

## Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells

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### Abstract

Artemisinin becomes cytotoxic in the presence of ferrous iron. Since iron influx is high in cancer cells, artemisinin and its analogs selectively kill cancer cells under conditions that increase intracellular iron concentrations. We report here that after incubation with holotransferrin, which increases the concentration of ferrous iron in cancer cells, dihydroartemisinin, an analog of artemisinin, effectively killed a type of radiation-resistant human breast cancer cell in vitro. The same treatment had considerably less effect on normal human breast cells. Since it is relatively easy to increase the iron content inside cancer cells in vivo, administration of artemisinin-like drugs and intracellular iron-enhancing compounds may be a simple, effective, and economical treatment for cancer. © 2001 Elsevier Science Inc. All rights reserved.

*Keywords:* Human breast cancer cells; Dihydroartemisinin; Holotransferrin

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### Introduction

Artemisinin is a sesquiterpene lactone isolated from the plant *Artemisia annua* L. and is being used for the treatment of malaria [1]. The artemisinin molecule contains an endoperoxide bridge that reacts with a ferrous iron atom to form free radicals [2,3]. Artemisinin is toxic to malaria parasites because they contain a high amount of iron in the form of heme molecules. Generation of free radicals leads to macromolecular damages and cell death [4]. In general, artemisinin and its analogs are relatively safe drugs with no obvious adverse reactions or noticeable side effects [5–9].

Compared to normal cells, most cancer cells have high rates of iron intake [10–11] and express a high cell surface concentration of transferrin receptors [12,13], which, after binding with holotransferrin, transport iron into the cell via a receptor-mediated endocytosis process

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[14]. In general, the aggressiveness and the proliferation index of cancer cells are positively correlated with transferrin receptor concentration [15,16]. Since cancer cells have higher iron influx via the transferrin receptor mechanism, cancer cells should be more susceptible to the cytotoxic effect of artemisinin, under conditions of high iron availability. We have previously reported that artemisinin selectively killed molt-4-lymphoblastoid cells (a human leukemia cell line) after incubation with holotransferrin [17], whereas the same treatment had significantly less effect on normal human lymphocytes. Furthermore, we found that oral administration of an artemisinin analog and ferrous sulfate retarded the growth of implanted fibrosarcoma tumors in the rat [18].

In the present study, we investigated the combined effect of dihydroartemisinin (a more water-soluble analog of artemisinin) and holotransferrin on human breast cancer cells *in vitro* and compared that with the response of normal human breast cells. The dihydroartemisinin/holotransferrin treatment is likely to be selectively toxic to breast cancer cells because breast cancer cells have 5–15 times more transferrin receptors on their cell surface than normal breast cells [13], and transferrin receptors are expressed on the cell surface of breast carcinoma cells but not on benign breast tumor cells [12]. Breast cancer cells have also been shown to take up more iron than normal breast cells [11]. Moreover, the dependence of breast cancer cells on iron intake is suggested by the finding that antibody to transferrin receptors can retard the growth of breast tumors [19]. We tested the treatment on two cell lines: HTB 125, a normal human breast cell line with epithelial cell morphology, and HTB 27, a human breast cancer cell line also with epithelial cell morphology. The HTB 27 cells are very radiation-resistant. We irradiated samples of HTB 27 cells with 500 rads of x-rays (culture medium was aspirated immediately before irradiation and then replenished), and found only a small change in cell counts at 8 and 16 hrs after irradiation (90 and 76% of the counts before irradiation, respectively) (data from three experiments).

## Materials and methods

HTB 125, a normal human breast cell line with epithelial cell morphology, and HTB 27, a human breast tumor cell line with epithelial cell morphology, were purchased from ATCC (Manassas, VA). Leibovitz L-15 medium for growing HTB 27 cells was purchased from GIBCO BRL (Rockville, MD). Hybrcare medium for HTB 125 cells was purchased from ATCC and epidermal growth factor was purchased from Beckton-Dickinson (Bedford, MA). Fetal bovine serum was purchased from ATCC.

HTB 125 cells (normal human breast cells) were grown at 37°C in a humid atmosphere of 5% CO<sub>2</sub> in Hybrcare supplemented with 10% fetal bovine serum and antibiotics. To the medium 30 ng/ml of fresh epidermal growth factor was added. HTB 27 cells (cancer epithelial cells) were grown at 37°C in a humid normal atmosphere in Leibovitz L-15 medium supplemented with 10% fetal bovine serum and antibiotics.

It was necessary to use different culture medium for each cell line as recommended by ATCC. If the same medium were used for both cell lines, one of the cell lines would not be growing in optimal culture conditions and that in turn might have affected our results. Therefore, considering that culture conditions for growth and maintenance were more important, we decided to use media recommended by ATCC for each cell type. However, it is unlikely that dihydroartemisin acts differently in the two different culture media.

