# Dihydroartemisinin induces radiosensitivity in cervical cancer cells by modulating cell cycle progression

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## ABSTRACT

**الأهداف:** التحقق من آثار العلاج الاشعاعي للارتيميسينين وتأثيره على الخلايا العنقية للسرطان.

**الطريقة:** أجريت دراسة مخبرية خلال الفترة من مايو 2009م حتى أغسطس 2012م في قسم طب الاشعاعي، جامعة سوتشو، سوتشو، الصين. تم تعيين خلايا هيلا و سيها لمجموعة الشاهد والارتيميسينين لمجموعة العلاج. كما أجريت مقايسة MTT و مقايسة مولد النسل، وتحليل الدورة الخلوية وموت الخلايا في الخطوط الخلوية لكلا المجموعتين.

**النتائج:** اعتمد الأثر المثبط لمادة الارتيميسينين على الخطوط الخلوية لخلايا هيلا و سيها على كلا من التركيز والوقت. زادت مادة الارتيميسينين الأثر الإشعاعي لخلايا هيلا وليس لخلايا سيها. تم تعزيز مرحلة موت وانقسام الخلايا بعلاج الارتيميسينين في خلايا هيلا. قلل الإشعاع مع الارتيميسينين من ظهور الكيناز وزيادة مادة السيلكين في خلايا هيلا.

**خاتمة:** أن مادة الارتيميسينين تبطل من مادة G2 في خلايا الهيلا والتي تقلل من توقف G2/M الناتج من الإشعاع، كما تستخدم كمحسس مشع فعال والذي يحسن من دخول الخلايا التالفه في الإنقسام.

**Objectives:** To investigate the radiosensitizing effects of dihydroartemisinin (DHA) and its underlying mechanisms in cervical cancer cells.

**Methods:** This experimental study was conducted between May 2009 and August 2012 in the School of Radiation Medicine and Protection, Soochow University, Suzhou, China. HeLa and Siha cells were assigned as the control group and DHA as treated group. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, clonogenic assay, cell cycle analysis, and apoptosis analysis were carried out in 2 cell lines of both groups.

**Results:** The inhibitory effect of DHA on the HeLa and Siha cell lines was dependent on both concentration and time. Dihydroartemisinin increased the radiosensitivity of HeLa cells, but not of Siha cells. Apoptosis and the gap2/mitosis (G2/M) phase transition induced by x-irradiation was enhanced by DHA treatment in HeLa cells. Irradiation, combined with DHA, decreased Wee1 expression while increasing Cyclin B1 expression in HeLa cells.

**Conclusion:** Dihydroartemisinin potently abrogates G2 checkpoint control in HeLa cells. It can relieve the G2/M arrest induced by irradiation; thus, it can be used as an effective radiosensitizer, which will probably promote the entry of more irradiation-damaged cells into mitosis.

