Effects of Artemisinin Dimers on Rat Breast Cancer Cells *In Vitro* and *In Vivo*

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Abstract. *Artemisinin has been shown to be an effective antimalarial and anticancer compound. Dimers of artemisinin have been synthesized and shown to be potent antimalarials compared with monomers. In the present study, we investigated the effect of two artemisinin dimers (dimer-alcohol and dimer-hydrazone) on rat mammary adeno-carcinoma cells (MTLn3) in vitro and in vivo compared with that of the artemisinin monomer dihydroartemisinin (DHA). We found that the dimers are considerably more potent than DHA in killing MTLn3 cells in vitro and suppressing the growth of MTLn3 breast tumors in vivo.*

The antimalarial artemisinin has been shown to have anticancer properties (1, 2). Presumably, cancer cells contain excessive free iron compared to normal cells. Artemisinin selectively kills cancer cells by forming free radicals intracellularly when reacted with iron (1). Recently, dimers of artemisinin have been synthesized in various laboratories. These dimers have been shown to have potent antimalaria and anticancer properties (3-20). In the present study, we investigated the anticancer activities of two artemisinin dimers synthesized in our laboratory, dimer-hydrazone (Dimer-Sal) and dimer-alcohol (Dimer-OH) and compared them with the artemisinin monomer dihydroartemisinin (DHA). These compounds were tested *in vitro* on rat breast adenocarcinoma MTLn3 cells and on the growth of MTLn3 breast tumors in the rat.

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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 initiation 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 experiments. All animal-use procedures had been reviewed and approved by the Animal Use and Care Committee of the University of Washington prior to experiments.

Synthesis of dimers. All starting materials and reagents for organic synthesis were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for artemisinin that was purchased from Shaanxi Sciphar Biotechnology Co., Ltd (Xi'an, P.R. China), and used without further purification. Matrix assisted laser desorption ionization time of flight mass spectrum (MALDI-TOF/MS) was recorded on a Bruker Biflex III MALDI-TOF/MS spectrometer. 1H-NMR spectra were recorded on a Bruker Datatronics Avance AV Series NMR operating at 300 MHz. Dimer-OH was synthesized according to a published procedure (16). Dimer-Sal was synthesized by reacting dimer-hydrazine and salicylaldehyde also according to a published procedure (12). Dimer-Sal: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.75 (br s, 1H), 8.31 (s, 1H), 7.28 (dt, J=7.8 and 1.5 Hz, 1H), 7.17 (dd, J=7.8 and 1.5 Hz, 1H), 6.99 (d, J=7.8Hz, 1H), 6.87 (t, J=7.8 Hz, 1H), 5.33(s, 1H), 5.27 (s, 1H), 4.21 (m, 1H), 2.8-2.6 (m, 3H), 2.4-2.1 (m, 3H), 2.07-1.77 (m, 8H), 1.7-1.5 (m, 3H), 1.5-1.15 (m including singlets at δ 1.37 and 1.33, 16H), 1.02-0.85 (m, 14H); LRMS (MALDI), m/z $[M+H]^+$=739.6. Chemical structures of Dimer-Sal, Dimer-OH and DHA are shown in Figure 1.

Procedures of in vitro experiment. MTLn3 cells (a gift from D. Jeffrey Segall of the Albert Einstein College of Medicine, Bronx, NY, USA) were grown in Eagle's alpha-modified minimum essential medium (MEM, Catalogue Number: 12561056, Invitrogen, Frederick, MD, USA) containing 10% fetal bovine serum (ATCC, Manassas, VA, USA) at 37˚C in an atmosphere of 5% CO_2 and 100% humidity. At approximately 95% confluency, cells were passaged in 7 T-25 flasks at a density of approximately 10,000 cells/mL. Each flask contained 5 ml of medium. After 24 h of incubation, the medium was replaced with fresh medium with 1 mg/mL of holotransferrin (Fortune Biologicals, Inc., Gaithersburg, MD, USA). Cells were further incubated for 1 h. For zero hour count, cells from one flask were trypsinized and counted using a hemocytometer. Cells from other flasks were treated with 0, 0.02, 0.05, 0.1, 0.5, and 2 μM of test compounds DHA, Dimer-Sal or Dimer-OH) freshly dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in each flask was 1%. Dihydroartemisinin was