After confluency, cells were trypsinized and plated in a ratio of 1:2 to a T-75 flask. For consistency, all experiments were done on cultures at 24 hrs after plating. Cells were counted in one set of flasks. Each flask contained approximately  $1.5 \times 10^6$  cells.

Medium from cultures was aspirated and 20 ml each of the same medium containing human holotransferrin (12  $\mu\text{M}$ , Sigma Chemicals, St Louis, MO) was then added to some flasks. Other flasks were replenished with the same volume of medium (without holotransferrin). Cells were incubated for 1 hr at 37°. At that time, the medium was again aspirated from the cell samples and 20 ml of fresh medium containing dihydroartemisinin (200  $\mu\text{M}$ , Calbiochem-Novabiochem, La Jolla, CA) was added to each of some flasks. Equal volume of the medium was added to each of the remaining flasks. Cells were further incubated for 8 or 16 hr at 37°C. Medium from a flask was then aspirated out and collected in a 50 ml tube. Attached cells were washed twice with 5 ml of PBS (phosphate buffered saline without calcium and magnesium) each time and these washes were collected along with the medium. Five milliliters of freshly made 0.025% trypsin (Amresco, Solan, OH) in PBS at 37°C was added for 5 min to detach cells from the flask. Detached cells from the flask were collected along with the medium and washes. Thus all cells, floating as well detached by trypsin, were collected together. Ten microliters of this cell suspension was loaded on each side of a hemocytometer and all cells (normal, as well apoptotic and necrotic) were counted. Apoptotic and necrotic cells were identified using the morphological criteria of Searle et al. [20]. Experiments were repeated three times. Cell viability was not determined because it is not correlated with cell loss as rapid cell disintegration was observed with the breast cancer cells.

Therefore, there were four treatment conditions: (1) cells treated with holotransferrin (1 mg/ml) alone; (2) cells treated with dihydroartemisinin (200  $\mu\text{M}$ ) alone; (3) cells treated with both holotransferrin and dihydroartemisinin; and (4) cells without drug treatment (control).

To study the replating efficiency of cells, samples of cells (HTB 125 and HTB 27) were treated with 'holotransferrin' or 'holotransferrin + dihydroartemisinin' as above for 8 hrs. They were then trypsinized and cells were counted at 24 hrs after replating.

## Results

Data of treatment with holotransferrin and dihydroartemisinin on human breast cancer cells (HTB 27) (upper graph) and normal human breast cells (HTB 125) (lower graph) are presented in Figure 1. Data in the upper part of Figure 1 show that holotransferrin had no significant effect on breast cancer cells (HTB 27), whereas dihydroartemisinin alone had only a small effect on the cells (cell count was decreased to 72% at 16 hrs). However, breast cancer cells treated with 'dihydroartemisinin + holotransferrin' showed a significant decrease (after 16 hrs of treatment, cell count was only 2% of that at time zero; Mann-Whitney U-test comparing 'dihydroartemisinin + holotransferrin' with 'control',  $p < 0.05$ ). A morphological examination of these cells showed that they were undergoing apoptosis and necrosis. However, during counting we only observed a small number of typical apoptotic or necrotic cells from cultures exposed to holotransferrin and dihydroartemisinin. We did observe numerous small apoptotic bodies. This could be due to floating and fragile nature of dead cells in monolayer cultures. Another reason could be due to rapid disintegration of such cells after holotransferrin and dihydroartemisinin treatment.

