTERT-RPE1 cells were seeded in triplicate in T-25 flasks at a cell density of 1.5x10<sup>6</sup>. Four hours later, cells were treated with different ART concentrations (final concentration, 0, 50 and 100 µg/ml). After 10 h, cells were washed twice with PBS and incubated with CD71-FITC or IgG1-FITC as control for 30 min at

4°C, according to the manufacturer's instructions. The CD71 expression levels were tested by FACSCalibur flow cytometry, and  $1 \times 10^4$  cells were acquired and analyzed for each sample, respectively, using the CellQuest-Pro software. The percentage of CD71 positive cells was defined as the CD71 expression levels.

**CD71-RNAi.** RB and hTERT-RPE1 cells were seeded in triplicate at  $3 \times 10^5$ /well in a 6-well plate. Transfections were performed at ~30-50% confluency. Cells were incubated in an antibiotic-free culture medium for 30 min before transfection. Cells were transfected with validated shRNAs at 100 nM; scrambled shRNA was used as a negative control, GAPDH was positive control shRNA, and 2 CD71 shRNAs (CD71-homo-1569 and CD71-homo-1865), using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions. Cells were cultured for 72 h and used in the following experiments. Silencing was confirmed using quantitative real-time PCR analysis (qRT-PCR). The specific sequences of these shRNAs were: scramble negative control: 5'-UUC UCC GAA CGU GUC ACG UTT-3' and 5'-ACG UGA CAC GUU CGG AGA ATT-3'; GAPDH positive control, 5'-GUA UGA CAA CAG CCU CAA GTT-3' and 5'-CUU GAG GCU GUU GUC AUA CTT-3'; CD71-homo-1569, 5'-GCC CAG AUG UUC UCA GAU ATT-3' and 5'-UAU CUG AGA ACA UCU GGG CTT-3'; CD71-homo-1865, 5'-GGC CAG CAA AGU UGA GAA ATT-3' and 5'-UUU CUC AAC UUU GCU GGC CTT-3'.

**Preclusion of off-target effect.** The BLAST program is a rapid sequence comparison tool that uses a heuristic approach to construct alignments by optimizing a measure of local similarity. It is widely used for nucleic acid and protein database searches (45). In our experiment, the CD71 shRNAs were blasted in the PubMed database to preclude off-target effect.

**Total RNA isolation and real-time PCR analysis.** Aurum total RNA mini kit was used to extract total RNA from cells treated with shRNAs according to the manufacturer's instructions; 10 µl of total RNA was used for cDNA synthesis by using iScript cDNA synthesis kit according to the manufacturer's instructions. Real-time PCR was performed sequentially by iQ SYBR Green SuperMix kit. The primer sequences were: CD71, forward, 5'-ATCTCGGTCATCAGGATTGC-3' and reverse, 5'-TTAAATGCAGGGACGAAAGG-3'; GAPDH, forward, 5'-CGCATCTTCTTGTGCAGT-3' and reverse, 5'-AATGAAGGGGTCGTTGATGG-3'.

**Intracellular concentrations of ART assay.** Intracellular ART concentration was performed as described by Okwelogu *et al* (47). Briefly,  $2 \times 10^7$  RB-Y79 cells were seeded in triplicate in T-75 bottle. After 4 h, cells were treated with different concentrations of ART (0, 15, 20, 25, 30, 35 and 40 µg/ml). After 24 h, cells were collected and resuspended in 500 µl PBS. Repetitive freeze thaw method using liquid nitrogen was used to extract cells, followed by alkaline hydrolysis treatment with 0.1 mol/l NaOH at 83°C for 1 h. The UV absorbance value at 237 nm was then registered by an ultraviolet spectrophotometer. An ART standard curve was drawn under the same conditions. Intracellular ART concentrations were calculated by standard curve (47).

**Cytotoxicity assay after CD71-RNAi.** To test the cytotoxicity on RB cells,  $3 \times 10^4$  cells were seeded in triplicate in 96-well plates at a density of  $1 \times 10^6$  cells/ml. After 4 h, cytotoxicity analysis was tested by flow cytometry as explained above.

**Cell cycle analysis.** RB and hTERT-RPE1 cells were seeded in T-25 culture bottle with  $5 \times 10^5$  cells. After 24 h, cells were treated with different ART concentrations (final concentration, 0, 5, 10, 15 and 20 µg/ml) for 24 h. Following collection, cells were washed twice with PBS and fixed with 80% ice-cold ethanol overnight at -20°C. The fixed cells were collected and incubated for 30 min in PBS containing 50 µg/ml RNase A at 37°C, stained with 50 µg/ml PI and 0.2% Triton X-100 for 10 min in the dark at room temperature. DNA content analysis were carried out using the FACSCalibur flow cytometer. G0/G1, S, G2/M cell cycle phase were analyzed with ModFit software (Verity Software House, Topsham, ME, USA).