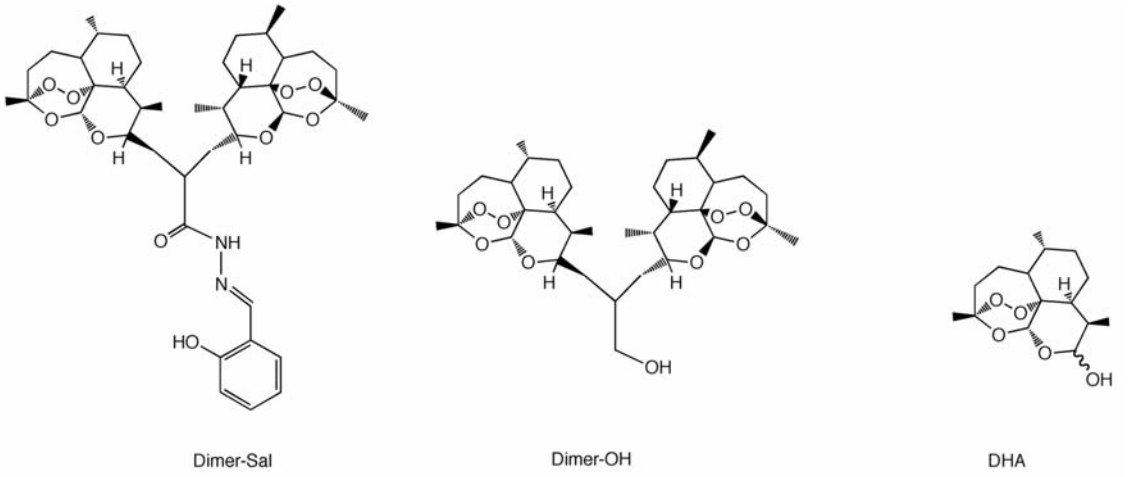

Figure 1. *Chemical structures of Dimer-Sal, Dimer-OH and DHA.*

a gift from Holley Pharmaceuticals (Chongqing, P. R. China). After 72 h of incubation under the conditions described above, cells from each flask were trypsinized and counted. Each dose response study was performed three times.

Procedures of in vivo experiment. MTLn-3 cells were grown in Eagle's alpha-modified MEM supplemented with 10% fetal bovine serum (ATCC). Subcutaneous breast tumors were produced by implanting approximately 10^6 cells from exponentially growing cultures into the flank of an animal. After implantation, rats were monitored on a daily basis to check for tumor development. When the tumors had grown to approximately 1 cm in diameter, daily drug treatment began.

Rats were randomly assigned to one of three drug-treatment groups: Dimer-Sal (n=9), Dimer-OH (n=7) and DHA (n=8). The dosage for each drug was 20 mg/kg/day. Drugs were dissolved in olive oil and intubated in a volume of 1 ml/kg using an 8-French feeding tube. A control group (n=13) was similarly given daily intubation of 1 ml/kg of olive oil. Daily drug treatment continued for 5 days. Tumor size was measured daily. The length, width and height (in mm) of the ellipsoidal tumor were measured with a caliper. Tumor volume was calculated using the formula: length × width2 × $\pi/6$. Data from each rat were expressed as the ratio of change in tumor volume from day one, *i.e.*, that was measured immediately before the first treatment was administered.

Data analysis. IC_{50} of Dimer-Sal, Dimer-OH and DHA on MTLn3 cells were calculated from the dose-response data. Data were analyzed using one-way ANOVA and differences between treatments were compared by the Newman-Kuel's test. Tumor growth curves from the treatment groups were compared using the nonparametric method of Krauth (21), comparing the levels of the curves (ao) using a one-tailed Mann-Whitney *U*-test. A difference at $p<0.05$ was considered statistically significant.

Results

Log dose-response curves of MTLn3 cells to Dimer-Sal, Dimer-OH and DHA are presented in Figure 2 a and b. The IC_{50} (mean±SD, n=3) of Dimer-Sal, Dimer-OH and DHA were 52±1, 43±1, and 360±180 nM, respectively. Thus, both Dimer-Sal and Dimer-OH are significantly more potent than DHA in inhibiting the growth of MTLn3 cells *in vitro*.