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Cervical cancer is a common high-risk gynecological malignancy, which is predominantly treated using radiotherapy.[1] Radiotherapy is effective in achieving localized control of cervical cancer. However, radio resistance has gained increasing attention for the treatment of cervical cancer, as it is one of the reasons for the clinical failure of radiotherapy. Nevertheless, the molecular mechanisms underlying this tumor radio resistance are not fully understood.[2] Artemisinin, a chemical compound extracted from the wormwood plant *Artemisia annua L.*, has been used for years to successfully treat malaria and viruses in humans. Various derivatives of artemisinin, such as artesunate, artemether, dihydroartemisinin (DHA) and arteether, have been identified.[3-5] Artemisinin and its analogs contain an endoperoxide bridge, which is activated by intraparasitic heme-iron to form free radicals. The generated free radicals kill malarial parasites by alkylating biomolecules.[3-6] In recent years, accumulating reports have demonstrated that the anticancer activities of artemisinin and its analogs both in vitro and in vivo[5-12] in cancers such as malignant glioma,[6] breast cancer,[7] melanoma,[8] and pancreatic cancer.[9] Dihydroartemisinin (DHA) is a water soluble, metabolically active, and is the safest and most effective antimalarial artemisinin derivative. Its anti-tumor effects have been recently reported.[5,6,9] However, the anticancer effects and radiosensitivity induced by DHA have not been reported for cervical cancer cells. Radiation therapy works by damaging the DNA of cancerous cells. DNA damage activates checkpoint pathways that inhibit the progression of cells through gap1 (G1) and gap2 (G2) phases and delay progression through S phase. These checkpoints provide cells with enough time to repair damaged DNA prior to resuming cell cycle progression.[13,14] Tumor cells that are mutated for tumor protein 53 (p53) lack an effective DNA damage-induced G1 arrest, and thus, the most prominent DNA damage-induced arrest occurs in G2 phase.[14] Signaling cascades can inhibit cyclin-dependent kinase (Cdk)/Cyclin B1 activities and progression from G2 into mitosis.[15] Wee1 kinase plays a crucial role in maintaining G2 arrest through its inhibitory phosphorylation of Cdc2.[16,17] After the induction of DNA damage, Wee1 activates and sustains the G2 arrest until the damaged DNA is sufficiently repaired.[16-18] This study aims to investigate the radiosensitizing effects of DHA and its underlying mechanisms. Human p53-mutant HeLa and p53 wild-type Siha cervical cancer cells were studied. Our results showed that DHA treatment enhanced the radiosensitivity of HeLa cells by suppressing Wee1 kinase and increasing Cyclin B1 protein levels after x-ray irradiation.

**Methods.** This study was conducted between May 2009 and August 2012 in School of Radiation Medicine and Protection, Soochow University, Suzhou, China. This experimental study was designed to explore the role of DHA in radiosensitivity of cervical cancer cells. The 2 cervical cancer cell lines HeLa and Siha were used. HeLa and Siha cells were assigned as a control group and DHA as a treated group. The effect of DHA on the survival rates of HeLa and Siha cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The radiosensitivity for these cell lines was determined using clonogenic assay. Alterations in cell cycle progression and apoptosis were analyzed by flow cytometry. The MTT assay, clonogenic assay, cell cycle analysis, and apoptosis analysis were carried out in the 2 cell lines of both groups. The ethical approval for the study was obtained from the Medical School of Soochow University.

*Reagents and cell culture.* Dihydroartemisinin was purchased from Sigma Chemical Co. (Sigma Chemical Co. St. Louis, USA) and was dissolved in dimethylsulfoxide (DMSO, Solon, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Media (DMEM) were obtained from the Life Technologies (Grand Island, USA). The 2 cervical cancer cell lines HeLa and Siha were maintained in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin G, 100 units/ml streptomycin sulfate; Gibco, Grand Island, USA). The cells were grown in a 37°C incubator at 5% $CO_2$. Both of the cell lines were obtained from the American Type Culture Collection.

*Cytotoxicity assay.* Cells (2×103) were seeded into 96-well plates in 0.1 ml of DMEM supplemented with FBS and were incubated for 24-hours. The cells were then treated with indicated concentrations of DHA and incubated for an additional 12, 24, 48 or 72-hours. The cells were incubated for 4-hours with 200 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Chemical Co. St. Louis, USA); the reagent was dissolved in DMSO. The absorbance was measured at 490 nm using a 96-well plate reader. All of the experiments were performed in triplicate.

*Clonogenic assay.* For standard clonogenic assays, cells were seeded into 6-well plates at 500-1,000 cells/well depending on the dose of radiation. Twenty-four hours after seeding, the cells were treated with DHA for 24-hours. The cells were irradiated using 6-MV X-rays from linear accelerators (Varian, Walnut Creek, USA) at a dose rate of 2 Gy/min; a 1.5-cm bolus was used as a compensator. The cells were then grown from 7-10 days to allow for colony formation and were subsequently

fixed and stained using crystal violet. Colonies consisting of 50 or more cells were counted as a clone.