**Statistical analysis.** All data are presented as means ± SD. The significance of the difference between groups was evaluated by Paired t-test and one-way repeated measures analysis of variance (ANOVA) and multiple comparisons with SPSS 17.0 software. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Specific ART cytotoxicity in the RB cell line.** The effect of ART in RB, either *in vitro* or *in vivo*, has not been tested. We compared the cytotoxicity of ART in an RB cell line (RB-Y79) vs. a normal retina pigment epithelium cell line (hTERT-RPE1). Several ART concentrations ranging from 12.5 to 200 µg/ml were used and the proportion of dead cell was measured using flow cytometric analysis (FCM) at 24 and 48 h as described in Materials and methods. At 12.5 µg/ml of ART, 17.5% of RB cells were dead after 24 h, as shown in Fig. 1A. A similar proportion was observed when 25 µg/ml ART was used. However, the average for RB cell death increased with higher ART doses. After 48 h, the number of RB dead cells increased 2-3 times significantly compared with 24 h treatment (Fig. 1B). ART killed only a small proportion of hTERT-RPE1 cells, with values slightly higher at 48 h than those observed with 24 h ART treatment.

This result shows that ART has a cytotoxic effect in a dose-dependent manner against the RB cell line with negligible effect on the normal retina cell line.

**ART cytotoxicity in the carboplatin-resistant RB cells.** Carboplatin is one of the chemotherapeutic drugs commonly used for RB treatment in the clinic (14). However, the effective drug concentration used in clinical treatment causes unwanted secondary effects (23). We compared the cytotoxic activity of carboplatin and ART in the RB and hTERT-RPE1 cell lines. We tested the cytotoxicity of 50 µg/ml carboplatin on both cell lines for 24 and 48 h (48,49). The results showed that only 15% of RB cells died after 24 h carboplatin treatment. This proportion was significantly lower than that at the same ART concentration ( $P < 0.001$ ) (Fig. 1A). After 48 h, carboplatin cytotoxicity increased up to 80%, similar to that observed at the same ART concentration (Fig. 1B).

