Cytotoxicity of Dihydroartemisinin Toward Molt-4 Cells Attenuated by *N-Tert*-butyl-alpha-phenylnitrone and Deferoxamine

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Abstract. *Derivatives of artemisinin, a compound extracted from the wormwood Artemisia annua L, have potent anticancer properties. The anticancer mechanisms of artemisinin derivatives have not been fully-elucidated. We hypothesize that the cytotoxicity of these compounds is due to the free radicals formed by interaction of their endoperoxide moiety with intracellular iron in cancer cells. The effects of N-tert-butyl-alpha-phenylnitrone (PBN), a spin-trap free radical scavenger, and deferoxamine (DX), an iron chelating agent, on the in vitro cytotoxicity of dihyroartemisinin (DHA) toward Molt-4 human T-lymphoblastoid leukemia cells were investigated in the present study. Dihydroartemisinin effectively killed Molt-4 cells in vitro. Its cytotoxicity was significantly attenuated by PBN and DX. Based on the data of our present and previous studies, we conclude that one anticancer mechanism of dihydroartemisinin is the formation of toxic-free radicals via an iron-mediated process.*

Artemisinin, a compound isolated from the wormwood *Artemisia annua L*, is a sesquiterpene lactone peroxide. Artemisinin analogs have been used as anti-malarials, with few side-effects. They have also been shown to have potent anticancer properties (1). The anticancer mechanisms of artemisinin are still being investigated. We have hypothesized that one anticancer mechanism of artemisinin is due to the generation of toxic-free radicals *via* the interaction between its endoperoxide moiety and intracellular iron (2, 3). A high amount of iron is required for DNA synthesis during mitosis in rapidly-dividing cancer cells. Cancer cells have a high concentration of cell surface transferrin receptors that enable a higher iron uptake compared to normal cells (4, 5). The high free iron content in cancer cells makes artemisinin selectively toxic to them in comparison with normal cells.

N-Tert-butyl-alpha-phenylnitrone (PBN) is a spin-trap compound that effectively sequesters free radicals both *in vivo* and *in vitro* (6, 7). Deferoxamine (DX), an iron-chelating agent, has been shown to inhibit DHA-induced apoptosis in HL-60 leukemia cells (8). Dihydroartemsinin (DHA) has been shown to have significant toxic effects toward cancer cells both *in vitro* and *in vivo* (9, 10). In the present study, PBN or DX was added to Molt-4 human lymphoblastoid cells incubated with DHA to inactivate the free radicals generated and to prevent the formation of free radicals, respectively, in order to test our hypothesis.

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Materials and Methods

Molt-4 cell cultures. Molt-4 cells (American Type Culture Collection (ATCC) Manassas, VA, USA) were cultured in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (ATCC). Cells were maintained at 37˚C in 5% CO_2/95% air and 100% humidity, and were split 1:2 at a density of approximately 1×10^6 cells/ml.

Treatment of Molt-4 cells with DHA, PBN, and DX. Stock solution of DHA (Holley Pharmaceuticals, Chongqing, China) was made in DMSO (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions of PBN (Sigma-Aldrich) and DX (CP20L1; a gift from Dr. R.A. Yokel, College of Pharmacy, University of Kentucky, Lexington, KY, USA) were prepared in complete RPMI.

Four treatment conditions: Control, DHA, PBN, and PBN plus DHA, were included in the PBN experiment. The control cultures were not treated with drugs. A similar set up was used for the DX experiment. Molt-4 cells, in complete RPMI-1640 medium, were first incubated for 24 h at 37˚C in a humid atmosphere of 5% CO_2 /95% air. One milliliter of cells each was put in a set of microfuge tubes and then 5 μl of previously prepared stock solutions were added to the tubes, yielding a final concentration of 12.2 μM DHA, and different concentrations of PBN (0.25, 0.5, 1.0 mM) or DX (10, 20 and 30 μM). The final DMSO concentration was maintained from 0.5% to 1% of cell volume because a high concentration of

DMSO can cause inhibition of apoptosis in certain cell types (11). Zero-hour cell counts were performed just before the addition of the test compounds. Cells were again counted at 24-, 48- and 72-h time points thereafter. To minimize damage to the cells during the experiment, indirect light was used. All experiments were performed three times.

