Development of a Dihydroartemisinin-resistant Molt-4 Leukemia Cell Line

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Abstract. *Artemisinin generates cytotoxic free radicals when it reacts with iron. Its toxicity is more selective toward cancer cells because cancer cells contain a higher level of intracellular-free iron. We previously reported that dihydroartemisinin (DHA), an active metabolite of artemisinin, has selective cytotoxicity toward Molt-4 human lymphoblastoid cells. A concern is whether cancer cells could develop resistance to DHA after repeated administration, thus limiting its therapeutic efficacy. In the present study, we developed a DHA-resistant Molt-4 cell line (RTN) by exposing Molt-4 cells to gradually increasing concentrations of DHA in vitro. The half-maximal inhibitory concentration (IC_{50}) of DHA for RTN cells is 7.1-times higher than that of Molt-4 cells. RTN cells have a higher growth rate than Molt-4 cells. In addition, we investigated the toxicities of two more potent synthetic artemisinin compounds, artemisinin dimer-alcohol and artemisinin-tagged holotransferrin toward RTN cells; RTN cells showed no significant cross-resistance to these compounds.*

Artemisinin, a compound isolated from the plant *Artemisia annua* L (sweet wormwood), is a well-known anti-malarial (1). In addition, artemisinin also has selective cytotoxicity against cancer cells (2). Artemisinin contains an endoperoxide bridge which reacts with intracellular free ferrous iron to generate free radicals, leading to cell death (3, 4). Iron is transported into cells *via* receptor-mediated endocytosis of the iron-containing plasma protein holotransferrin. Cancer cells have a higher concentration of cell surface transferrin receptors (5, 6).This allows cancer cells to pick-up more iron, required for their rapid cell division. Higher iron contents in cancer cells therefore make them more susceptible to artemisinin cytotoxicity.

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There are numerous *in vitro* (7-9), *in vivo* (10, 11), and cancer patient (12-14) studies supporting the anticancer activity of artemisinin and its derivatives. We have reported that dihydroartemisinin (DHA), a major active metabolite of artemisinin, has selective cytotoxicity against Molt-4 human lymphoblastoid leukemia cells by inducing apoptosis (15). Other studies have also shown that DHA induces apoptosis in other cancer cell lines (16, 17). Artemisinin and its derivatives, including DHA, might be effective cancer chemotherapeutic agents.

A major problem with chemotherapy is that cancer cells can develop drug resistance. Mechanisms explaining this elucidated so far include increased drug efflux, enhanced drug inactivation and DNA repair, apoptosis defects, insufficient drug delivery, and target receptor modification (18-21). Drug resistance occurs to most anticancer agents, thus, it is possible that cancer cells could become resistant to DHA after repeated administration.

In an effort to overcome chemoresistance to DHA, experimental studies using *in vitro* models of DHA-resistant cancer cell lines might provide insight into the effective usage of artemisinin (22). In the present research, we established a DHA-resistant Molt-4 cell line (RTN) by continuously exposing Molt-4 cells to gradually increasing concentrations of DHA *in vitro*. We examined the characteristics of the RTN cell line and compared its responses to DHA and two other synthetic artemisinin derivatives artemisinin dimer-alcohol (dimer-OH) (11) and artemisinin-tagged holotransferrin (ART-TF) (23) that have higher potencies against cancer cells than DHA does.

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). They 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. At a cell density of 6×10^5 cells/ml, they were diluted to a density of 6×10^4 cells/ml approximately 24 h prior to experiments.