*Measurement of apoptosis.* Cells were treated with DHA for 24 hours prior to treatment with 6 Gy irradiation. Apoptosis was measured using propidium iodide (PI)/ annexin-V double-staining (Kaigen, Nanjing, China). The cells were harvested at 24-hours after treatment with DHA. Apoptotic fractions were measured using flow cytometry (Beckman, USA).

*Cell cycle progression analysis.* Cells were treated for 24 hours with DHA and irradiated at the indicated doses. Twenty-four hours after irradiation, both the floating and the attached cells were harvested by centrifugation at 1000 rpm for 5 min. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed with 70% ice-cold ethanol. Prior to flow cytometric analysis, the cells were treated with 0.25 mg/ml RNase A and 50 μg/ml propidium iodide for 30 min at 37°C. The treated cells were then passed through 35-μm cap strainers coupled to 12×75-mm Falcon tubes, and 10,000 cells per sample were collected for flow cytometric analysis.

*Western blot.* Cells were treated with DHA for 24-hours and then irradiated at the indicated doses. Cells in a 6-well-culture cluster were washed twice with ice-cold PBS and then directly lysed in 200 μl of cell lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 μM pepstatin A) in each well. The lysates were boiled, centrifuged at 10,000 rpm, and then loaded onto a 12% SDS-PAGE gel. The samples were electrophoresed for 2-hours and then transferred onto Millipore Immobilon transfer membranes (Millipore Billerica, USA) using a Bio-Rad electro blotting apparatus (Biorad, Hercules, USA). After blocking with 5% nonfat milk in PBS-Tween-20 for one hour at room temperature, the membranes were blotted with the appropriate Wee1, Cyclin B1, and Cdc2 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:1,000 -1:2,000 dilution. The membranes were then incubated with the appropriate horseradish peroxidase-coupled secondary antibody at a 1:2000 dilution for one hour at room temperature. The blots were washed with Tris buffered saline with Tween 20 (TBST) and then incubated in detection reagent (ECL Advance Western Blotting Detection Kit, Amersham Bioscience, Freiburg, Germany), followed by exposure to a Hyperfilm ECL film (Pierce, Rockford, IL, USA). Beta-actin served as the loading control and was detected using a mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology).

*Statistical analysis.* All analyses represent experiments that were performed in at least triplicate. The results were evaluated by one- or 2- way analysis of variance using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) Version 17.0 to determine the significance. The sensitizer enhancement ratios (SER) were measured using Sigmaplot software according to the multi-target single hit model. The significance level was taken as $p<0.05$.

## Results.

*The cytotoxicity of DHA on human cervical cancer cells.* To evaluate the cytotoxic effects of DHA on cultured human cervical cancer cells, we treated the HeLa and Siha cells with different concentrations of DHA for different treatment times and measured cell viability using an MTT assay. The inhibitory effects elicited by DHA on the cells were dependent on both concentration and time (**Figures 1A and 1B**). Comparatively, DHA induced more cytotoxicity in Siha cells than in HeLa cells. To evaluate the ability of DHA to sensitize tumor cells to radiation, moderately toxic doses (that reduced cell viability to approximately 85%) were applied. When applied at 20 μmol/L, DHA induced approximately 15% inhibition of HeLa cell viability, which was equivalent to the effect of 100 μmol/L DHA on Siha cells. These concentrations were used for subsequent experiments.

