NOTE *Internal Medicine*

Erythrophagocytosis Enhances Heme-Dependent Cytotoxicity of Antimalarial Drugs in Canine Histiocytic Sarcoma Cell Line DH82

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ABSTRACT. Antimalarial drugs, dihydroartemisinin (DHA) and artesunate (ATS), exhibit iron-dependent cytotoxicity in tumor cells. We hypothesized that erythrophagocytic uptake of heme-iron enhances the cytotoxicity of DHA and ATS. Erythrophagocytic (EP) treatment of the canine histiocytic sarcoma cell line DH82 markedly increased the cytotoxicity of DHA and ATS compared to controls. Succinyl acetone, an inhibitor of intracellular heme synthesis, decreased the cytotoxicity of DHA and ATS in normal cells, but this change was not observed in EP cells. These results suggest that exogenous heme derived from erythrocytes can enhance the cytotoxicity of DHA and ATS. Furthermore, our study suggests that heme could be a novel component of tumor treatment in veterinary medicine.

KEY WORDS: artesunate, DH82, dihydroartemisinin, erythrophagocytosis.

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Artemisinin (ART) is extracted from the plant *Artemisia annua* and has long been used for the treatment of chills and fevers in traditional Chinese medicine [8]. ART and its derivatives (ARTs), including artesunate (ATS), artemether, arteether and dihydroartemisinin (DHA), are widely used in combination for the treatment of malaria [8]. ATS and DHA exert antitumor effects on various human cancers and canine sarcoma [5, 7, 12, 21]. These compounds exert their antimalarial activity through the iron-mediated cleavage of endoperoxide bridges and generate free radicals. However, the mechanisms by which ARTs damage neoplastic cells remain unknown. It has been postulated that the production of free radicals by iron compounds, such as transferrin-binding iron, ferrous iron and heme (Fe^{2+} protoporphyrin IX), enhances the cytotoxicity of ARTs *in vitro* [1, 4, 13, 20]. In particular, heme was reported to be more reactive with ART than with hemoglobin, hemin (Fe^{3+} protoporphyrin IX) and inorganic iron *in vitro* [19].

Here, we focused on heme-mediated cytotoxicity, which is thought to underlie the proposed anti-tumor activity of ARTs. Specifically, we hypothesized that heme derived from erythrocytes might enhance the cytotoxicity of ARTs in erythrophagocytic (EP) cells. To assess this possibility, we evaluated the cytotoxicity of ARTs in a canine histiocytic sarcoma cell line DH82.

Calcium-ionophore A23195 (Sigma-Aldrich, St. Louis, MO, U.S.A.), DHA (Sigma-Aldrich), ATS (Sigma-Aldrich) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) (Moostine; Naprod Life Sciences, Mumbai, India) were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich). Vincristine sulfate (Oncovin; Nihonkayaku, Tokyo, Japan) was dissolved in saline. 5-Aminolevulinic acid (ALA) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in H_2O, and succinyl acetone (SA) (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS). Stock solutions were prepared as follows: 1 mM of calcium-ionophore A23195, 0.1 M of DHA and ATS, 16 mg/m*l* of CCNU, 0.2 mg/m*l* of vincristine, 0.3 M of ALA and 0.1 M of SA. These solutions were stored at −80°C until required.

We used the DH82 cell line (ECACC, Salisbury, U.K.), derived from the bone marrow of a male, 10-year-old golden retriever suffering from histiocytic sarcoma. DH82 cells possess a macrophage-like morphology, are able to phagocytize latex particles, adhere to plastic and are positive for Fc-γ receptor [18]. Cells were grown in 75-cm^2 culture flasks (Greiner Bio-one, Tokyo, Japan) with Eagle's minimal essential medium (EMEM; DS Pharma Biomedical, Osaka, Japan) supplemented with 15% fetal bovine serum (FBS; Life Technologies Japan Ltd., Tokyo, Japan), 1% non-essential amino acids (NEAAs; DS Pharma Biomedical), 0.15% $NaHCO_3$, 200 mg/*l* streptomycin and 100,000 U/*l* penicillin as a medium and incubated at 37°C/5% CO_2.