Cell counts. Cell numbers were counted from a 10-μl aliquot from each sample using a hemocytometer. Cells were thoroughly mixed by vortex before an aliquot was taken for counting. Dead cells were not counted because of their rapid elimination (12). An increase in count of cells treated with drug combination relative to that of cells treated with DHA alone was used as the index to indicate the inhibitory effect of PBN and DX on cytotoxicity of DHA.

Data analysis. Data are expressed as the percentage cell count from time zero. Time-response curves were compared using the method of Krauth (13). The levels of the curves, ao of the orthogonal polynomial coefficient, were compared using the Mann-Whitney *U*-test. A *p*-value at <0.05 is considered statistically significant.

The percentage reduction in DHA toxicity by each concentration of PBN or DX was calculated using the formula

$$\left[1-\left(\frac{Control-(DHA+PBN\ or\ DX)}{Control-DHA}\right)\right]\times 100$$

Dose-response curves were plotted. The coefficient of correlation (r) of linear regression was determined for each curve.

Results

The percentage cell counts from time zero of the four treatment groups in the experiments with 0.25, 0.5, 1.0 mM PBN as a function of incubation time are plotted in Figure 1A-C, respectively. Dihydroartemisinin (12.2 μM) significantly reduced cell counts when compared with those of the control. Co-treatment with PBN significantly attenuated the cytotoxicity of DHA at all three concentrations studied (PBN-plus-DHA *vs*. DHA, $p<0.05$). There was no significant difference between the group treated with PBN alone and the control group except for 0.25 mM PBN, in which a small but significant decrease in cell count was observed.

The percentage cell counts from time zero of the four treatment groups in the experiments with 10, 20, and 30 μM DX as a function of incubation time are plotted in Figure 1D-F, respectively. Co-treatment with DX significantly attenuated the cytotoxicity of DHA at all three concentrations studied (DX-plus-DHA *vs*. DHA, $p<0.05$). There was no significant difference between cells treated with DX-alone and the control.

The percentage reduction in DHA toxicity by PBN was plotted against the concentrations of PBN (0.25 mM, 0.5 mM, and 1.0 mM) and is shown in Figure 2. A significant dose-response relationship was found (r=0.985, df=3, $p<0.001$). The percentage reduction in DHA toxicity by DX was plotted against the concentrations of DX (10 μM, 20 μM, and 30 μM) in Figure 3. Even though a significant correlation was found (r=0.823, df=3, $p<0.05$), the data actually showed a plateau effect at the dose-range studied.

Discussion

Our results show that both PBN and DX significantly increased the DHA-treated Molt-4 cell count when compared with DHA treatment alone. They show that an anticancer activity of DHA is related to intracellular iron concentration and free radical formation. Our results support the hypothesis that the endoperoxide moiety in DHA interacts with free intracellular iron to generate toxic free radicals. These results are consistent with those of previous research (2, 3, 14, 15). *N-Tert*-butyl-alpha-phenylnitrone, up to 1 mM concentration, as shown in a previous study, has no significant toxicity toward cancer cells *in vitro* (16). Thus, 1 mM was the highest concentration of PBN used in this study.

Iron is required for mitosis. The depletion of iron by a high concentration of DX could also inhibit Molt-4 cell division (17). We observed a decrease (statistically non-significant) in cell counts when the cells were treated with 30 μM of DX alone, as shown in Figure 1F. In another experiment, we have found that DX at 50 and 100 μM could significantly reduce Molt-4 cell growth (data not shown). Therefore, the highest concentration of DX used in this study was 30 μM. Two processes, namely, free radical- mediated apoptosis by DHA and reduced cell division as a result of iron depletion could play a role in determining cell counts in the DX experiment. Even with possible inhibition of cell division as a result of iron depletion, the DX plus DHA treatment group still had a higher percentage cell count than the group treated with DHA alone in the same experiment.