*The effect of DHA on the radiosensitivity of HeLa and Siha cells.* To investigate the effect of DHA on the radiosensitivity of HeLa and Siha cells, we performed an in vitro clonogenic cell survival assay using DHA treatment plus radiation. HeLa cells treated with 20 μmol/L DHA plus x-ray irradiation exhibited significantly lower clonogenic survival rates than cells treated with radiation alone. The sensitizer enhancement ratios (SER) were 1.47 for cells treated with radiation plus DHA, compared to cells treated with radiation alone (**Figure 2A**). Siha cells treated with radiation plus 100 μmol/L DHA exhibited a SER of 1.06, compared to cells treated with radiation alone (**Figure 2B**). The data were further analyzed using the 2-way ANOVA to test the interaction effect between DHA and radiation. Our results indicated that interaction effect between DHA and radiation was statistically significant ($p=0.001$) for HeLa cells, suggesting that DHA treatment could sensitize cells to x-irradiation. However, in Siha cells, the interaction effect between DHA and radiation was not statistically significant ($p=0.25$). Taken together, these results demonstrated that treatment with DHA could increase the radiosensitivity of human HeLa cells with mutant p53 but not of Siha cells with wide-type p53.

***The effect of DHA combined with X-irradiation on cell cycle progression.*** To determine whether the observed DHA-induced radio sensitization was associated with changes in cell cycle progression, HeLa and Siha cells were cultured in DMEM without serum for 24 hours prior to the addition of DHA alone or combined with 6 Gy x-irradiation. As shown in **Figures 3A** & **3B**, radiation induced a G2 arrest in the p53-mutant HeLa cells and a G1 arrest in p53 wild-type Siha cells. Combined treatment with DHA and x-irradiation decreased the population of HeLa but not Siha cells arrested in gap2/mitosis (G2/M) phase. This result clearly indicates that DHA abrogates the DNA damage-induced G2 checkpoint but elicits no effect on the G1 checkpoint.

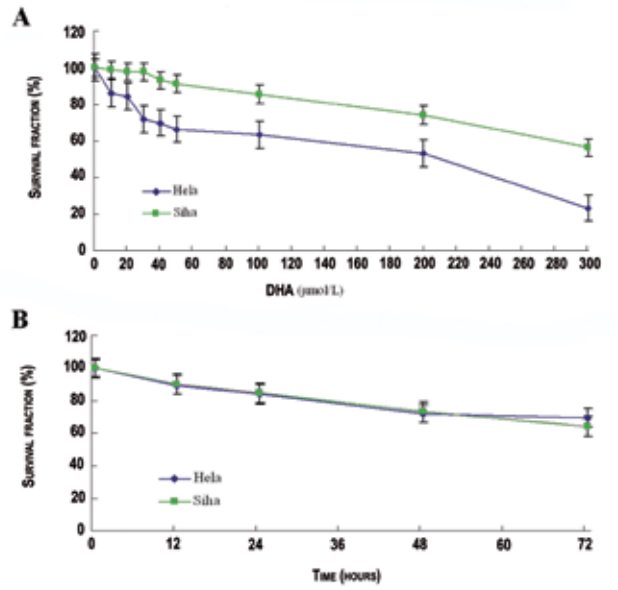


**Figure 1 -** Graph showing the **A)** Dihydroartemisinin (DHA)-induced cytotoxicity in HeLa and Siha cells. HeLa and Siha cells were exposed to the indicated concentrations of DHA for 24-hours. Cell survival was assessed using an MTT assay. **B)** The DHA-induced cytotoxicity in HeLa (20 μmol/L DHA) and in Siha cells (100 μmol/L DHA). HeLa and Siha cells were exposed to DHA for different time points. Cell survival was assessed using an MTT assay. The data are shown as the mean values ± standard error of the mean (±SEM) for 3 independent experiments.