We collected 5 m*l* of blood from a healthy beagle dog into tubes containing heparin. This study was approved by the Kitasato University Animal Committee. Red blood cells (RBCs) were separated by centrifugation (400 × *g*, 10 min), and then, buffy coat and plasma were aspirated and PBS was added (3 m*l*). This procedure was repeated 3 times. After the third wash, the supernatant was aspirated, and the number of cells was enumerated using a Bulker-Turk hemocytometer (Sunlead Glass Corp., Saitama, Japan). Delaby *et al.*

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reported that the aging treatment for mouse RBCs, using the 2.5 mM calcium chloride ($CaCl_2$) (Wako Pure Chemical Industries, Ltd.) and 0.5 μM calcium-ionophore A23195 with incubation at 30°C at 16 hr, increased the expression of phosphatidylserine on the cell surface, which acts as a signal for reticuloendothelial cells to be phagocytized [3]. Collected canine RBCs (1×10^8 cells/m*l*) were suspended in HEPES buffer (10 mM HEPES, 140 mM NaCl and 0.1% BSA, pH 7.4) supplemented with 0–0.4 mM $CaCl_2$ and 0 or 0.5 μM calcium-ionophore A23195. The cell suspension was incubated at 30°C for 16 hr; thereafter, the supernatant was aspirated, and RBCs were washed with PBS and then centrifuged (200 × *g*, 10 min). After 2 washes, supernatant was aspirated, cell density was measured and 1×10^8 canine RBCs were added to DH82 cells and then incubated in 6-well culture plates (Greiner Bio-One) at a density of 5×10^5 cells/well overnight. After 3 hr of co-culture, the medium was removed, and cells were washed with 1 m*l* of PBS. Then, 1 m*l* of hemolysis buffer (140 mM NH_4Cl and 17 mM Tris, pH 7.6) was added to the well and left for 3 min for lysis of non-ingested RBCs. Thereafter, the remaining cells were washed with PBS once and collected using trypsin. We stained the smear cells with Diff-Quick (Sysmex, Kobe, Japan), and the number of phagocytic cells containing RBCs was divided by 500 cells and calculated the percentage of EP cells as the EP ratio.

EP cells were suspended in EMEM with 15% FBS, and viable cells were counted using the trypan blue exclusion method [16]. Then, cells were seeded onto 6-well plates at 3×10^5 cells/well. We added drugs or chemicals 1 hr after cell seeding and incubated them for 48 hr. Non-EP cells were used as the controls. The same vehicle solution used to dissolve compounds was added to each untreated control. In this study, we used 0 to 50 μM DHA and ATS, 0 to 50 μg/m*l* CCNU, 0 to 25 *n*g/m*l* vincristine, 1 mM ALA and 0.5 mM SA. Changes in cell number were analyzed by trypan blue staining. The 50% inhibitory concentration (IC_{50}) was calculated on the basis of mean viable cell count standardized to the number of control cells.

All data are presented as the mean ± standard deviation (mean ± SD) from individual experiments repeated at least 3 times. Differences between groups were analyzed using Student's *t*-test with a *P*-value less than 0.05 considered statistically significant.

In our preliminary experiment, we performed EP treatment as described for mouse RBCs above, but observed a remarkable degree of hemolysis in canine RBCs. This indicates that the optimal conditions for EP treatment of canine RBCs are different from those used to treat mouse RBCs. When we changed the $CaCl_2$ concentration to 0.1 mM, the maximum mean EP ratio was 72.2 ± 3.6% (Table 1, Fig. 1). Therefore, we used 0.1 mM $CaCl_2$ and 0.5 μM calcium-ionophore for