Neither PBN nor DX, at the dose ranges studied, could fully block DHA cytotoxicity toward Molt-4 cells. A possible explanation for DX has been given above. Perhaps higher concentrations of PBN would be needed to achieve complete blockade. Carbon-based free radicals are formed from the reactions of the endoperoxide moiety of DHA with iron. Subsequently, different species of free radicals can be formed. It has been shown that the use of a single free radical scavenger alone cannot fully-block the cytotoxicity of DHA toward Molt-4 cells (18). A combination of different types of free radical scavengers may be needed to completely block the toxicity of DHA.

Other properties of the artemisinin molecule may contribute to its toxicity toward cancer cells. It has been shown that artemisinic acid, which does not contain an endoperoxide moiety, had some cytotoxicity toward cancer cells (19). Other cellular processes affected by artemisinin also may lead to anticancer effects, for example, it has been reported that DHA inhibited cancer growth by activating the

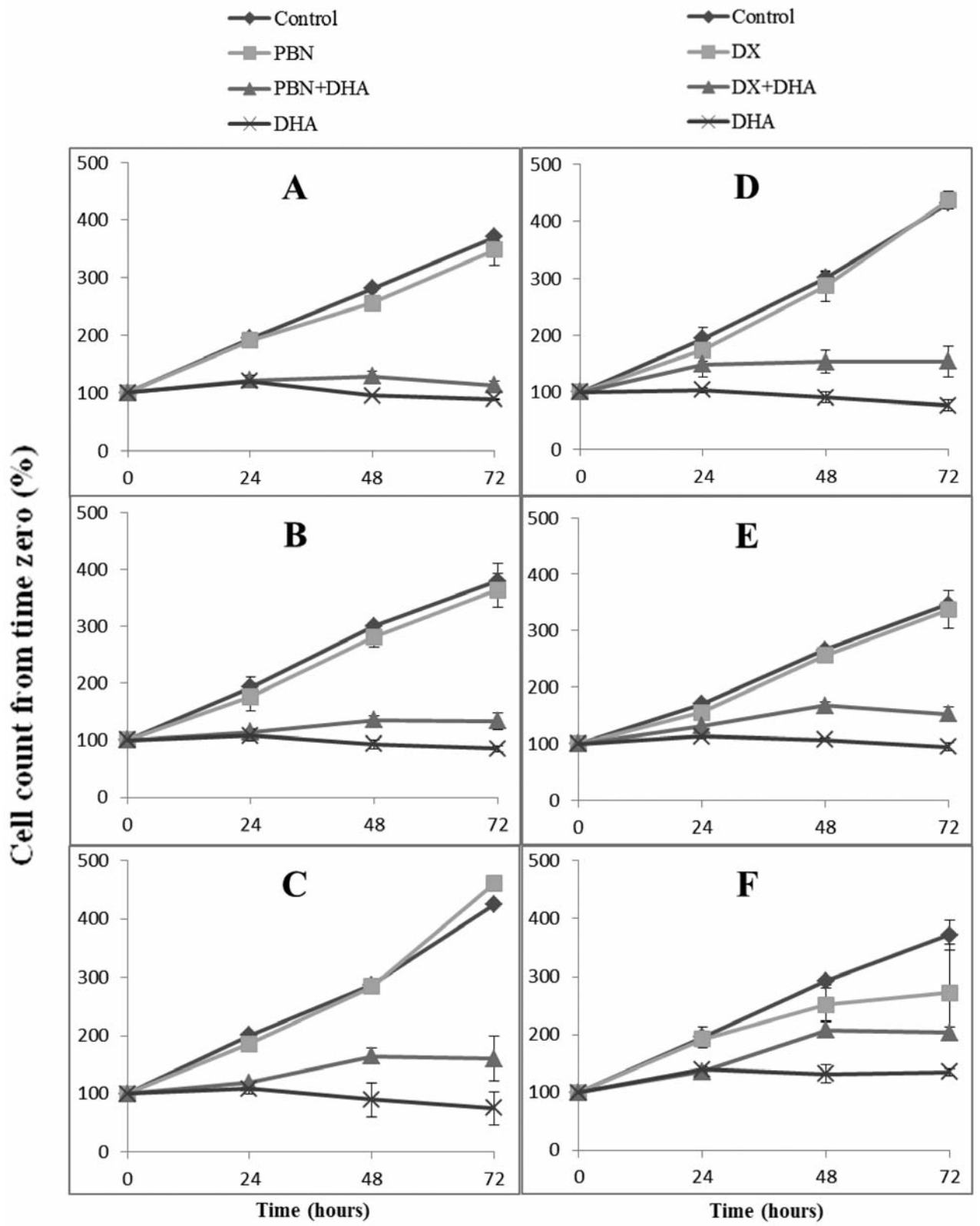

Figure 1. *Graphs showing the mean percentage cell count (±SEM, N=3) of Molt-4 cells at the 0, 24, 48, and 72-h time points with different treatment combinations of dihydroartemisinin (DHA), N-Tert-butyl-alpha-phenylnitrone (PBN), and deferoxamine (DX). Graphs show the effects of 0.25 mM (A), 0.5 mM (B), and 1 mM (C) PBN added along with 12.2 μM DHA and of 10 μM (D), 20 μM (E), and 30 μM (F) DX added along with 12.2 μM DHA.*

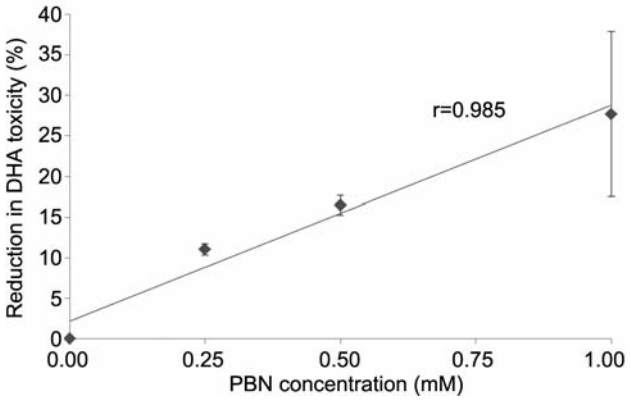

