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Additive Cytotoxic Effects of Dihydroartemisinin and Sodium Salicylate on Cancer Cells

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Abstract. *Background/Aim: The standard methods of chemotherapy in cancer treatment are expensive and pose serious health effects. The present study investigates an alternative chemotherapy by testing the combined treatment of two drugs on leukemia cells: dihydroartemisinin (DHA) and sodium salicylate (SS). Materials and Methods: Cells were divided into 4 treatment groups: a control, treatment with DHA-only, treatment with SS-only, and treatment with both DHA and SS. Cells were counted immediately before the addition of any reagents (0-h count), and at 24, 48, and 72 h after treatment. Results and Conclusion: At low concentrations, the combination of DHA and SS significantly reduced cancer cell proliferation, although no synergistic interaction between the two drugs was found. Even without a clear synergistic interaction, the combination of DHA and SS provides a safe and affordable form of cancer treatment.*

The standard methods of chemotherapy and radiation in cancer treatment pose serious adverse health effects and can be very costly. Therefore, the search for alternative chemotherapies is imperative and the drug Artemisinin has shown great potential in cancer research. Artemisinin is a compound extracted from the sweet wormwood plant, *Artemisia annua* L. It has been shown to selectively kill cancer cells *in vitro*, in mice xenograft models, and in a select number of human patients. Our group and others have found that the artemisinin analog, dihydroartemisinin (DHA), kills cancer cells by apoptosis (1, 2). Previous studies have also tested DHA with various other low-toxicity drugs and assessed the efficacy of these combinations in killing cancer cells. The present study tests the combination of DHA with sodium salicylate (SS) - the sodium salt of aspirin. In preliminary tests, this combination was found to induce apoptosis in the majority of Molt-4 cells (a human lymphoblastoid leukemia cell line) in culture at a reasonably low concentration of 40 μM (unpublished data). The purpose of the current study was to test even lower concentrations of SS and DHA and assess the resulting cytotoxicity on MOLT-4 cells.

Although this study did not aim at testing a mechanism of interaction between DHA and SS, a brief search into the literature of these drugs provides a compelling theory on how they might interact to reduce proliferation of cancer cells. This theory is based on three main ideas: i. DHA induces apoptosis in cancer cells (1, 2). ii. The kinase N1F-kappa B inhibits apoptotic pathways and boosts expression of multi-drug resistance (mdr1), which expels many drugs from the cell, and iii. SS is a type of non-steroidal anti-inflammatory drug (NSAID) and NSAIDs inhibit NF-kappa B activity by binding to I kappa kinase-beta which is a central mediator of NF-kappa activation (3). These facts encourage the idea that SS could enhance the cytotoxicity of DHA by binding to 1 kappa kinase-beta and preventing NF-kappa B activation; thus allowing DHA to remain in the cell and induce successful apoptosis.

Materials and Methods

Materials. All chemicals were from Sigma (Sigma-Aldrich, St. Louis, MO) unless mentioned otherwise. Molt-4 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (ATCC, Manassas, VA). Cells were maintained at 37˚C in 5% CO_2/95% air and 100% humidity, and were split 1:2 at a concentration of approximately 1×10^6 cells/mL.

Stock solution of DHA (Holley Pharmaceuticals, Chongqing, China) was prepared in DMSO and Stock solutions of SS was made in complete RPMI and frozen at –20˚C.

Methods. Molt-4 cells in medium were divided into 4 treatment groups: a control, treatment with DHA-only, treatment with SS-only, and treatment with both DHA and SS. The control group cells were incubated with the solvent dimethyl sulfoxide (DMSO) at a concentration of 1% but without DHA or SS. The second treatment group was treated with the same amount of solvent in addition to 5.87 μM DHA. The third treatment group included 1% solvent and 4.89 μM SS. The last treatment group included 1% DMSO, 5.87 μM

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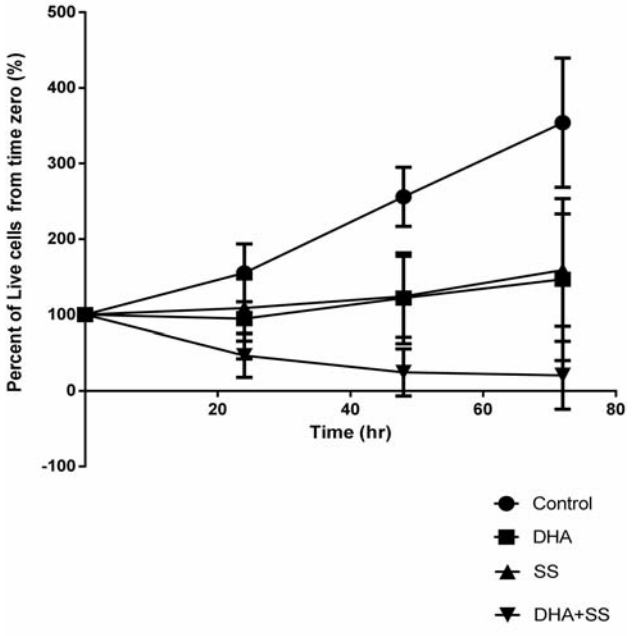

Figure 1. *Comparison of four cell treatments over a period of 72 h. Cells were divided in four treatment groups and counted at 24-h increments for 72 h after administering the designated treatment. The numbers of live cells present at each 24-h increment are shown as percentages from time 0 (100%). Note the large proliferation of cells in the control group compared to the decrease in percentage of live cells in the DHA+SS treatment.*

