DNA Damage in Dihydroartemisinin-resistant Molt-4 Cells

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Abstract. *Artemisinin generates carbon-based free radicals when it reacts with iron, and induces molecular damage and apoptosis. Its toxicity is more selective toward cancer cells because cancer cells contain a higher level of intracellular free iron. Dihydroartemisinin (DHA), an analog of artemisinin, has selective cytotoxicity toward Molt-4 human lymphoblastoid cells. A major concern is whether cancer cells could develop resistance to DHA, thus limiting its therapeutic efficacy. We have developed a DHA-resistant Molt-4 cell line (RTN) and found out that these cells exhibited resistance to DHA but no significant cross- resistance to artemisinin-tagged holotransferrin (ART-TF), a synthetic artemisinin compound. In the present study, we investigated DNA damage induced by DHA and ART-TF in both Molt-4 and RTN cells using the comet assay. RTN cells exhibited a significantly lower level of basal and X-ray-induced DNA damage compared to Molt-4 cells. Both DHA and ART-TF induced DNA damage in Molt-4 cells, whereas DNA damage was induced in RTN cells by ART-TF, and not DHA. The result of this study shows that by the cell selection method, it is possible to generate a Molt-4 cell line which is not sensitive to DHA, but sensitive to ART-TF, as measured by DNA damage.*

Artemisinin, a well-known anti-malarial, is a natural sesquiterpene lactone isolated from the plant *Artemisia annua* L. (1). Artemisinin and its derivatives also have been reported to exhibit selective anticancer activity *in vitro* (2, 3), *in vivo* (4, 5), and in patients with cancer (6-8). We have reported that dihydroartemisinin (DHA), an analog of artemisinin, has selective cytotoxicity against Molt-4 human lymphoblastic leukemia cells by inducing apoptosis (9).

Artemisinin contains an endoperoxide moiety that could react with intracellular free ferrous iron to generate carbon-based free-radicals. These free radicals can induce molecular damage, including DNA damage, which eventually accounts for its cytotoxicity (10). In mammalian cells, iron is transported *via* receptor-mediated endocytosis of the iron-carrying plasma protein holotransferrin. Cancer cells express higher levels of transferrin receptors compared to normal cells and pick-up more iron, which is required for rapid cell division (11, 12). Having a higher intracellular iron level, therefore, makes cancer cells more susceptible to artemisinin cytotoxicity. Due to its high specificity against cancer cells, artemisinin and its derivatives, including DHA, are potentially effective cancer chemotherapeutic drugs.

However, for successful chemotherapy, drug resistance is considered to be a major obstacle. Drug resistance to most anticancer agents occurs; hence it is likely that cancer cells could also develop resistance to artemisinin-like compounds. Increased tolerance to DNA damage and increased DNA-damage repair might significantly contribute to drug resistance (13, 14). In an effort to overcome such chemoresistance, it is critical to investigate DNA damage in human cancer cell lines in response to artemisinin-like compounds.

The most widely accepted method for assessing DNA damage is the comet assay (15). The major advantage of the comet assay over other methods of measuring DNA damage is that it has the capability to identify DNA damage at the single-cell level. Another significant advantage is that it has the sensitivity for detecting low levels of DNA damage (16, 17). Moreover, only a small number of cells need to be processed, allowing for analysis of various experimental conditions within a short period of time.

We established a DHA-resistant Molt-4 cell line (RTN) and found that the half maximal inhibitory concentration (IC_{50}) of DHA for RTN cells is 7.1-times higher than that for Molt-4 cells, and it has a higher growth rate than Molt-4 (18). We have also shown that RTN cells do not exhibit resistance to artemisinin-tagged holotransferrin (ART-TF) (19), an artemisinin compound we developed in our laboratory. ART-TF was 76-times more effective in killing RTN cells, when compared with DHA (18).

In the present study, we investigated the level of DNA-damage induced by DHA and ART-TF in both Molt-4 and RTN cells using the alkaline comet assay that measures DNA single-strand breaks in cells. In this assay, cells with high

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levels of DNA damage display extended migration of DNA from the nucleus when a low electrical current is applied (20). Quantification based on size and the intensity of the comet tail provides a comparative index of DNA damage induced by DHA and ART-TF on Molt-4 and RTN cells.

Materials and Methods

Chemicals. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

Molt-4 cell culture. Molt-4 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in RPMI-1640 media (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (ATCC) at 37˚C with 5% CO_2 in air and 100% humidity.

Development of a DHA-resistant RTN cell line. DHA-resistant RTN cell line was developed as previously described (18). Briefly, Molt-4 cells were exposed to increasing concentrations of DHA (Holley Pharmaceuticals, Chongqing, China): three times each for 24 h at 25, 50, 75, and 100 μM. At each step, after exposure to DHA, cells were washed by centrifugation. Pellets were re-suspended in DHA-free RPMI-1640 medium. Until the surviving cells exhibited normal exponential growth, cells were then exposed again to DHA. After all the steps, the surviving cells were washed and cultured.