Figure 2. *Dose-response curve of Molt-4 cells to N-Tert-butyl-alpha-phenylnitrone (PBN). Graph shows the percentage reduction of dihydroartemisinin (DHA) toxicity (mean±SEM) by different concentrations of PBN (0 mM, 0.25 mM, 0.5 mM, and 1 mM).*

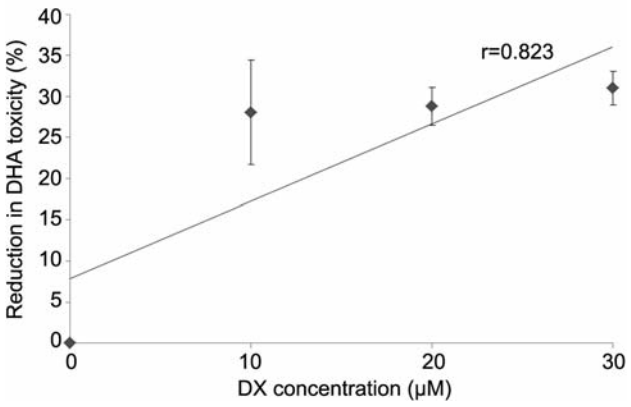

Figure 3. *Dose-response curve of Molt-4 cells to deferoxamine (DX). Graph shows the percentage reduction of dihydroartemisinin (DHA) toxicity (mean±SEM) by different concentrations of DX (0 μM, 10 μM, 20 μM, and 30 μM).*

p38 mitogen-activated protein kinase (8), and by accelerating the degradation of c-MYC onco-protein and induction of apoptosis (20). Research has shown that artemisinin and its derivatives cause damage to macromolecules including DNA (15) and proteins (20-22), which may also lead to cell death, but the mechanisms of this are still not fully-understood.

In conclusion, both chelation of iron by deferoxamine, and scavenging of free radicals by PBN attenuated the cytotoxicity of DHA toward Molt-4 cells. Our results indicate that iron-mediated free radical formation plays an important role in the mechanism of artemisinin cytotoxicity toward cancer cells. Future research should be carried out to explore the relationship between artemisinin compound-generated free radicals and cellular macromolecular damage, as well as other activities of artemisinin that are toxic to cancer cells, in order to get a better picture of the anticancer mechanisms of artemisinin.

Conflicts of Interest

The Authors declare no conflicts of interest related to this research.

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