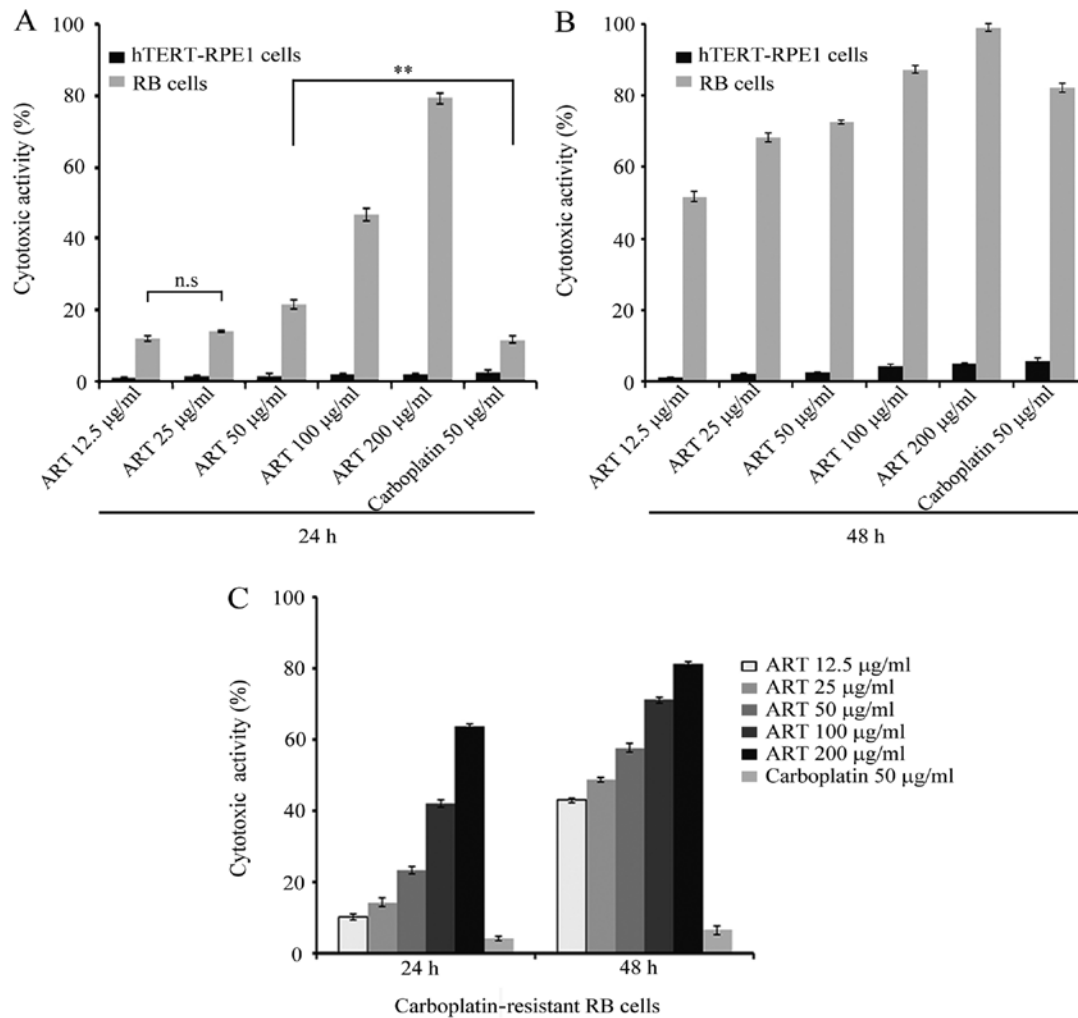


Figure 1. Artesunate (ART) cytotoxicity in retinoblastoma (RB), normal retina and carboplatin-resistant RB (RB-R) cells. (A) RB-Y79 and hTERT-RPE1 cells were treated with different concentrations of ART. After 24 h, cells were stained with propidium iodide (PI) (1  $\mu\text{g/ml}$ ) and 10,000 cells were measured respectively by flow cytometry on a BD FACSCalibur. Cytotoxicity is expressed as the percentage of dead cells, relative to the total cell number in the culture corrected by subtracting the natural cell death observed in the untreated control culture. Results are presented as the mean  $\pm$  SD (n=3, \*\*P<0.01). (B) Cytotoxicity of ART on RB-Y79 and hTERT-RPE1 cells at 48 h. Results are presented as the mean  $\pm$  SD (n=3). (C) RB-R cells were treated with different concentration of ART, cells were stained with PI (1  $\mu\text{g/ml}$ ) and 10,000 cells were measured respectively by flow cytometry on a BD FACSCalibur. Cytotoxicity at 24 and 48 h is expressed as the percentage of dead cells, relative to the total cell number in the culture corrected by subtracting the natural cell death observed in the untreated control culture. The percentage of dead cells was calculated compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD (n=3).

The generation of drug-resistant cells is considered an important factor in the failure of chemotherapeutic cancer treatment. A distinctive characteristic of RB is the fact that it has high expression levels of the drug-resistant proteins that have been suggested to confer resistance (at least in RB cell lines) to drugs used commonly in the clinic for cancer treatment (21,23,50,51). The relationship between the expression of those proteins and the clinical outcome after chemotherapy treatment in RB remains unclear (50,51). Nevertheless, we next explored whether ART was capable of killing RB-R cells. We generated an RB cell line unresponsive to 40  $\mu\text{g/ml}$  of carboplatin in the laboratory (as described in Materials and methods). This cell population was shown to be unresponsive to 24 h treatment with 40  $\mu\text{g/ml}$  of carboplatin (data not shown). Therefore, we tested the cytotoxicity in RB-R cells at different ART concentrations and 50  $\mu\text{g/ml}$  carboplatin for 24 and 48 h. As shown in Fig. 1C, ~15% of the RB-R cells were killed by 12.5  $\mu\text{g/ml}$  ART, in contrast to the 5% cell death observed in

50  $\mu\text{g/ml}$  of carboplatin treatment. ART cytotoxicity on RB-R cells increased with higher ART concentrations. After 48 h of treatment, the cytotoxicity was significantly greater with all the ART concentrations tested. However, carboplatin cytotoxicity on RB-R remained low and similar at any incubation time tested (Fig. 1C).

Taken together, our results suggest that ART is effective against RB cells in a dose-dependent manner and, more importantly, it is capable of effectively killing RB-R cells.

*Relative CD71 expression levels in RB and hTERT-RPE1 cell lines.* We next explored whether CD71 expression was related to ART activity in RB cells. We quantitatively compared CD71 protein expression at the cell membrane in RB and hTERT-RPE1 cells (Fig. 2). The relative level of CD71 (in the absence of ART) in RB cells reached ~70%, displaying values 2 times lower in hTERT-RPE1 cell lines (Fig. 2B). Next, RB and hTERT-RPE1 were incubated with 50  $\mu\text{g/ml}$  ART and