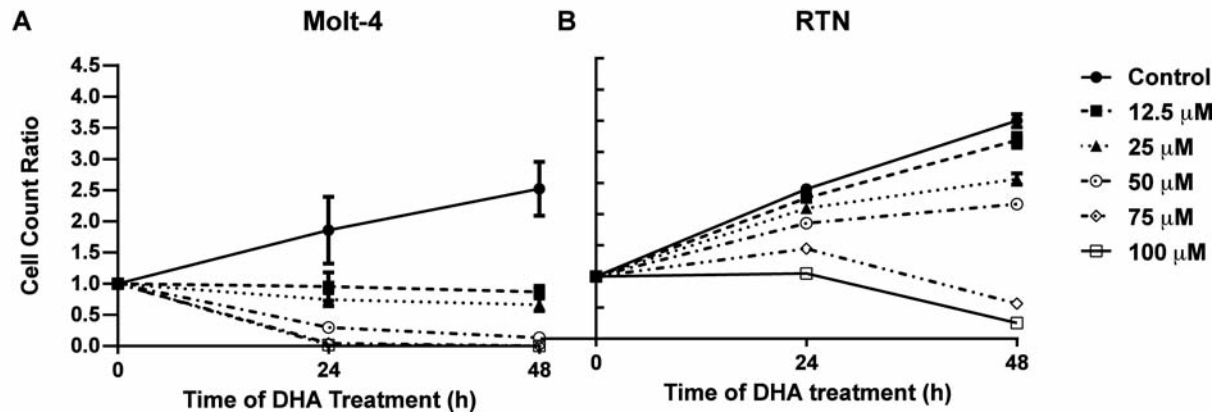

Figure 1. *Time–response plots for different concentrations of dihydroartemisinin (DHA) on Molt-4 (A) cells and RTN cells (B) at different concentrations. Cell count ratio is defined as:* $\left(\frac{\textit{Cell count at time point}}{\textit{Cell count at time zero}}\right)$

Development of a DHA-resistant RTN cell line. Molt-4 cells were first exposed to 25 μM DHA in RPMI-1640 medium for 24 h. Cells then were washed by centrifugation at 870 × *g* for 5 min in a microfuge (Sorvall Microspin, model 245). Pellets were then re-suspended in fresh RPMI-1640 medium. Until the surviving cells completely recovered and showed a normal exponential growth, cells were then exposed again to 25 μM DHA for 24 h. This process was repeated two more times. The cells were then exposed to increasing concentrations of DHA: three times each (3×24 h) at 50 μM, 75 μM, and 100 μM. Surviving cells were washed and cultured in a DHA-free medium. The resulting cell line was named RTN.

Drug testing of Molt-4 and RTN cells. Molt-4 and RTN cells were pre-incubated for 24 h, which allowed cells and media to be conditioned prior to drug treatment at a density of approximately 1×10^5 to 1.5×10^5 cells/ml. 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 (Holley Pharmaceuticals, Chongqing, China), dimer-OH (synthesized as previously described) (11) and ART-TF (synthesized as previously described) (23) to compare their cytotoxicities.

To compare the cytotoxicity of DHA on Molt-4 and RTN cells, cells were incubated with different concentrations of: DHA (12.5, 25, 50, 75, and 100 μM), dimer-OH (0.5, 1, and 2 μM) and ART-TF (1, 2, and 4 μM). Control samples had no drug treatment. Dihydroartemisinin and dimer-OH were dissolved in dimethyl sulfoxide (DMSO) and ART-TF in phosphate-buffered saline (PBS) before adding to cell samples. The final concentration of DMSO in samples was 1%. Molt-4 and RTN cell numbers were counted using a hemocytometer immediately prior to drug treatment (0 h) and at 24 and 48 h of drug treatment. To assess cell viability, we used trypan blue exclusion. Only viable cells were counted.

Data analysis. Each experiment was conducted three times. The mean and standard deviation were calculated. Responses were calculated as the ratio of viable cell counts at 24 and 48 h time points relative to the viable cell count at 0 h. Log dose–response and time–response curves were plotted using the ratios. GraphPad Prism 6.03 software (La Jolla, CA, USA) was used for statistical analysis. Time response curves were compared by the method of Krauth (24). The a0 of the orthogonal polynomial coefficient of the curves, were compared using the Mann–Whitney *U*-test. Log dose–response curves of drug treatment were plotted. Half maximal inhibitory concentrations (IC_{50}s) were determined and compared using the two-tailed Student's *t*-test. A difference at $p<0.05$ was considered statistically significant.

