Artemisinin-Transferrin Conjugate Retards Growth of Breast Tumors in the Rat

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Abstract. *Background: Artemisinin is a compound isolated from the wormwood Artemisia annua L. It reacts with iron and forms cytotoxic free radicals. It is selectively more toxic to cancer than normal cells because cancer cells contain significantly more intracellular free iron. Previously, we found that covalently tagging artemisinin to transferrin enhanced the selectivity and toxicity of artemisinin toward cancer cells in vitro. In the present research, artemisinin-transferrin conjugate was tested in a rat breast cancer model. Materials and Methods: Breast tumors were induced in rats by subcutaneous implantation of rat MTLn3 breast cancer cells. Once tumors were formed, daily intravenous injections of artemisinin-transferrin conjugate were administered. Results: The conjugate significantly retarded the growth rate of breast tumors in the rat. No significant side effect was observed in the rats during treatment. Conclusion: Artemisinin-transferrin conjugate could be developed into a potent therapeutic agent for cancer in humans.*

Artemisinin is a small molecule (MW 282) isolated from the wormwood plant *Artemisinin annue* L. It contains an endoperoxide moiety that reacts with atomic iron to form cytotoxic free radicals. Artemisinin is used as an antimalarial and kills malaria parasites by reacting with heme iron inside the parasite (1). We first proposed that artemisinin could be a selective anticancer compound (2) because cancer cells contain significantly more free iron than normal cells. In an *in vitro* experiment, we found that artemisinin selectively killed human leukemia cells and was significantly less toxic to normal lymphocytes. In addition, increasing intracellular iron by addition of the iron carrying plasma protein transferrin to the culture medium further enhanced the toxicity of artemisinin toward leukemia cells (2). A subsequent experiment confirmed that artemisinin is more toxic to human breast cancer cells than to normal human breast cells and the effect was enhanced by addition of transferrin (3). We demonstrated that artemisinin induces apoptosis in cells by reacting with intracellular iron (4).

Transferrin is transported into cells *via* a receptor-mediated endocytotic process. Iron is released from transferrin once it is transported inside a cell. Cancer cells, in general, express more cell surface transferrin receptors and uptake significantly more iron than do normal cells (5, 6). We proposed that the selectivity and toxicity of artemisinin toward cancer cells could be further enhanced by covalently tagging artemisinin to transferrin. Thus, artemisinin would be endocytosed into cancer cells as a pro-drug. Once inside the cell, when iron is released from transferrin, it would react immediately with artemisinin and cause the formation of cytotoxic free radicals. We covalently tagged artemisinin to the carbohydrate moiety of transferrin molecules. We found that the conjugate compound was more potent and selective than artemisinin in killing cancer cells *in vitro* (7-9). More recent research confirmed that artemisinin-tagged transferrin was transported into cancer cells *via* transferrin receptors and indeed induced apoptosis (10). In the present experiment, artemisinin-transferrin conjugate was tested in a rat model of breast cancer. As a comparison, we also tested dihydroartemisinin, an analog of artemisinin, on this animal cancer model.

Materials and Methods

Animals. Female Fisher-344 rats (Charles River Laboratories, Wilmington, MA, USA), ranging in body weight from 130 to 150 g at the start of experiments, were used. Experiments were carried out in a specific pathogen-free laboratory. Rats were fed Purina rat chow and given water *ad libitum* during the course of the experiment. All animal-use procedures had been reviewed and approved by the Animal Use and Care Committee of the University of Washington prior to experiments.

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Synthesis of artemisinin-conjugate of rat transferrin. Purification of rat transferrin (rTf) from rat serum (Innovative Research, Inc., Novi, MI, USA) was conducted as described elsewhere (11, 12).

Purified rTf (2.7×10^{-4} M, 900 μl in 0.1 M sodium acetate, pH 5.5) was mixed with 500 mM sodium periodate (225 μl) (Sigma-Aldrich) (final concentration, 100 mM) for 2 h at room temperature. The mixture was applied to a Sephadex G-25 column (1.8×25 cm) at 4˚C, and oxidized rTf was eluted with 0.1 M sodium acetate buffer (pH 5.5).