Results of the *in vivo* experiment are presented in Figure 3. Compared to the control, Dimer-Sal ($p<0.01$), Dimer-OH ($p<0.01$), and DHA ($p<0.05$) significantly retarded the growth of MTLn3 tumors in the rat. Furthermore, both Dimer-Sal (Dimer-Sal *vs*. DHA, $p<0.01$) and Dimer-OH (Dimer-OH *vs*. DHA, $p<0.01$) are significantly more potent than DHA. In addition, the response to Dimer-OH was significantly different from that to Dimer-Sal ($p<0.01$). The effect of Dimer-OH on tumor size appeared to last longer.

Discussion

We have shown in both *in vitro* and *in vivo* experiments that artemisinin dimers are more potent than the monomer in regard to cancer cell toxicity and suppression of tumor growth. There has been one more animal cancer study of artemisinin dimers. Galal *et al*. (8) reported that daily subcutaneous injection (25-50 mg/kg/day) of a dimer caused a significant growth delay of HL-60 human leukemia xenografts in the mouse.

Artemisinin dimers have been tested in many different cancer cell lines and found to be effective in either suppressing their growth or causing cell death (apoptosis) (*e.g.* 3, 4, 20). In general, cancer cell cytotoxicity of dimers is more potent than that of the monomers. The increase in potency varies from 10- to 100-fold (4, 10, 13, 20). Artemisinin dimers have also been shown to be as or even more potent than some chemotherapeutic agents, such as doxorubicin (19). However, the dimers have been shown to be much less toxic to normal cells than cancer cells (13, 18). Posner *et al*. (17) reported a high therapeutic index (>150) for some of the dimers they synthesized. The highly selective cytotoxicity of artemisinin