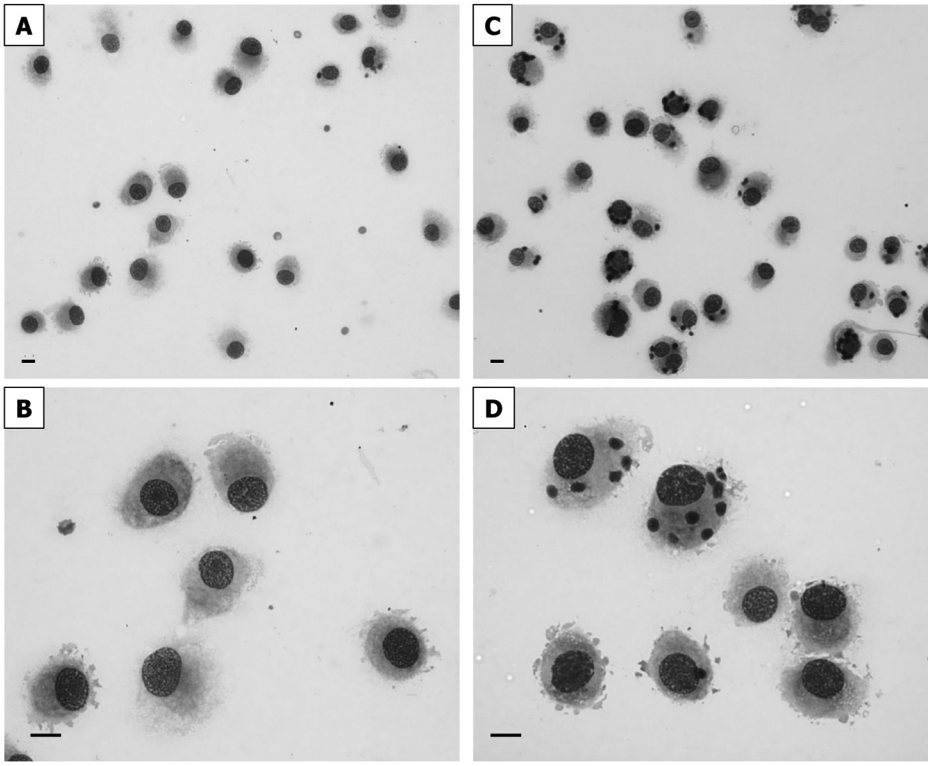

Fig. 1. Microscopic image of erythrophagocytosis by DH82 cells incubated for 3 hr with canine RBCs which treated with 0.5 μM calcium-ionophore A23195 (A, B) or 0.5 μM calcium-ionophore A23195 and 0.1 mM $CaCl_2$ (C, D) at 30°C for 16 hr. Then, DH82 treated for 3 min with hemolysis buffer. Low (A, C) and high (B, D) magnification images. Bar=10 μm.

Table 1. Erythrophagocytic (EP) ratio of DH82

$CaCl_2$ (mM)	Calcium-ionophore (μM)	EP ratio (%)
0	0	< 5
0	0.5	14.0 ± 2.0
0.05	0.5	24.1 ± 5.9
0.1	0.5	72.2 ± 3.6
0.2	0.5	66.8 ± 2.4
0.3	0.5	69.0 ± 6.1
0.4	0.5	60.0 ± 5.3

Cells were co-cultured for 3 hr with canine RBCs. RBCs were incubated at 30°C for 16 hr in HEPES buffer supplemented with $CaCl_2$ at various concentrations, and with or without 0.5 μM calcium-ionophore A23195 was then performed.

the aging treatment for canine RBCs for this experiment.

We then measured the proliferation of EP cells and control cells. No significant difference in cell count was observed after 48 hr (Fig. 2). Therefore, EP treatment does not seem to affect the proliferation of DH82 cells. Figure 3 shows the changes in cell viability following treatment with DHA, ATS, CCNU and vincristine. CCNU and vincristine were selected as control drugs, because they are known to be cytotoxic to canine histiocytic sarcoma [14, 17]. The viability of EP cells was significantly decreased by DHA and ATS treatment compared with control cells. The IC_{50} of EP and control cells was 0.7 and 16.9 μM with DHA, 0.8 and 9.8 μM with ATS, 3.0 and 2.8 μg/m*l* with CCNU and 6.6 and 7.3 *n*g/m*l* with vincristine, respectively. Thus, the values of

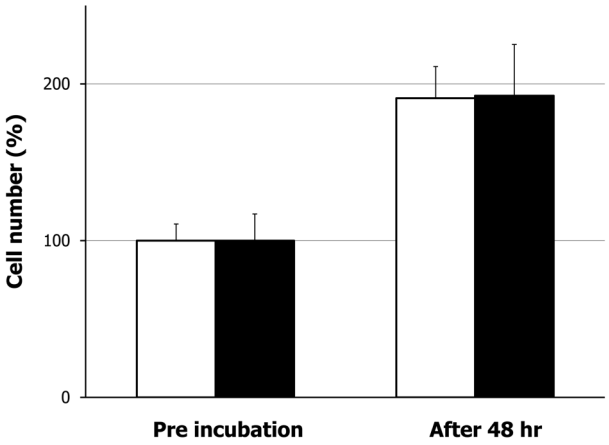

Fig. 2. Cell proliferation of DH82. Cells were incubated for 48 hr, and the cell number was calculated before and after incubation. Values are presented as the mean ± SD, and the number of cells pre-incubation was normalized to 100%. Each bar shows control cells (open bar) and EP cells (black bar).