Artelinic acid hydrazide solution in dimethyl sulfoxide (DMSO) (3.6×10^{-2} M, 117 μl) was mixed with the oxidized rTf (8.5×10^{-5} M, 900 μl) for 24 h at room temperature. After the reaction period, the mixture was centrifuged (2 min at 7,800×g) and the supernatant was applied to a Sephadex G-25 column at 4˚C, and eluted with DPBS (Dulbecco's phosphate-buffered saline) (pH 7.2). The eluted sample was concentrated using a Microcon YM-10 centrifugal filter device (Millipore, Billerica, MA, USA). The resulting artemisinin-tagged rTf was characterized by a matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometer (TOFMS) to determine the molecular weight. The average number of artemisinin units per rTf was ca. 3.9, calculated based on the difference in mass between rTf (74866.5) and artemisinin-tagged rTf (ART-Tf) (76544.0).

Procedures of in vivo experiment. MTLn-3 cells, a rat mammary adenocarcinoma cell line, were grown in Eagle's alpha-modified minimal essential medium (MEM) (Invitrogen, Calsbad, CA, USA) supplemented with 10% fetal bovine serum. Subcutaneous breast tumors were produced by implanting approximately 10^6 cells from exponential cultures into the flank of an animal. After implantation, rats were monitored on a daily basis to check for tumor development and body weight. When the tumors had grown to approximately 1 cm in diameter, daily drug treatment began.

Rats were randomly assigned to one of the following treatment groups: Group 1: daily intravenous injection (*via* a tail vein) of the artemisinin-transferrin conjugate at 1.0 mg (13 nmol)/day dissolved in phosphate buffer (pH 7.4) in a volume of 0.1 ml; Group 2: intravenous injection of 0.1 ml of the buffer alone; Group 3: daily oral intubation of 20 mg/kg of dihydroartemisinin (DHA; Holley Pharmaceuticals, Fullerton, CA, USA) suspended in olive oil and intubated in a volume of 1 ml/kg using an 8-French feeding tube; Group 4: daily intubation of 1 ml/kg of olive oil alone. Daily drug treatment continued for 5 days. Tumor size was measured daily. The length and width of the ellipsoidal tumor were measured with a caliper. Tumor volume was calculated using the formula: length x width^2 x $\pi/6$. Data from each rat were expressed as percentage change in tumor volume from day one, which was measured immediately before the first treatment was administered.

Data analysis. Tumor growth curves from the treatment groups were compared using the nonparametric method of Krauth (13), comparing the levels of the curves (a_o) using a one-tailed Mann-Whitney *U*-test. A difference at $p<0.05$ was considered statistically significant.

Results

Results of daily intravenous injection of the artemisinin-transferrin conjugate on growth of breast tumors in the rats are shown in Figure 1. The treatment significantly retarded the growth of tumors ($p=0.0039$) compared with control. Actually, an initial decrease in tumor size was observed.

Results of oral administration of DHA are shown in Figure 2. Dihydroartemisinin also significantly retarded tumor growth ($p=0.026$) compared with control, but to a lesser extent than that observed with the artemisinin-transferrin conjugate.

In both experiments, no significant differences in body weights or visible side-effects were observed between drug-treated and control animals.

Discussion

Our earlier *in vitro* study (7) showed that artemisinin-transferrin conjugate is more potent than artemisinin in killing cancer cells. This is also true in this rat breast cancer model. We cannot make direct comparison based on our results in this experiment because only one single dose of each drug was studied and the drugs were administered to the rats by different routes because the conjugate cannot be given orally and DHA cannot be administered intravenously. However, since only 13 nmol of the conjugate was administered to a rat daily, the amount was sufficient to stop the tumors from growing, whereas an oral dose of 20 mg/kg/day DHA, which is a considerably high dose, only retarded tumor growth by approximately 25% .

In order to treat cancer, the killing rate of a drug on cancer cells should be at least the same as or faster than the growth rate of the cells in the tumor. Artemisinin and its analogs have short half-lives in the body (14). Therefore, they are not sufficiently efficient for cancer treatment unless they are given frequently or at high doses. Another drawback is that artemisinin becomes less effective after repeated administration due to induction of degradation enzymes (15, 16). One way to circumvent this is to design artemisinin compounds with high potency, selectivity and long half-lives. The artemisinin-transferrin conjugate may provide an answer to this problem. The conjugate takes advantage of the transferrin receptor mechanism to deliver artemisinin into cancer cells *via* endocytosis. Artemisinin-transferrin conjugate probably has a very long half-life similar to that of transferrin that can remain in the circulation for days. In addition, tagging artemisinin to a macromolecule may also enhance its targeting to solid tumors due to the enhanced permeability and retention effect (17).