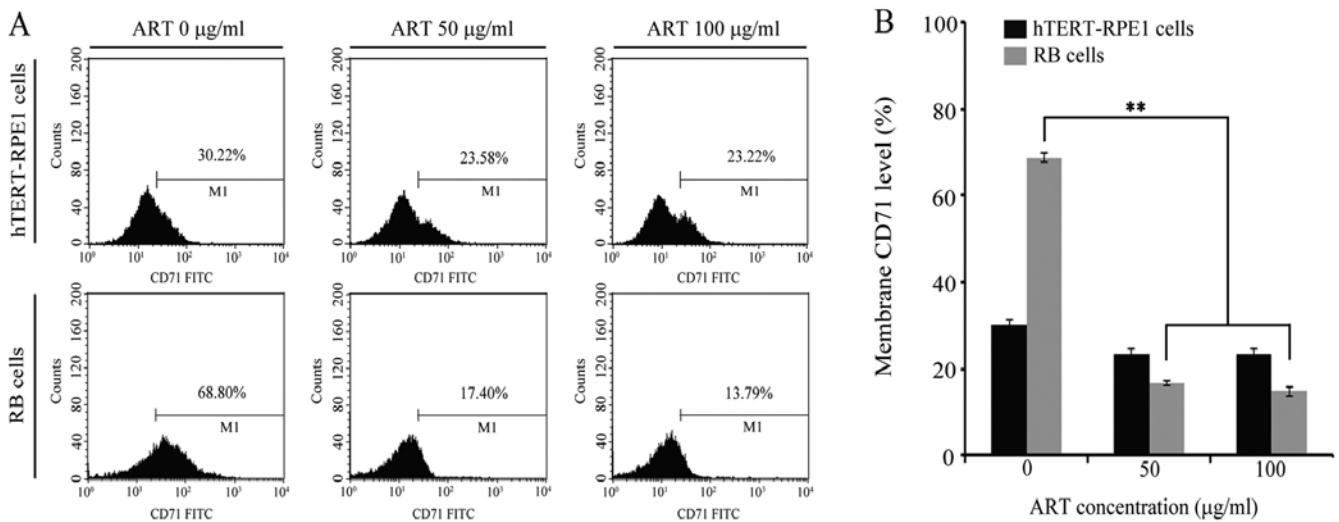


Figure 2. Membrane CD71 expression level in retinoblastoma (RB) Y79 and hTERT-RPE1 cells. (A) RB-Y79 and hTERT-RPE1 cells were treated with different concentrations of artesunate (ART). After 10 h, cells were stained with CD71-FITC and IgG-FITC as isotype control. Ten thousand cells were acquired and CD71 expression was determined by flow cytometry on a BD FACSCalibur. Flow cytometry scatter plots are shown. (B) Membrane CD71 expression in RB-Y79 and hTERT-RPE1 cells was expressed as the percentage of CD71 positive cells relative to cells acquired by flow cytometry compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD (n=3, \*\*P<0.01).

100  $\mu$ g/ml ART for 10 h. We first set up a curve dose response in RB cells and the cytotoxicity was tested at different times ranging from 4 to 24 h (data not shown). Based on the results, we selected the proper time for the drug to cause <5% death. Therefore, we considered 10 h to be an appropriate length of time for the drug to exert molecular action without killing the cells. The CD71 level at the cell membrane was measured in  $1 \times 10^4$  cells in any experimental conditions by FCM (Fig. 2). As shown in Fig. 2, ART had no effect on CD71 at the cell membrane in hTERT-RPE1 cell lines. However, CD71 protein at the membrane decreased 4 times when RB cells were incubated with ART, regardless of the dose, as demonstrated by FCM (Fig. 2). These results show that ART induced the CD71 downregulation at the cell membrane in RB cells.

*ART internalization depends partly on CD71.* In order to get an insight into the specific cytotoxicity in the RB cell line and its relation with CD71, we quantified the intracellular ART concentration. Therefore, we proposed that if CD71 is involved in the internalization of ART, then it would be expected that: i) cells with the highest surface expression of CD71 will have higher intracellular levels of ART, and ii) that suppressing CD71 expression in RB cells will render these cells unresponsive to ART. To verify these, intracellular ART concentration was measured by FCM in RB and hTERT-RPE1 cells after the addition to the media culture of increasing doses of ART ranging from 15 to 40  $\mu$ g/ml. The results showed that intracellular ART in RB cells increased with the extracellular ART dose. However, low levels of ART were found inside the hTERT-RPE1 cell lines (Fig. 3A) regardless of the dose used.

Since both the RB cell line and its normal counterpart, the hTERT-RPE1 cell line, have different levels of CD71 expression at the cell membrane, we further explored whether the CD71 protein expression levels were correlated with ART internalization in the RB cell line. To address this issue, we knocked down CD71 in the RB cell line by using the RNA

interference technique (RNAi). We used siRNAs validated by the commercial company (GenePharma Co., Ltd.) and they were blasted in the PubMed database against CD71 to preclude off-target effect. Two non-overlapping shRNAs were used for the CD71 and scramble shRNA was used as a negative control (as explained in Materials and methods). For siRNA validation, we used a quantitative RT-PCR. The qRT-PCR was carried out using the housekeeping GAPDH mRNA in parallel to facilitate comparison with the relative levels of CD71 transcripts purified from RB RNAi-treated cells. After 2 days of siRNA treatment, undetectable CD71 mRNA amplification product was obtained; only a negligible CD71 mRNA amount was detected at a very high cycle number. This result showed that the mRNA knockdown efficiency is acceptable (Fig. 3C and D) under our experimental conditions. The intracellular ART concentration was quantified by FCM in control RNAi and CD71 RNAi-treated cells. Intracellular ART increased with higher ART concentration in the culture media in control RB cells but only a low level of ART was found inside the cells in the CD71 knockdown conditions (Fig. 3B). These results suggest that CD71 may be involved in ART internalization.

*Specific ART cytotoxicity is mediated by CD71.* We next explored whether CD71 was implicated in ART cytotoxicity. Cytotoxicity was evaluated in RB and hTERT-RPE1 cells in both CD71 RNAi and scrambled control conditions (Fig. 3E and F). A low proportion of cell death was observed in control RNAi in hTERT-RPE1 cells, showing values similar to those observed in Fig. 1A at 50 and 100  $\mu$ g/ml ART. No differences in cell death were observed in the CD71 knockdown cells in the 2 ART concentrations tested. Moreover, significantly higher values of cell death were measured in RB cells ranging from 20 to 50% at 50 and 100  $\mu$ g/ml ART, respectively, consistent with the data from Fig 1A. Conversely, a significant 2.5-fold reduction in cytotoxicity was evident in the CD71 RNAi-treated RB cells. These results suggested that