Treatment of Molt-4 and RTN cells. Molt-4 and RTN cells were pre-incubated for 24 h, at a density of approximately 1 to 1.5×10^5 cells/ml, allowing the cells and media to be conditioned prior to drug treatment. Cells were aliquoted (1 ml) into microfuge tubes prior to drug treatment. At this time, cells were in the log phase of growth. Molt-4 and RTN cells were treated with DHA and ART-TF [synthesized as previously described (19)]. DHA was dissolved in dimethyl sulfoxide (DMSO) and ART-TF in phosphate-buffered saline (PBS). Both Molt-4 and RTN cells were exposed to three treatment conditions: control, 6.2 μM DHA, and 6.2 μM ART-TF; control samples had no drug treatment. The final concentration of DMSO and PBS in the samples was 1% and 10%, respectively. The cells were incubated with the drugs for 24 h and DNA damage was immediately measured using the comet assay. Each experiment was conducted three times.

Comet assay. Comet assay was conducted as previously described (16, 21). In brief, treated Molt-4 and RTN cells were embedded in 0.7% agarose and placed on microscopic slides. One Molt-4 and one RTN cell slide without any drug treatment were immediately irradiated three times with 200 rad of X-rays using a Kelley-Koett device (Covington, CT, USA) at a rate of 100 rad/min for 2 min. X-ray irradiation served as a positive control. The cells on slides were then immersed in a lysing solution [1.25 M NaCl, 0.01% sodium lauroyl sarcosinate, 50 mM tetra-sodium salt of ethylenediaminetetra-acetic acid (EDTA), 10 mM Tris, 0.5 mg/ml proteinase K, 1 mg/ml reduced glutathione; pH 10] at 37˚C for 1 h. Slides were put in an alkaline solution (300 mM NaOH, 1 mM EDTA and 0.2% DMSO; pH 13.5) for 20 min, followed by electrophoresis for 20 min (18 V, 0.48 mA). The slides were then immersed twice in 2 mg/ml cetlyltrimethylammonium bromide solution (40 mM Tris; pH 7.4) for 10 min and three times in 75% ethanol solution (20 mM Tris; pH 7.4) for 10 min. After drying overnight, the slides were stained with YOYO-1 dye (Molecular Probes, Eugene, OR, USA).

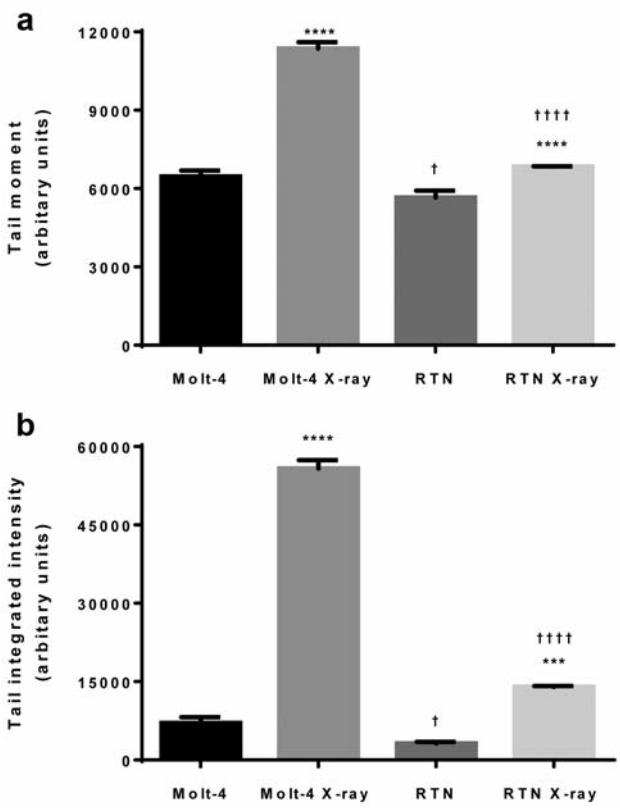

Figure 1. *Mean 'tail moment' (a) and mean 'tail integrated intensity' (b) of the four different treatment groups: Molt-4 control, X-ray-treated Molt-4, RTN control, and X-ray-treated RTN. Error bars denote SEM. ***p<0.001, ****p<0.0001 compared to respective untreated control (Molt-4/RTN) cells; †p<0.05, compared to Molt-4; ††††p<0.0001 compared to Molt-4 X-ray-treated cells.*