IC_{50} were approximately 24- and 12-fold higher for DHA and ATS, respectively, in EP cells compared to controls. These results indicate that EP treatment of DH82 increased the cytotoxicity of DHA and ATS, but did not increase that of CCNU and vincristine.

To examine the mechanism of cytotoxicity of DHA and ATS for EP cells, we used ALA and SA to regulate intra-

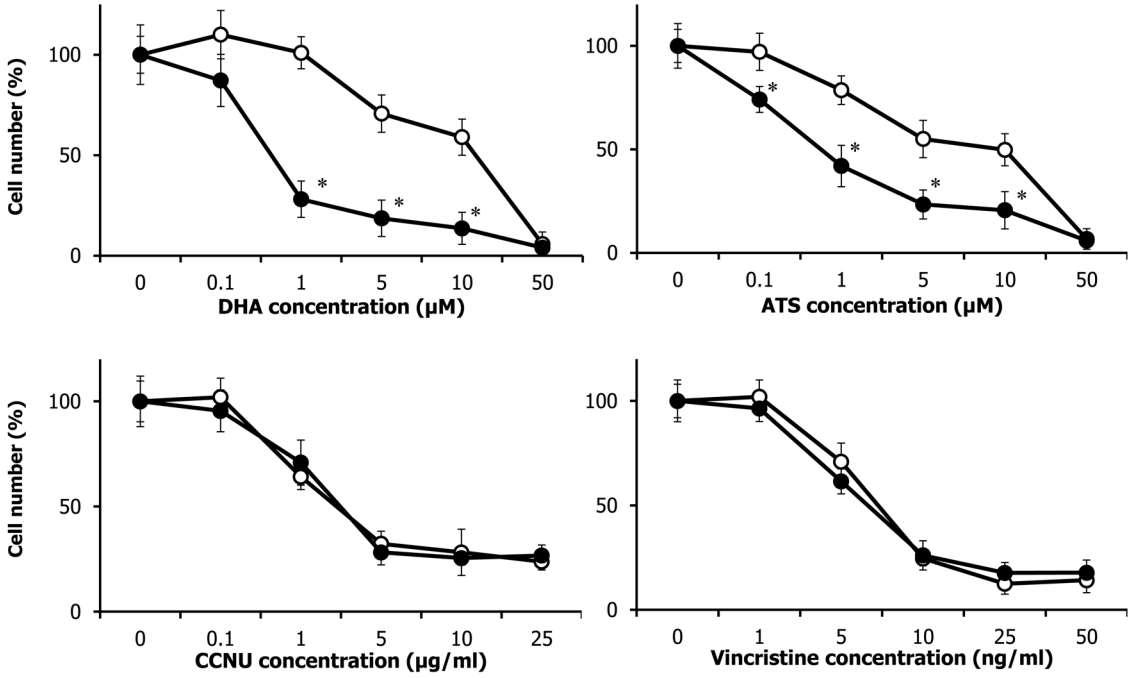

Fig. 3. Changes in viability of EP cells following 48 hr incubation with various concentrations of DHA, ATS, CCNU and vincristine. ○; control cells and ●; EP cells. Values are presented as the mean ± SD. **P*<0.05 versus the same drug concentration of control cells.