Results

Figure 1 shows the growth responses to DHA of Molt-4 cells (Figure 1A) and RTN cells (Figure 1B) with time. A dose-dependent growth inhibition by DHA was observed in both Molt-4 and RTN cells. In addition, in control samples (*i.e.* cultures not treated with drug), RTN cells grew significantly faster than did Molt-4 cells ($p<0.05$).

Figure 2 shows the log dose responses to DHA of Molt-4 and RTN cells at the 48-h time point. The IC_{50}s (mean±SD, n=3) of DHA for Molt-4 cells and RTN cells were 5.8±1.7 μM and 41.0±0.2 μM, respectively. RTN cells had a significantly higher IC_{50} value (by 7.1-fold) than Molt-4 cells ($p<0.0001$). Thus, this result indicates that RTN cells had acquired resistance against DHA.

Figure 3 shows log dose-responses of Molt-4 and RTN cells to Dimer-OH at the 48-h time point. The IC_{50}s (mean± SD, n=3) of dimer-OH for Molt-4 cells and RTN were 4.6±1.0 μM and 5.9±2.6 μM, respectively. There was no significant difference between these two values ($p>0.05$).

Figure 4 shows log dose-responses to ART-TF of Molt-4 cells and RTN cells at the 48-h time point. IC_{50} (mean±SD, n=3) of ART-TF on Molt-4 cells and RTN cells were 0.74±0.22 μM and 0.54±0.04 μM, respectively. There was no significant difference between these two values ($p>0.05$).

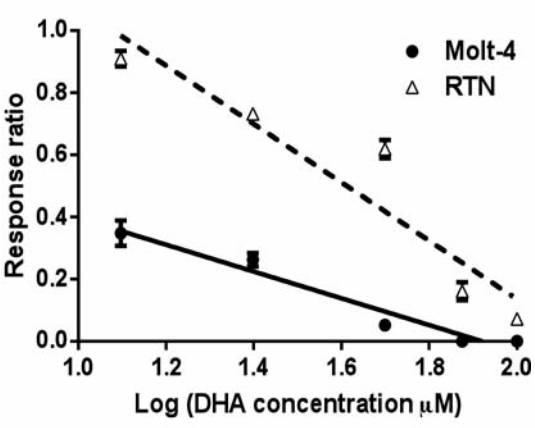

Figure 2. *Log dose–response plots for Molt-4 cells and RTN cells to dihydroartemisinin (DHA) at the 48-h time point. Graph shows the ratio (mean±SD, n=3) at different log concentrations of DHA (12.5, 25, 50, 75, and 100 μM). Ratio is defined as:* $\left(\frac{\textit{Cell count of drug treated sample}}{\textit{Cell count of control}}\right)$

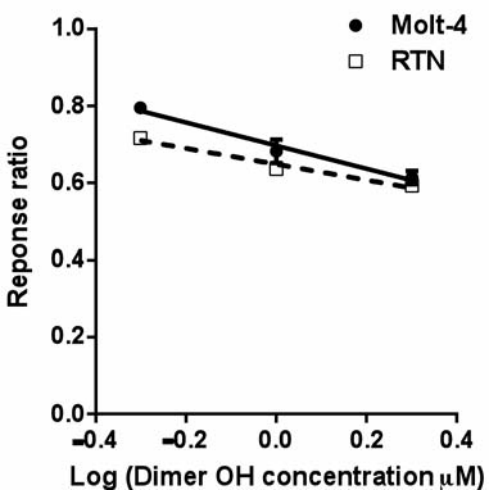

Figure 3. *Log dose–response plots for Molt-4 cells and RTN cells to artemisinin dimer-alcohol (dimer-OH) at the 48-h time point. Graph shows the ratio (mean±SD, n=3) at different log concentrations of dimer-OH (0.5, 1, and 2 μM).*