Comet assay slide analysis. The VisCOMET image analysis software (Impulse Bildanalyse GmbH, Gilching, Germany) was used to assess DNA damage on microscopic slides. Two parameters, tail integrated intensity and tail moment (Olive) (22), were chosen as the primary indices to quantify DNA damage. 'Tail integrated intensity (Singh)' is an index that incorporates the length and breadth of the tail. The algorithm to measure the tail integrated intensity in VisComet starts from the beginning of the tail and examines each vertical scan line until the end of the tail is reached. From each vertical scan line, the product of the breadth, position and total intensity is accumulated. Each experiment was repeated three times. One slide was prepared in each replicate and 66 cells were scored from each slide. The average 'tail moment' and 'tail integrated intensity'(arbitrary units) for the 66 cells in each slide were used in data analysis.

Data analysis. Data of 'tail integrated intensity' and 'tail moment' are presented as the mean±SEM, with n=3 for each treatment group. GraphPad Prism 6.03 software (La Jolla, CA, USA) was used for

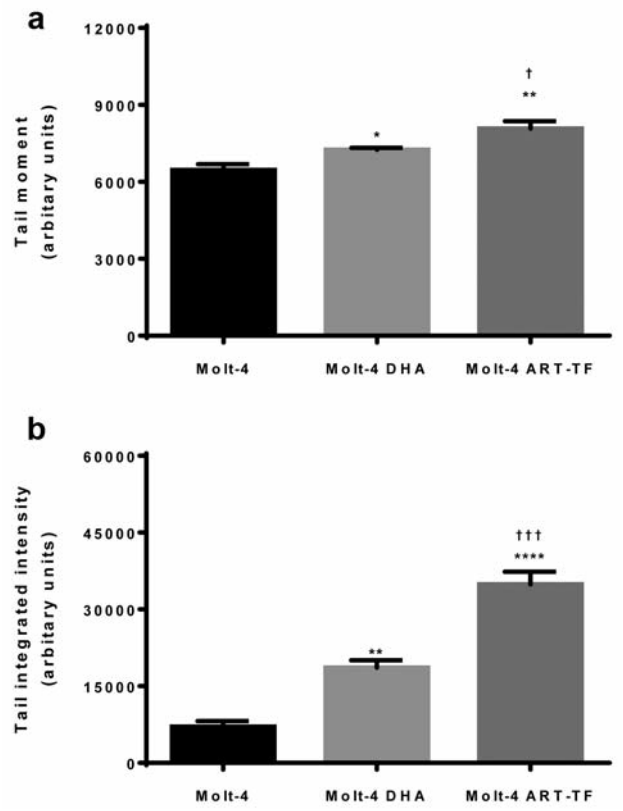

Figure 2. *Mean 'tail moment' (a) and mean 'tail integrated intensity' (b) of control Molt-4 cells and those treated with 6.2 μM dihydroartemisinin (DHA) and 6.2 μM of artemisinin-tagged holotransferrin (ART-TF) for 24 h. Error bars denote SEM. *p<0.05, **p<0.01, ****p<0.0001 compared to control Molt-4 cells; †p<0.05; †††p<0.001 compared to DHA-treated Molt-4 cells.*

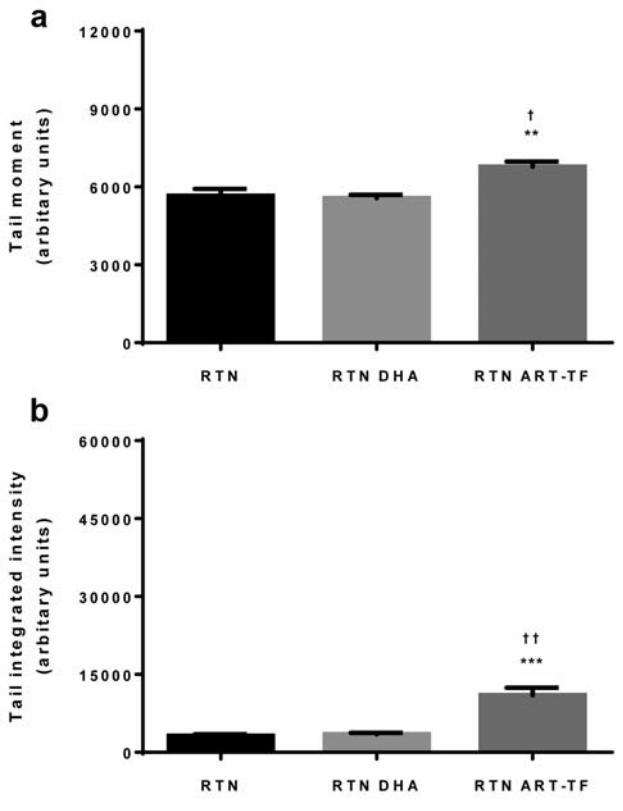

Figure 3. *Mean 'tail moment' (a) and mean 'tail integrated intensity' (b) of control RTN cells and those treated with 6.2 μM dihydroartemisinin (DHA) and 6.2 μM of artemisinin-tagged holotransferrin (ART-TF). Error bars denote SEM. **p<0.01, ***p<0.0001 compared to control RTN cells; †p<0.05; ††p<0.01 compared to DHA-treated RTN cells.*

statistical analysis. One- or two-way ANOVA followed by the Newman-Keuls multiple comparison test were used in data analysis. A difference at $p<0.05$ was considered statistically significant.