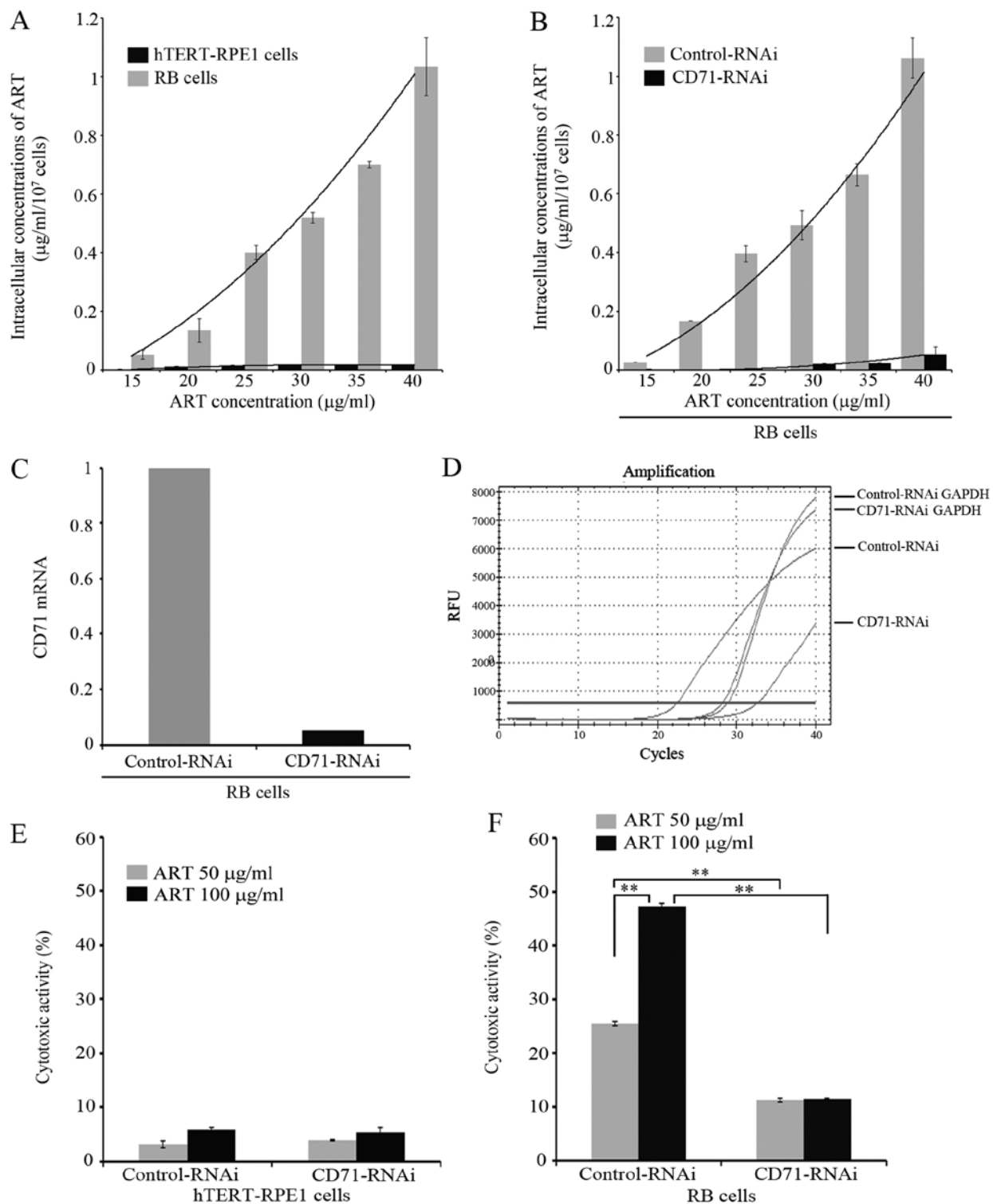


Figure 3. Intracellular concentration of artesunate (ART) and cytotoxicity assay on retinoblastoma (RB) Y79 and hTERT-RPE1 cells after CD71-RNAi. (A) RB-Y79 and hTERT-RPE1 cells were treated with different concentrations of ART. After 24 h, intracellular concentration of ART was determined by ultraviolet spectrophotometer at 237 nm. The intracellular concentration of ART was calculated according to standard curve. Results are presented as the mean  $\pm$  SD (n=3). (B) RB-Y79 cells after CD-71 RNAi were treated with different concentrations of ART. After 24 h, intracellular concentration of ART was determined by ultraviolet spectrophotometer at 237 nm. The intracellular concentration of ART was calculated according to standard curve. Results are presented as the mean  $\pm$  SD (n=3). (C) RB-Y79 cells were transfected with scrambled negative control shRNA, GAPDH positive control shRNA and CD71-homo-1569/1865 shRNAs and detected by qRT-PCR. CD71 mRNA level in CD71-RNAi RB-Y79 cells was calculated compared to the controls treated with scrambled shRNA using GAPDH mRNA as control. (D) CD71 mRNA real-time curves of different groups treated with scrambled negative control shRNA, GAPDH positive control shRNA and CD71-homo-1569/1865 shRNAs in RB cells by qRT-PCR were performed. (E) hTERT-RPE1 cells after CD71-RNAi were treated with different concentrations of ART. After 24 h, cells were collected, stained with propidium iodide (PI) (1  $\mu\text{g/ml}$ ) and 10,000 cells were measured respectively by flow cytometry on a BD FACSCalibur. Cytotoxicity is expressed as the percentage of dead cells, relative to the total cell number in the culture corrected by subtracting the natural cell death observed in the untreated control culture. Results are presented as the mean  $\pm$  SD (n=3). (F) RB-Y79 cells after CD71-RNAi were treated with different concentrations of ART. After 24 h, cells were collected, stained with PI (1  $\mu\text{g/ml}$ ) and 10,000 cells were measured respectively by flow cytometry on a BD FACSCalibur. Cytotoxicity is expressed as the percentage of dead cells, relative to the total cell number in the culture corrected by subtracting the natural cell death observed in the untreated control culture. Results are presented as the mean  $\pm$  SD (n=3, \*\*P<0.01).