Discussion

We have established a DHA-resistant Molt-4 cell line termed RTN. Previously, Lu *et al*. developed a DHA-resistant colon carcinoma cell line (HCT116/R) after 45 exposure cycles to DHA (25). They exposed colon carcinoma cells to DHA for 72 h before a washing process for the next increment of DHA concentration. The IC_{50} of the resulting DHA-resistant cells was 4.3-times higher than that of the parent cells. We exposed Molt-4 cells to 25, 50, 75, and 100 μM of DHA for three 24-h cycles for each concentration, with a total of 12 cycles. In our case, a 7.1-fold increase in IC_{50} was found for the resistant cells. It is possible that different cancer cell lines have different susceptibilities to drug-resistance development. Bachmeier *et al*. reported that after 24 h of incubation with artesunate, resistance developed in MDA-MD-231 but not in MDA-MB-468 breast cancer cells (26). Sadava *et al*. reported two small cell lung cancer cell lines with different susceptibilities to artemisinin (27). The IC_{50} of artemisinin for one cell line (H69VP) was 10 times higher than that for the other (H69). Interestingly, pretreatment with transferrin overcame artemisinin-resistance in the H69VP cells.

Our previous results showed that both dimer-OH and ART-TF are more potent than DHA in killing Molt-4 cells (11, 23). This is also true for RTN cells. When comparing IC_{50} values, dimer-OH was significantly more potent than DHA by 7-fold ($p<0.001$), and ART-TF by 76-fold ($p<0.0001$). Furthermore, ART-TF was more potent in killing the cells than dimer-OH. The IC_{50} value of ART-TF for RTN cells was significantly lower than that of dimer-OH by 11-fold ($p<0.05$).

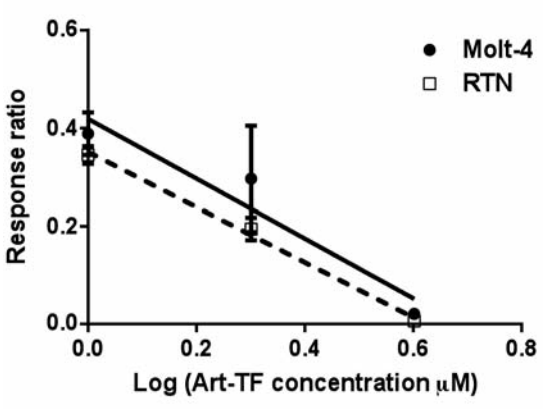

Figure 4. *Log dose–response plots for Molt-4 cells and RTN cells to artemisinin-tagged holotransferrin (ART-TF) at the 48-h time point. Graph shows the ratio (mean±SD, n=3) at different log concentrations of ART-TF (1, 2, and 4 μM).*

We also found that RTN cell count ratio increased faster than that of the Molt-4 cells. There could be two possible explanations for this: RTN cells have a higher division rate; or there is a decreased tendency of RTN cells to die, *e.g*., *via* apoptosis. Since cells that divide at a higher rate should be more susceptible to the toxicity of DHA, the second explanation seems more likely.

It is interesting that RTN cells exhibited resistance to DHA, but not to dimer-OH and ART-TF. This may indicate that these compounds have different mechanisms of action

from that of DHA. Dihydroartemisinin and dimer-OH probably enter cells by diffusion and can reach organelles such as the mitochondria and trigger apoptosis. However, dimer-OH can cause molecular cross-linkings, which are highly cytotoxic. ART-TF is transported into cells *via* receptor-mediated endocytosis. Its cytotoxicity is probably related to membrane damage in endosomes, plasma membrane, and lysosomes. Thus, in the use of artemisinin and its derivatives for cancer treatment, compounds with different mechanisms of action can be used to circumvent development of resistance. In addition, as suggested in malaria treatment using endoperoxides (28), compounds of different chemical structures can be used to overcome resistance.

Conflicts of Interest

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

Acknowledgements

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