Artemisinin can retard cancer growth but not completely stop or reduce the growth. This is shown by the data of this experiment on breast cancer and on fibrosarcoma in rats reported previously by us (18). In most studies on the effect of artemisinin analogs on tumor growth in animals, an approximately 20-60% reduction in growth was generally observed in colorectal carcinoma xenografts (19), hepatoma

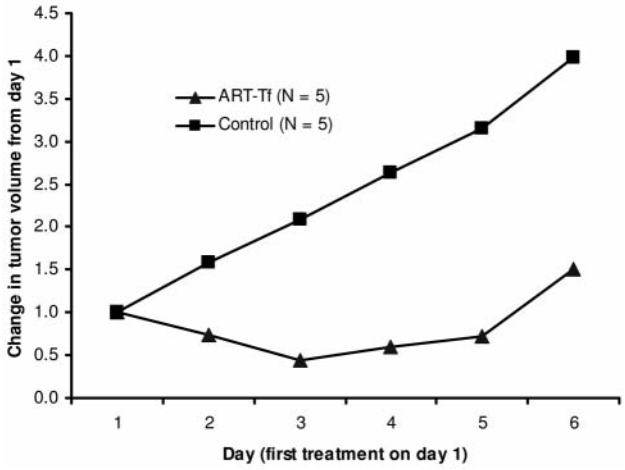

Figure 1. *Effect of five daily intravenous injections of artemisinin-transferrin conjugate (ART-Tf) (13 nmol/day i.v. dissolved in 0.1 ml of phosphate buffer) on growth of breast tumor in the rat. Controls were similarly injected with 0.1 ml of phosphate buffer alone.*

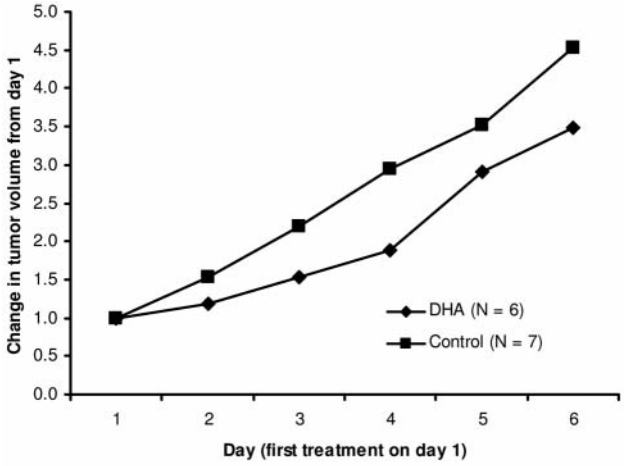

Figure 2. *Effect of five daily administrations of dihydroartemisinin (DHA) (20 mg/kg/day p.o. suspended in olive oil and intubated at a volume of 1 ml/kg) on growth of breast tumor in the rat. Controls were similarly intubated with 1 ml/kg of olive oil alone.*

xenograft (20), ovarian cancer (21), and HL-60 human leukemia xenograft (22). Exceptions are a study by Willoughby *et al*. (23) in which a complete elimination of prostate cancer xenograft in mice was reported after treatment with artemisinin, and that of Wang *et al*. (24) in which no significant effect on the growth of implanted Lewis lung cancer in mice was found with artemisinin treatment. Once the tumor is established, artemisinin is not very effective in reversing the progress. However, artemisinin is effective in the prevention of cancer when the target cells are still small in number. This is shown in our study (25) on the prevention of breast cancer development in rats and a study by Disbrow *et al*. (26) on formation of papillomavirus-induced tumor in the dog.

However, artemisinin compounds have been shown to have anti-angiogenesis (27-29), anti-inflammatory (30, 31) and anti-metastasis (20, 32, 33) properties, all of which are favorable anticancer properties. These properties most likely are not mediated by transferrin receptor mechanisms. Thus, artemisinin-transferrin conjugate, even with potent cytotoxicity towards cancer cells, would probably not have these other anticancer properties. A promising artemisinin compound with high cancer cell toxicity and also prossessing these anticancer properties may come from a group of dimeric compounds being developed by various investigators (34, 35).

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