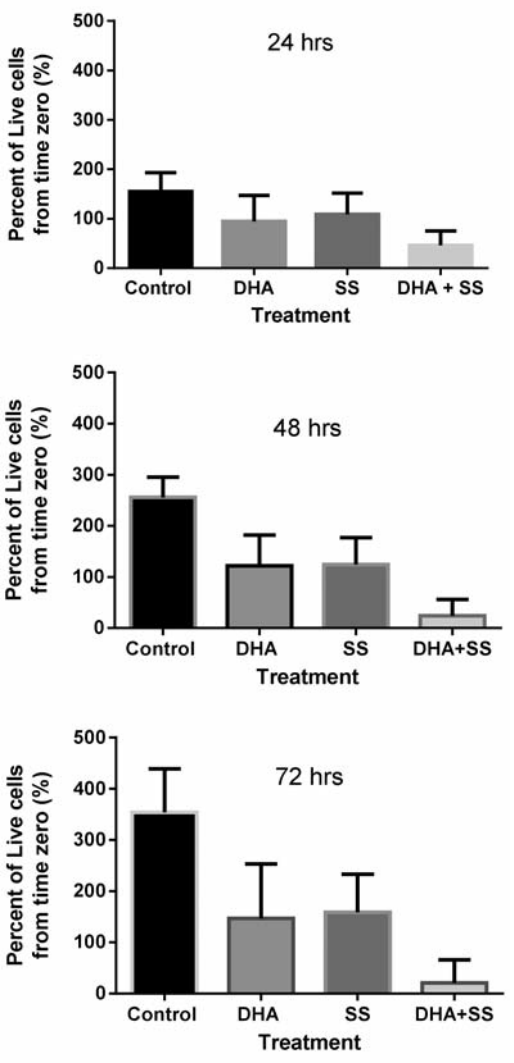

Figure 2. *Comparing cell counts between treatment groups at 24, 48, and 72 h. This Figure displays the same data as Figure 1, but allows better comparison at each 24-h count.*

DHA and 4.89 μM SS. Thus, the four treatment groups reflected cells that were only treated with solvent (no artemisinin or aspirin), only artemisinin, only aspirin, and finally a combination of artemisinin and aspirin. The specific concentrations of DHA and SS were determined based on previous work. Trypan blue was used to count viable cells using a hemocytometer. Live cells were counted immediately before the addition of any reagents (0-h count), and then at 24, 48, and 72 hours after the addition of the designated reagents. Experiments were performed in triplicate using this design.

Results

As is shown in Figure 1, untreated Molt-4 cells in the control group proliferated by 354% over the course of 72 h. The cells treated with DHA multiplied to 147% of the original cell count, those treated with SS increased to 159%, and cells treated with DHA and SS together showed a decrease in cell numbers to 20.6% of the original cell count after 72 h. Figure 2 shows the same data as Figure 1, but allows for better comparison at each 24-h count. A repeated-measures 2-way ANOVA test was used to analyze the data, and we found that the difference in cell count between the control group and DHA (5.87 μM) was significant as early as 24 h after treatment (p=0.014). Treatment with SS (4.89 μM)-alone did not result in a significant difference in cell count until 48 h after treatment (p=0.0001). Using a Newman-Keuls multiple comparisons test however, we found that the combination of DHA and SS resulted in a significant difference in cell number as early as 24 h (p<0.05). By the 72-h count, all three drug treatments (DHA-alone, SS-alone, and DHA+SS) showed significant difference in cell counts from the control group, and the Newman-Keuls comparison tests showed significant differences between the separate DHA and SS treatments *versus* the DHA+SS combined treatment (p<0.05). Time response plots were also compared by the Krauth method (4) and the levels of the curves and the a0 of the orthogonal polynomial coefficient were compared using the Mann-Whitney *U*-test (4). From this test,

the effects of DHA-alone and DHA+SS were significant with a $p<0.05$, but the treatment of cells with SS alone did not show a significant difference from the control cell group.

However, the 2-way ANOVA test also indicated that there was no interaction between DHA and SS at any point in the 72-h treatment period. This implies that the combination of DHA and SS simply has additive effects *versus* a synergistic effect; *i.e.* SS does not enhance DHA efficacy, but rather just adds to the suppression of cell proliferation.

Discussion

Our results indicate that when DHA and SS are used in combined treatment of Molt-4 cells, the effect on cell proliferation is additive *versus* synergistic. That is, treatment with both DHA and SS suppresses the proliferation of cancer cells to a greater extent than either drug alone. However, our results do not reflect any interaction such that one drug improves the efficacy of the other. We have come up with three possibilities to explain these results: (i) DHA and SS have separate mechanisms for reducing cancer cell proliferation (ii) the drug concentrations we used were too low for an interaction to occur or be detected or (iii) an interaction depends on specific timing of drug treatment, of which we did not explore in this study. Further studies are required to determine which of these possibilities correctly describe the DHA+SS relationship in order to optimize the efficacy of this drug combination. If (i) is the case, then the combination of DHA+SS simply provides effective cancer treatment, and perhaps reduces the need to rely on one drug alone. To explore option (ii), further testing of various DHA and SS concentrations is required to explore the optimum efficacy of this drug combination and determine if there is a threshold concentration at which an interaction occurs. Finally, possibility (iii) stems from information presented in the introduction, which predicts synergism when the cells are first exposed to SS before being treated with DHA. This hypothesis is based on the idea that SS may increase the efficacy of DHA by inhibiting NF-kappa B; ultimately leading to an increase in apoptosis of cancer cells (3).

Conclusion

Although the mechanism and interaction of dihydroartemisinin and Sodium salicylate is still not well-understood, the present study clearly indicates the potential of these drugs as a safe and inexpensive form of cancer treatment.

Conflicts of Interest

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

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