***The effect of DHA and x-irradiation on Wee1, Cyclin B1 and Cdc2 proteins.*** One of the most important driving forces for the G2-M progression is the Cdc2/Cyclin B1 protein complex. Cdc2 is both positively and negatively regulated by phosphorylation. Thr-161 phosphorylation is required for Cdc2 kinase activity, whereas Thr-14 and Tyr-15 phosphorylation inhibits its kinase activity. Wee1 is the major kinase that phosphorylates Cdc2 on Tyr-15.[19-21] Therefore, inhibition of Wee1 can decrease Cdc2 Tyr-15 phosphorylation and lead to the activation of Cdc2 kinase. Thus, we investigated whether DHA treatment modulated the expression of Wee1, Cyclin B1 and Cdc2 after x-irradiation of HeLa and Siha cells. As shown in **Figure** 4, the number 5, 6 or 12 Gy x-ray irradiation induced the expression of Wee1. The addition of DHA prior to irradiation resulted in decreased Wee1 and increased Cyclin B1 expression in HeLa cells (**Figure** 4, left panel). Therefore, the decrease of Wee1 might abrogate the arrest of cells in G2, resulting in increased amounts of unrepaired, damaged DNA in cells that prematurely enter mitosis.

In the Siha cells, the relative expression of Wee1 and Cyclin B1 remained unaltered. The Cdc2 protein levels were unstable in both HeLa and Siha cells. These results indicated that the combined treatment with DHA and radiation reduced Wee1 and increased Cyclin B1 expression, abrogating the G2/M arrest induced by radiation. These radiosensitizing effects were observed in the p53-mutant HeLa cells but not in the p53 wild-type Siha cells. The expression of Cdc2 remained unchanged

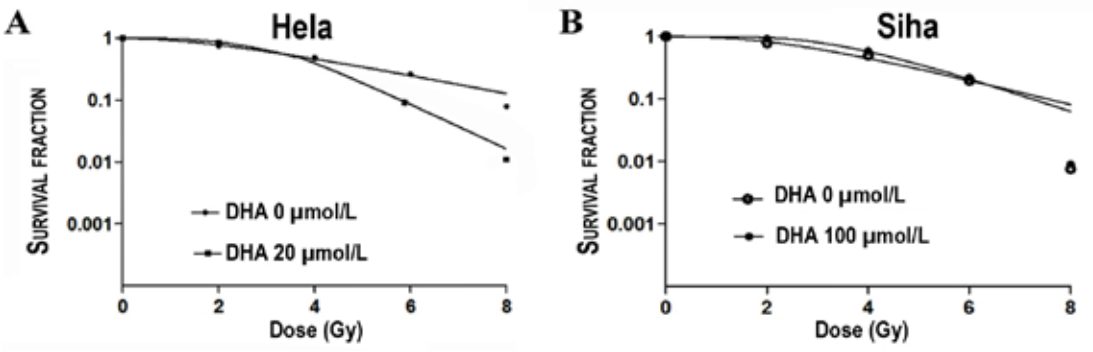


**Figure 2 -** The dihydroartemisinin (DHA)-induced radiosensitivity in HeLa **A)** and **B)** Siha cells. Clonogenic cell survival curves were generated for HeLa and Siha cells that were treated with the indicated concentrations of DHA for 24-hours and then were exposed to 2, 4, 6 or 8 Gy irradiation (IR). The survival data were normalized to that of the unirradiated control group. The sensitizer enhancement ratios (SER) was calculated for HeLa or Siha cells that were treated with 20 or 100 μmol/L DHA prior to x-irradiation. The values shown are the mean values ± standard error for 3 independent experiments.