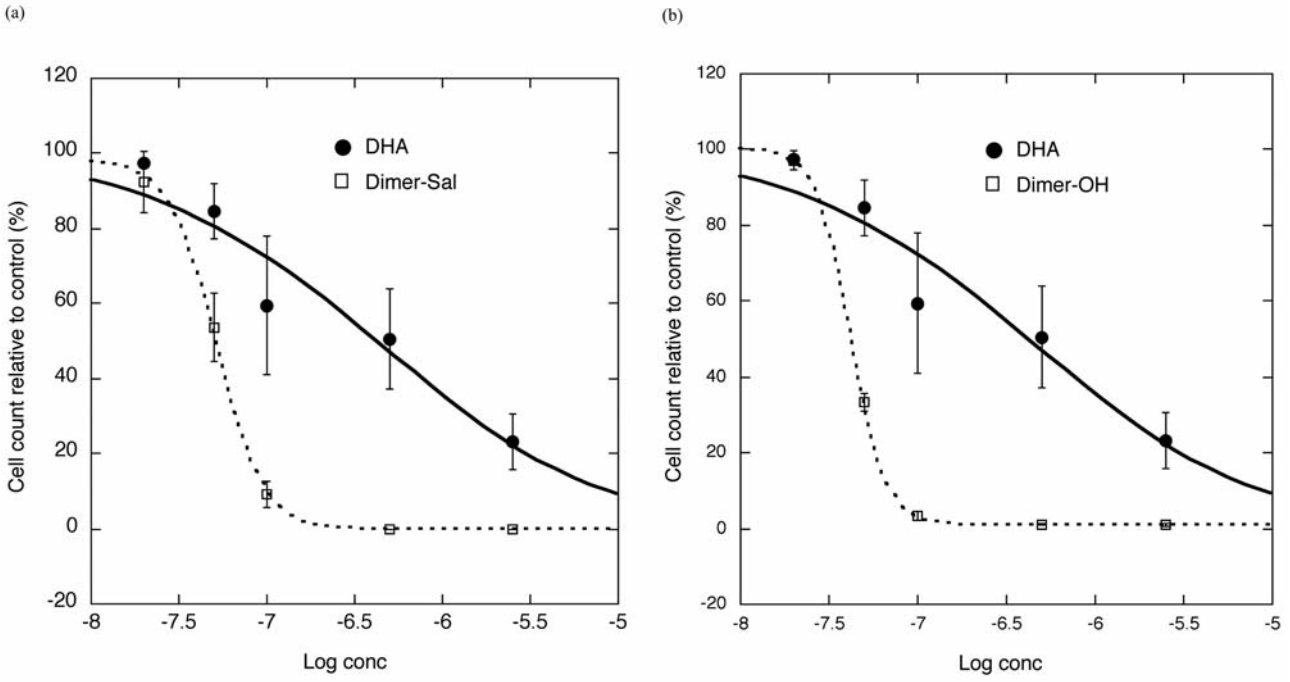

Figure 2. *Log dose-response plots of DHA and (a) Dimer-Sal and (b) Dimer-OH effects on MTLn3 cells in vitro.*

dimers towards cancer cells makes them an attractive option for development of cancer treatment.

The mechanism of action of artemisinin dimers on cancer cells is not known. However, it must be pointed out that the presence of two endoperoxides in one molecule would not guarantee its effectiveness towards cancer cells. Other factors also play a role on its potency. For example, not all dimers tested were found to have an effective antiproliferative effect on cancer cells and those that do also have different potencies towards different cancer cell lines (*e.g.* 8, 12, 19). In the present study, we also found that Dimer-OH has a slight but significantly more potent effect than Dimer-Sal *in vitro* and *in vivo*.

Beekman *et al*. (5) speculated that the spatial positions of the active groups are an important consideration. They found that non-symmetric DHA dimers are more potent than symmetric dimers in killing EN2 cancer cells. The linkers of the dimers also play an important role. Chadwick *et al*. (7), in testing their C10 carba artemisinin dimers, found that changing the number of carbon atoms in the linker changed the potency of the dimer in killing HL-60 cells: dimers with more carbon atoms in their linkers were more active. Jung *et al*. (11) reported that linker size affected the potency of their artemisinin dimers. Jeyadevan *et al*. (10), from their study on artemisinin phosphate ester dimers, concluded that the nature of the linker in the dimers played an important role in their antiproliferative effect on cancer cells. Furthermore, it is also not known why the dimers are more potent than monomers. One possibility is that dimers, with two active groups, after activation by iron, can form cross-linking of biological molecules, which could cause a more devastating effect on cellular functions leading to cell death. Interestingly, Beekman *et al*. (6) concluded that the ether linkage of their artemisinin dimers was the component that kills cancer cells, whereas the endoperoxides only played a minor role. However, Stockwin *et al*. (20) found that both the antioxidant L *N*-acetylcysteine and the iron-chelator desferroxamine were able to block the

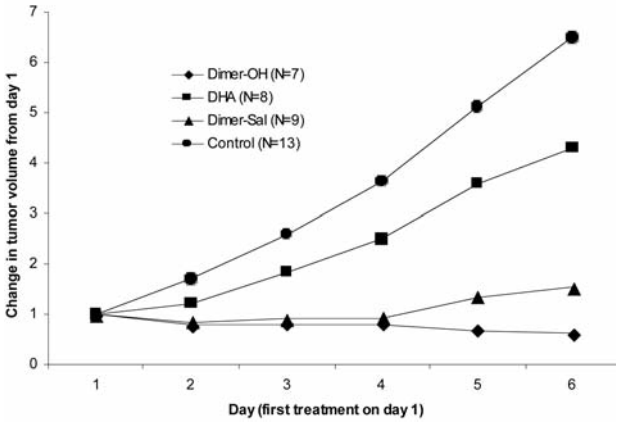

Figure 3. *Effects of Dimer-Sal, Dimer-OH and DHA on growth of MTLn3 tumors in the rat. Data from each rat were expressed as the ratio of change in tumor volume from day one, which was measured immediately before the first treatment was administered.*

cancer cell cytotoxicity of their dimers, which would suggest an involvement of the endoperoxide moieties. They suggested that formation of reactive oxygen species causes endoplasmic reticulum stress leading to apoptosis.

Therefore, artemisinin dimers cannot be considered as a single group of compounds with similar general properties. The arrangement of atoms in the molecules, the chemical characteristics of the linkers, and the *in vivo* pharmacokinetics of a compound can determine the cytotoxic effectiveness and action of the compound on cancer cells.

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