Results

We measured basal and X-ray-induced levels of DNA damage in Molt-4 and RTN cells by measuring 'tail moment' and 'tail integrated intensity' (Figure 1). Using these two parameters, we found that Molt-4 cells exhibited significantly greater DNA damage than RTN cells both under control and X-ray-treated conditions (tail moment: control: Molt-4 *vs*. RTN: $p<0.05$, and X-ray: Molt-4 *vs*. RTN: $p<0.0001$; tail integrated intensity: control Molt-4 *vs*. RTN: $p<0.05$ and X-ray: Molt-4 *vs*. RTN: $p<0.0001$). A two-way ANOVA of the data showed a significant interaction effect ($p<0.0001$) indicating that Molt-4 and RTN cells responded significantly differently to X-rays as measured by both tail moment and tail integrated intensity.

To determine the difference in the level of cell type-specific DNA damage in Molt-4 and RTN cells treated with DHA and ART-TF, Molt-4 and RTN cells were treated with the same concentration (6.2 μM) of DHA and ART-TF for 24 h and the cells' tail moment and tail-integrated intensity were measured. DHA and ART-TF were able to induce DNA damage in Molt-4 cells compared to the untreated control (Figure 2). ART-TF induced more DNA damage compared to DHA in Molt-4 cells in terms of tail moment and tail-integrated intensity (tail moment: control *vs*. DHA: $p<0.05$, control *vs*. ART-TF: $p<0.01$; DHA *vs*. ART-TF: $p<0.05$; tail integrated intensity: control *vs*. DHA: $p<0.01$, control *vs*. ART-TF: $p<0.0001$, DHA *vs*. ART-TF: $p<0.001$).

In RTN cells, there was no significant difference ($p>0.05$) in tail moment and tail integrated intensity between untreated and DHA-treated RTN cells (Figure 3). In addition, ART-TF induced significantly greater DNA damage by both parameters when compared to untreated and DHA-treated RTN cells (tail moment: control *vs*. ART-TF: $p<0.01$, DHA

vs. ART-TF: $p<0.05$; tail integrated intensity: control *vs*. ART-TF: $p<0.001$, DHA *vs*. ART-TF: $p<0.01$). Thus, it appears that DHA did not cause a significant change but ART-TF was able to induce a significant increase in DNA damage in RTN cells.

Discussion

Previously, Li *et al*. reported that artesunate, a semi-synthetic derivative of artemisinin, induced DNA damage in VC8 cells (23), and Berdelle *et al*. reported that artesunate induced DNA damage in the human glioblastoma cell line LN-229, as measured by the comet assay (24). In this study, we used the comet assay to determine DNA damage in Molt-4 and RTN cells, and investigated the level of DNA-damage induced by DHA and ART-TF. Previous studies have developed various cancer cell lines that are resistant to artemisinin and its derivatives (25-27). To our knowledge, this is the first time that the level of DNA damage in an artemisinin-resistant cancer cell line was studied. The comet assay results revealed that compared to Molt-4 cells, RTN cells are more resistant to DNA damage both in a normal state and under X-ray irradiation. This may be due to an enhanced DNA repair capability in RTN cells.

However, there was no significant DHA-induced DNA damage in the RTN cells. This may be due to enhanced DNA repair (13, 14), reduced intracellular DHA due to enhanced efflux mediated by cell surface transporters (28), or enhanced antioxidant molecules/enzymes that reduce DHA-generated reactive oxidative species (29, 30).

It is interesting to observe that RTN cells are resistant to DHA but susceptible to ART-TF in terms of DNA damage. The data support our previous finding that ART-TF is more effective in killing RTN cells compared to DHA (18). One possible explanation could be that DHA and ART-TF have different mechanisms of action. While DHA enters the cells by diffusion, ART-TF is transported into cells *via* receptor-mediated endocytosis (31). It would be more difficult to eliminate ART-TF once in the cells. Further molecular studies are needed to determine the mechanisms of cancer cell killing by ART-TF.

Conflicts of Interest

The Authors declare no conflicts of interest with regard to this research.

Acknowledgements

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