ART cytotoxicity could be explained in part by the relatively high CD71 protein expression in the RB cell line.

*ART induces G1 phase cell cycle arrest in human RB cells.* An association between ART cytotoxicity and cell cycle was previously reported (35,39). We sought to determine whether ART has an effect on cell cycle in RB cells. In order to examine this, we analyzed the cell cycle phases in RB cells and in a normal retina cell line treated for 24 h with 5, 10, 15 or 20  $\mu\text{g/ml}$  of ART. Since we intended to detect any alteration in cell cycle without killing the cells, we used ART concentrations that cause <10% cell death after 24 h of incubation (Fig. 1A). The cell cycle was unaffected by ART in the normal cell line at any drug concentration tested (Fig. 4). Markedly, a G0/G1 arrest was observed when RB cells were incubated with ART at 5  $\mu\text{g/ml}$  (Fig. 4A, D and G) with additional effects at higher ART concentrations (Fig. 4A and G). Moreover, S phase was significantly affected by ART incubation (Fig. 4B and E) depicting values nearly 20% lower than untreated RB cells but reaching values similar to the hTERT-RPE1 untreated cells ( $P < 0.05$ ) (Fig. 4E). ART effect on S phase seems to be independent of the drug concentration used in the experiment (Fig. 4B). On the contrary, mitotic phase seems to be unaffected regardless of the ART dose used in our assay. These findings suggest that ART has an effect on G0/G1 and S phase but no effect on the G2/M phase in the RB cell line.

## Discussion

This study presents evidence, for the first time, that artesunate (ART) exerts a strong and selective cytotoxicity against retinoblastoma (RB). First, we showed that ART cytotoxicity in RB cells increases in a dose-dependent manner while the same doses cause negligible cell death in normal retina cell lines. Secondly, ART is more effective in RB than carboplatin with a markedly strong cytotoxic effect on carboplatin-resistant RB (RB-R) cells. Thirdly, ART internalization in RB cells is dependent upon the expression of the transferrin receptor (CD71) and, finally, ART influences cell cycle progression by arresting RB cells in G1 and decreasing the proportion of RB cells in the S phase.

In 1992, Deng *et al* (52) first found that artemisinin and its derivatives (Arts) have cytotoxicity in a murine leukemia cell line (P388) (33) and, in the same year, Sun *et al* (53) found that Arts have cytotoxicity in a human hepatoma cell line (SMMC-7721) and in human gastric carcinoma cells (SGC-7901) but only limited cytotoxicity in the normal embryonic lung cell line (WI-38).

The first evidence of ART antitumor activity was reported by Woerdenbag *et al* (54) and Yang *et al* (55). Since then, research has focused on understanding the anti-neoplastic properties of ART. ART has antitumor activity in a wide range of cell lines with variable efficacy from one cancer cell line to another (56). For example, ART seems to be less effective in breast cancer (MCF-7), gastric cancer (MKN) or some prostate cancer cell lines (such as PC-3) (57,58) compared with other cancer cells (59). This study provides the first evidence that ART acts in an RB cell line, and it is specific for RB with a negligible effect on normal retina cells. A comparison of ART vs. carboplatin cytotoxicity showed that ART is active at concentrations

similar to those of established antitumor drugs (59). Moreover, this study showed that ART cytotoxicity is higher than carboplatin at the same dose. It has been reported that the Arts are effective against a wide range of resistant cancer cell lines including doxorubicin, methotrexate and hydroxyurea-resistant lines with no cross-resistance (44,60). In the present study we showed that ART is effective in RB-R cells in a dose-dependent manner. These characteristics make ART a suitable candidate as an anti-neoplastic drug for RB treatment.

This study also showed that ART cytotoxicity increases with longer incubation times. However, the serum concentration of Arts declines quickly, with a half-life of the order of an hour (30). Our experimental evidence has been collected *in vitro* and it is therefore only indicative of the pharmacological behavior of the drug *in vivo*. The short half-life of ART in serum forces clinicians to administer the drug at least daily and typically several times a day. For example, the WHO-approved adult dose of ART of 2.4 mg/kg given at 0, 12 and 24 h for malaria management. Nevertheless, in this study we found a very low cytotoxicity on normal retina cell lines even after 48 h of ART incubation. Taking into account the long treatment required for cancer management, we cannot preclude a harmful effect on a daily administration during the long period needed to manage an aggressive cancer. However, clinical trials have shown promising results in cancer patients. ART administration to a laryngeal squamous cell carcinoma patient during nine months showed a 70% tumor reduction prolonging and improving the quality of life of the patient (61).