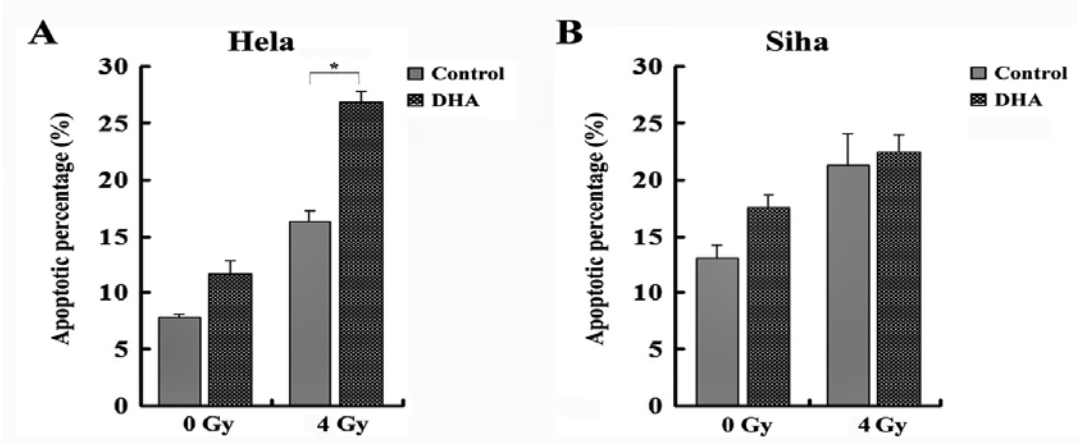


**Figure 3 -** Induction of apoptosis by dihydroartemisinin (DHA) and radiation in **A)** HeLa and **B)** Siha cells. Apoptosis was measured using propidium iodide (PI)/annexin-V double-staining. Statistical analysis between the groups were determined by using analysis of variance, **p*<0.05.

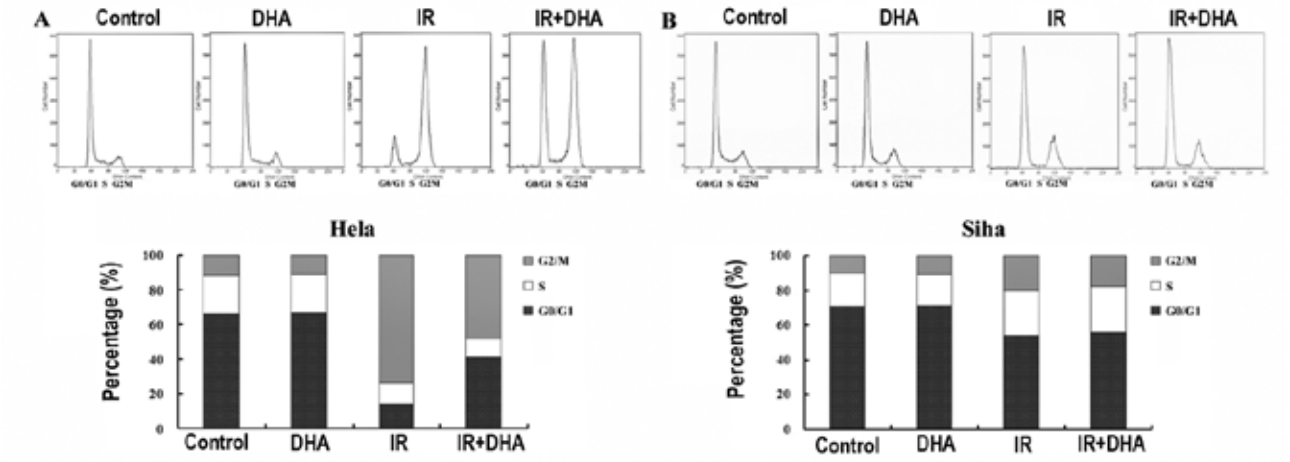


**Figure 4 -** The effect of dihydroartemisinin (DHA) and radiation on **A)** HeLa and **B)** Siha cell cycle progression. Cells were treated with or without 20 μmol/L (HeLa) or 100 μmol/L DHA (Siha) for 24-hours prior to exposure to 6 Gy irradiation (IR). After 24-hours, both attached and floating cells were harvested, and the cell cycle distributions were analyzed.

in both cell types regardless of radiation treatment, suggesting that DHA might affect Cdc2 kinase activity indirectly through Wee1 rather than the Cdc2 protein level directly.