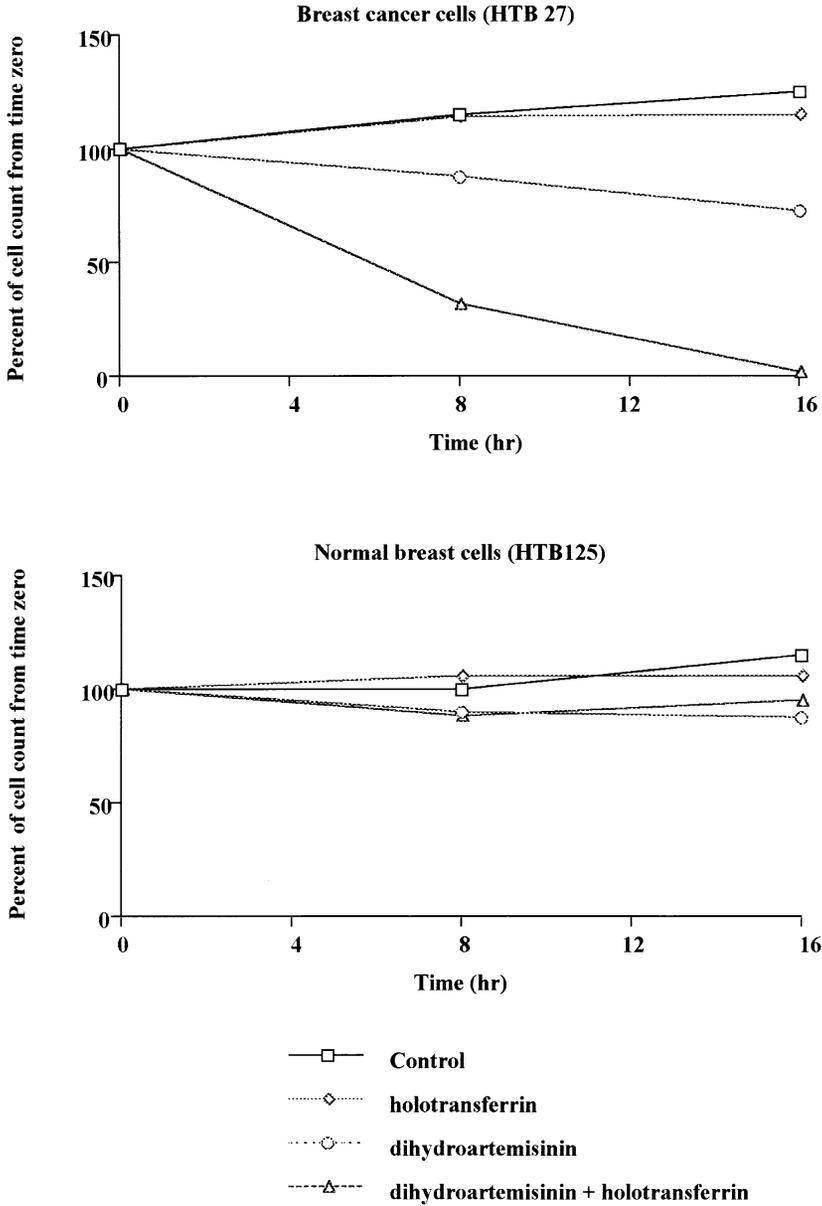


Fig. 1. Effects of treatment with holotransferrin and dihydroartemisinin (200  $\mu$ M) on human breast cancer cells (HTB 27) (upper graph) and normal human breast cells (HTB 125) (lower graph). Data are presented as percentage of cell count at time zero (100%), which is 1 hr after addition of holotransferrin and immediately before the addition of dihydroartemisinin. Each data point represents the mean of data from three experiments.

The lower part of Figure 1 shows the responses of the normal breast cells (HTB 125). The different treatments (holotransferrin; dihydroartemisinin; and ‘holotransferrin + dihydroartemisinin’) had non-significant effect on these cells.

Figure 2 presents the data of replating of human breast cancer cells (upper graph) and nor-