Sustained proliferation and growth of malignant cells require a high iron metabolism for cell survival and cancer progression (62). Transferrin receptor 1 (CD71) plays a key role in the uptake of iron and regulation of its intracellular concentration (41,62). Most cancer cells exhibit an increment in transferrin receptor expression compared with their normal counterpart. However, CD71 expression levels in cancer cells depend on the cell line. For some cancer cell lines (such as the astrocytoma U373 cell line) the transferrin receptor expression is lower than for others cell lines (such as the leukemia cells, CCRF-CEM), while it is still higher than its normal (non-malignant) counterpart (37,63-65). In the present study, we found that 70% of the RB cells expressed CD71 protein at the plasma membrane and it is more than 2 times higher than in normal retina cells, suggesting that the CD71 receptor could be a potential target for the ART cytotoxic activity in RB cells.

Accordingly, experiments from other groups showed that the use of a monoclonal antibody directed against the transferrin receptor was able to block artemisinin action in neoplastic cells (37). This raises the question of the functional relationship between ART cytotoxicity and the receptor. It is widely accepted that iron content and metabolism are relevant in the selective antitumor activity of artemisinins (42,43,66). Consistent with this, we showed that ART decreased CD71 levels in the cell membrane. A recent study by Ba *et al* (66) showed that CD71 internalization is mediated by the artemisinin-derived compound, DHA, and this internalization may disrupt cellular iron uptake, leading to cell growth arrest and cell death. However, in our study we found that the internalization of ART depends on the CD71 expression and, consequently, it influences the ART cytotoxicity, indicating that ART is internalized by an endocytic pathway together with CD71,