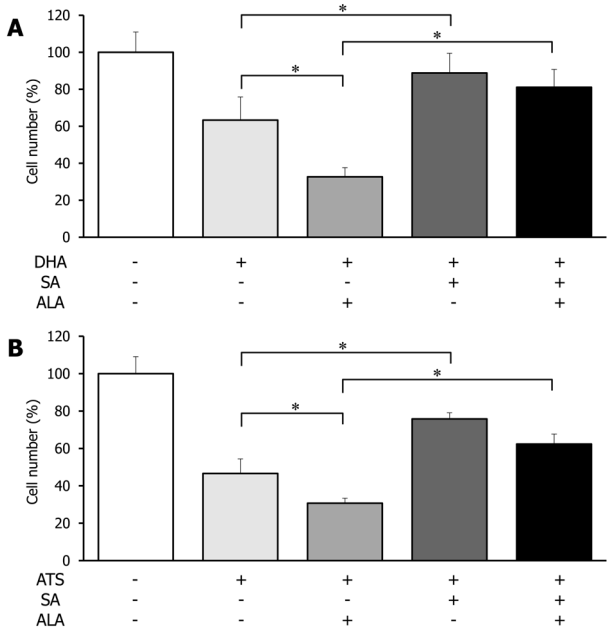

Fig. 4. Effect of SA and ALA on the cytotoxicity of DHA (A) and ATS (B) in control cells. Cell viability was calculated after 48 hr incubation. Values are presented as the mean ± SD. **P*<0.05.

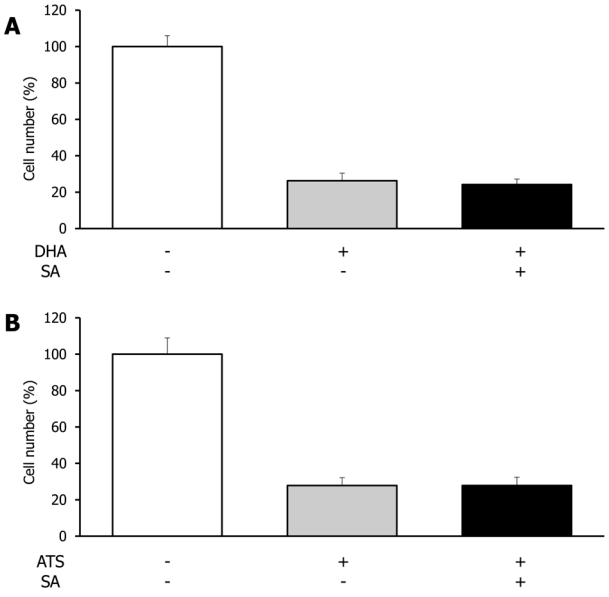

Fig. 5. Effect of SA on the cytotoxicity of DHA (A) and ATS (B) in EP cells. Cell viability was calculated after 48 hr incubation. Values are presented as the mean ± SD. **P*<0.05.

cellular heme synthesis. ALA is a heme precursor and has been used as an agent to increase heme synthesis [6], and SA inhibits the enzyme aminolevulinate dehydratase, thereby preventing heme synthesis [2]. In a previous study, the cytotoxicity of DHA and ATS was increased upon addition of ALA; SA inhibited this effect in human leukemia (Molt-4), breast cancer (MDA-MB-231) and prostate cancer (PC-3) cell lines [20]. Therefore, the mechanism of cytotoxicity of ART is thought to be related to intracellular heme levels. Consistent with previous results, the cytotoxicities of DHA and ATS were enhanced by ALA in the control cells, and this effect was inhibited by SA (Fig. 4). We thus inferred that DH82 cells were susceptible to heme-mediated cytotoxic activity. Interestingly, the attenuation of cytotoxicity of DHA and ATS following SA treatment was not observed in the EP cells (Fig. 5), probably because the EP cells obtain hemoglobin from phagocytized RBCs, which is then resolved into heme and globin, and this exogenous heme might increase the cytotoxicity of ARTs directly.

In conclusion, we found that the cytotoxicity of DHA and ATS was likely increased by heme derived from engulfed RBCs in the DH82 cell line. This finding suggests that heme is a suitable component of novel antitumor therapies in veterinary medicine. Cancer cells have an increased capacity to synthesize heme and increased requirements for iron in order to sustain rapid cell proliferation, and this is the basis for photodynamic therapies for cancer [9, 11]. In addition, erythrophagocytosis of tumor cells was reported to be a feature of canine histiocytic sarcoma [10, 15], and therefore, the findings of our study suggest a strategy for tumor-specific targeting based on the selective elevation of heme in these cells. However, further studies must be performed in order to determine the efficacy with which ARTs induce canine tumor cell death. Such studies, which should be performed in other cell types as well as *in vivo* models, will help define the likely clinical efficiency of ARTs-dependent heme targeting therapies.

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