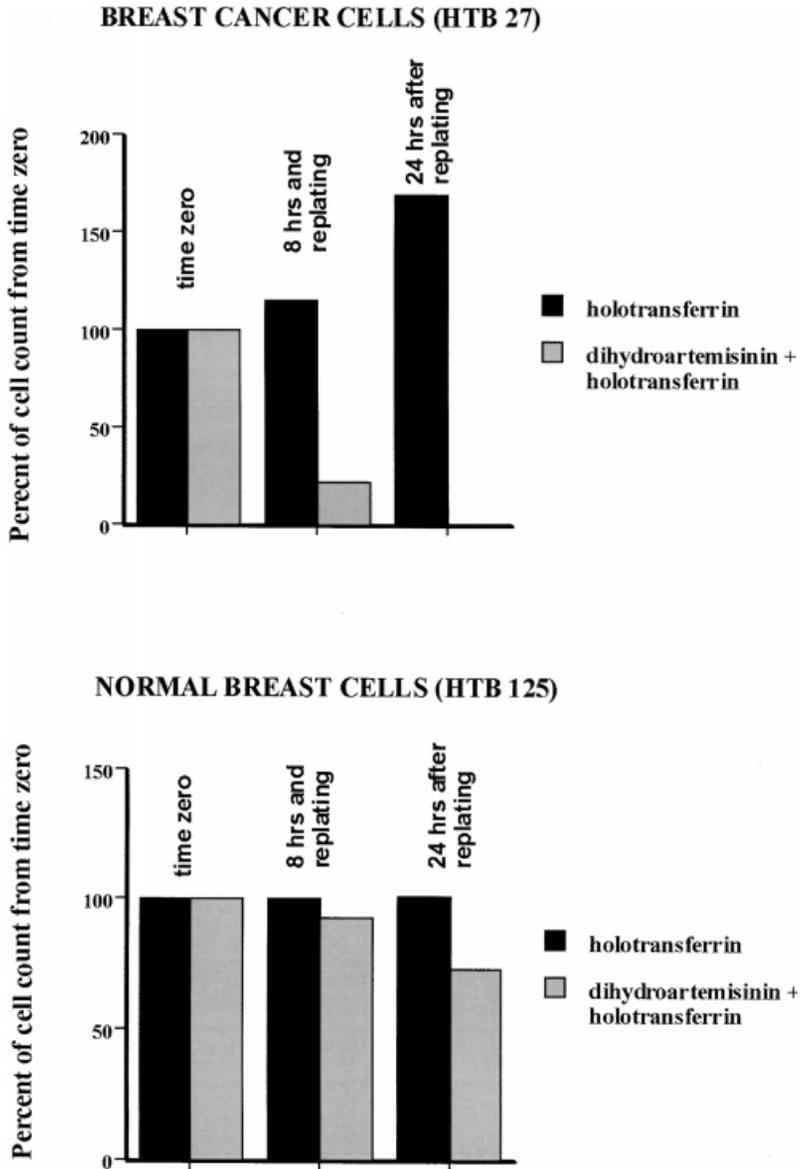


Fig. 2. Replating of human breast cancer cells (HTB 27) (upper graph) and normal human breast cells (HTB 125) (lower graph) at 8 hrs after addition of dihydroartemisinin to the medium (i.e., ‘time zero’, when cell counts were presented as 100%). Cell counts were made at 24 hrs after replating. Data of ‘holotransferrin + dihydroartemisinin’-treated samples were compared with those of ‘holotransferrin’ alone treated samples. Each data point represents the mean of data from three experiments.

mal human breast cells (lower graph) at 8 hrs after addition of dihydroartemisinin to the medium. Data in the upper part of Figure 2 show that breast cancer cells treated with holotransferrin alone for 8 hrs are viable, and that active cell proliferation occurred. At 24 hrs after replating, cell counts had increased to 150% of time zero. However, after ‘holotransferrin +

dihydroartemisinin' treatment, the cell count was zero at 24 hrs after replating. This indicates that breast cancer cells were completely non-viable after 8 hrs of treatment with holotransferrin and dihydroartemisinin.

Data on replating of normal breast cells are shown in the lower part of Figure 2. Cells from both 'holotransferrin' and 'holotransferrin + dihydroartemisinin' treatments grew normally after replating. There was a slight decrease in cells treated with 'holotransferrin + dihydroartemisinin', suggesting that the treatment did damage some of the normal breast cells.

## Discussion

These data indicate that 'holotransferrin + dihydroartemisinin' treatment is selectively toxic to human breast cancer cells, and with relatively low toxicity on normal human breast cells. This may suggest a simple and novel method for the treatment of breast cancer: iron content in breast tumors can be increased by oral administration of an iron salt, such as ferrous sulfate or ferrous citrate, thus making the cells more susceptible to the cytotoxic effect of artemisinin-like compounds.

Artemisinin and its analogs have been used for the treatment of more than one million cases of malarial infection [21]. More potent analogs of artemisinin and similar compounds are also available and are being developed [22–25]. However, it must be pointed out that different analogs should be tested for their suitability for cancer treatment. High doses of the analogs arteether (20 mg/day for 8 days) have been reported to induce neurological deficits in the dog, and artemether (12.5 to 50 mg/kg/day for 28 days) to induce neurological syndromes in the rat [26], but oral and subcutaneous administration of the synthetic analog RO42-1611 (arteflene) to rats at 400 mg/kg/day for 4 weeks was well tolerated and did not induce any mutagenic effect [27].

Since only one-third of the transferrin molecules in the circulation are normally saturated with iron, the concentration of holotransferrin (iron-loaded transferrin) can be increased by administration of an iron salt orally. This, in turn, will lead to an increase in iron transport in cancer cells and will also enhance the cells' susceptibility to artemisinin. This has been feasible in our previous *in vivo* experiment [18]. Thus, using a proper dosing schedule, oral or parenteral administration of artemisinin analogs plus a ferrous salt has the potential to be a powerful anti-cancer therapy. The treatment can be an economical addition or alternative for traditional chemotherapy, and may also be useful for the prevention of cancer and its metastasis. Furthermore, the effectiveness of artemisinin and its analogs can be enhanced by increasing oxygen tension, decreasing intake of antioxidants, and blockade of peroxidase and catalase by drugs such as micronazole [28–30]. Thus, the anticancer efficacy of artemisinin/intracellular-iron enhancer treatment can be explored in future studies.

## Acknowledgment

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