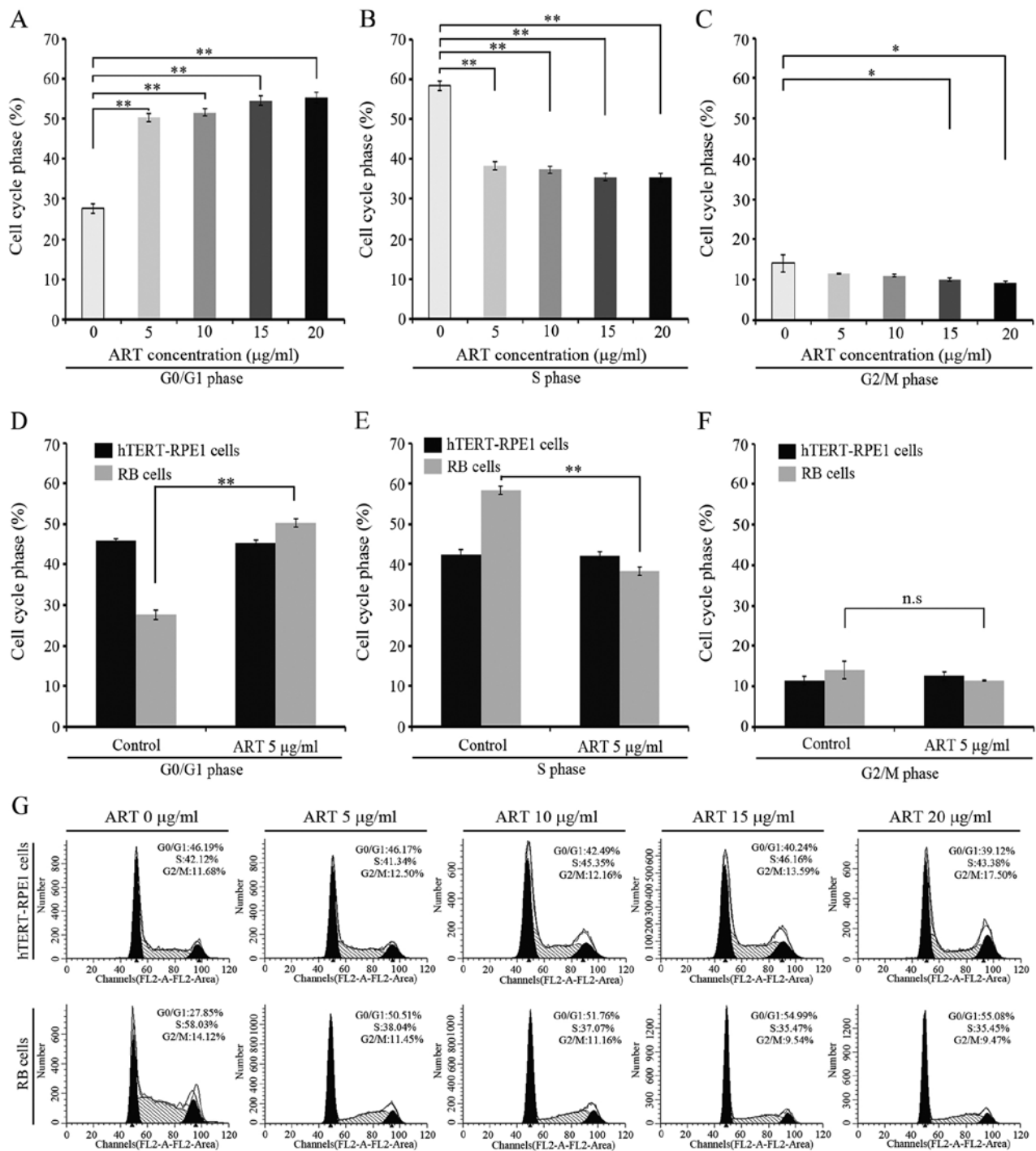


Figure 4. Cell cycle analysis on retinoblastoma (RB) and hTERT-RPE1 cells. (A) RB-Y79 cells were treated with different concentrations of artesunate (ART) for 24 h; 15,000 cells were acquired and G0/G1 cell phase analysis were detected by flow cytometry on a BD FACSCalibur. The percentage of G0/G1 cell phase was analyzed using ModFit software compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (B) RB-Y79 cells were treated with different concentrations of ART for 24 h; 15,000 cells were acquired and S cell phase analysis were detected by flow cytometry on a BD FACSCalibur. The percentage of S cell phase was analyzed using ModFit software compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (C) RB-Y79 cells were treated with different concentrations of ART for 24 h; 15,000 cells were acquired and G2/M cell phase analysis was detected by flow cytometry on a BD FACSCalibur. The percentage of G2/M cell phase was analyzed using ModFit software compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (D) RB-Y79 and hTERT-RPE1 cells were treated with 5  $\mu\text{g/ml}$  ART; after 24 h, 15,000 cells were acquired and G0/G1 cell phase analysis was detected by flow cytometry on a BD FACSCalibur. The percentage of G0/G1 cell phase was analyzed using ModFit software compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (E) RB-Y79 and hTERT-RPE1 cells were treated with 5  $\mu\text{g/ml}$  ART; after 24 h, 15,000 cells were acquired and S cell phase analysis were detected by flow cytometry on a BD FACSCalibur. The percentage of S cell phase was analyzed using ModFit software compared to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (F) RB-Y79 and hTERT-RPE1 cells were treated with 5  $\mu\text{g/ml}$  ART; after 24 h, 15,000 cells were acquired and G2/M cell phase analysis was detected by flow cytometry on a BD FACSCalibur. The percentage of G2/M cell phase was analyzed using ModFit software comparing to the controls treated with the corresponding amounts of medium. Results are presented as the mean  $\pm$  SD ( $n=3$ ,  $**P<0.01$ ). (G) RB-Y79 and hTERT-RPE1 cells were treated with different concentrations of ART; after 24 h, cell cycle was detected by flow cytometry on a BD FACSCalibur. The percentage of different cell phase was analyzed using ModFit software. Column diagrams are shown.



probably in a similar way that transferrin is used to internalize iron into the cell (41). If this mechanism is involved in the action of ART, then reducing the expression of CD71 should render the cancer cells unresponsive to ART (since ART will not be internalized and will be rendered ineffective). Knocking down the CD71 receptor by RNAi lowered the cytotoxicity associated to ART, in accordance with Efferth *et al* (37) who used antibody to block the transferrin receptor (44). However, reducing the CD71 expression did not abolish ART-mediated cytotoxicity in RB. This residual ART cytotoxicity may indicate that the cytotoxicity mediated by CD71 might not be the only mechanism by which ART is internalized and/or exerts its cytotoxic action (44,67). On the other hand, the residual CD71 at the membrane may be sufficient for enough amounts of ART to enter the cell and exert its action.

Finally, in agreement with other studies, low doses of ART are sufficient to alter cell cycle progression (68). RB cells are arrested in the G1 phase according to our data. In addition, the proportion of cells in the S phase decreased significantly as it is expected if the iron metabolism is affected (44).

Arts may act via multiple mechanisms. The toxicity of artemisinin-related compounds is attributed to iron-mediated oxidative damage by generating reactive oxygen species (ROS) and/or carbon-centered radicals (69,70). Both products may play an important role in inducing DNA damage, mitochondrial depolarization and apoptosis (40,44). Then, cancer cells may suffer more severe damage due to the elevated iron levels that support their high cellular metabolism (71-73). Transferrin receptor plays a key role since it increases the iron level inside the cell. Cancer cells have elevated levels of this receptor at the plasma membrane (37,74). Ba *et al* (66) showed that in a hepatoma and breast cancer cell line, DHA (an ART derivative) acts through regulating cell-surface TfR-1. They proposed that in the presence of ART, the CD71 receptor is internalized through a non-classical endocytic pathway. In doing so, the iron uptake is altered. We showed here that ART is internalized by the transferrin receptor CD71 (and probably together with the receptor).

We propose that ART uses the CD71 endocytic pathway to be internalized into the cell, reducing CD71 levels at the plasma membrane, therefore blocking iron uptake, damaging the cells by a mechanism independent of oxidative damage. However, our results do not exclude that ART exerts additional cytotoxic actions once it is inside the cell. For example, it has been shown that ART may act on the activation of the mitochondrial intrinsic apoptotic pathway leading to cell death (75), among others (60,76-78).

In summary, the present study showed that ART has a strong cytotoxic effect on RB cells with low cytotoxicity on normal retina cells. We propose that ART is a sound and potentially safe candidate to treat RB. A randomized study *in vivo* may provide further insight into the efficiency of the treatment.

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