**Discussion.** Recently, artemisinin and its derivatives have been shown to exhibit anti-cancer properties through their ability to reduce cell number in a variety of solid tumors in vitro and in vivo.[5-9,22] Dihydroartemisinin reacts with ferrous iron to generate free radicals, leading to macromolecular damage and subsequent cell death.[5-9] Cancer cells usually express more cell surface transferrin receptors and uptake more iron compared to normal cells, rendering them more vulnerable to the cytotoxic effects of artemisinin.[5,23] Once inside the cell, iron released from transferrin can react immediately with artemisinin or and its derivatives, resulting in the formation of cytotoxic free radicals.[7,8] In this study, we have shown that DHA elicits inhibitory effects on human cervical cancer cells in a concentration- and time-dependent manner. Comparatively, DHA induced a stronger inhibitory effect on HeLa than on Siha cells. Moreover, DHA increased radiosensitivity and promoted the apoptosis of the p53-mutant HeLa cells but not of the wild-type p53 Siha cells. In HeLa cells, combined DHA and radiation treatment decreased and increased Wee1 and Cyclin B1 expression levels, respectively, impairing the irradiation-induced G2/M arrest. However, in the Siha

cells, the combined treatment with DHA and radiation did not alter the expression levels of Wee1 or Cyclin B1.

Artemisinin and its analogs are effective radio sensitizers that enhance radiation-induced DNA damage and block DNA repair pathways after irradiation.[6,24] In this study, DHA can decrease the cell G2/M arrest induced by radiation, which probably resulted in more irradiation-damaged cells entering into mitosis phase. However, DHA elicited no significant inhibition effect on G1/S arrest. Irradiation exposure can induce a G2/M arrest in HeLa cells through increased and decreased expression of Wee1 and Cyclin B1, respectively. This cell cycle arrest and protein expression change was reversed by treatment with DHA. Siha cells might be arrested in G1/S phase, thus accounting for the lack of changes in protein expression and cell cycle progression. The expression of Cdc2 exhibited no significant changes in either HeLa or Siha cells that were treated with a combination of DHA and irradiation, suggesting that DHA might affect Cdc2 indirectly through Wee1. Wee1 is part of an intricate network of kinases and phosphatases that regulate the G2 checkpoint,[16,25] the abrogation of this checkpoint by Wee1 inhibition results in mitotic catastrophe. DHA treatment resulted in similar abrogation of the radiation-induced G2 arrest in HeLa cells.

The tumor suppressor and transcription factor p53 is a major regulator of cellular defense against neoplastic transformation and cancer development.[26] p53 is often regarded as a genome guardian that mediates cellular response to stressful conditions.[27,28] The p53 gene is mutated in more than 50% of all human cancers.[26,29] Defects in p53-dependent pathways are correlated with tumor resistance to radiation and chemotherapy.[30] DNA-damaging agents often induce cell cycle arrest in G1 or G2 phases,[31,32] which are facilitated by checkpoint mechanisms that provide time for the repair of sub-lethal DNA damage prior to the resumption of cell cycle progression.[33] Due to the defective signaling that results from the mutation of p53, many cancer cells do not exhibit a functional G1 arrest and are more dependent upon the G2 checkpoint for response to DNA damage.[34] Therefore, the abrogation of the G2 checkpoint has emerged as a potential therapeutic strategy because it promotes premature mitotic entry and subsequent cell death.[16,34] The findings of this study, which used paired p53-positive and p53-negative cancer cells, support the hypothesis that abrogation of the G2 checkpoint by targeting the Cyclin B1 and Wee1 kinases represents an effective therapeutic approach against p53-null cancer cells. Wild-type p53 might protect the genome from accumulating DNA damage and transmitting genetic mutations to subsequent daughter cells.[27-29]

***Study limitations.*** A limitation of this study is that more paired p53-positive and p53-negative cancer cells are need to be used to confirmed the role of DHA in radiosensitivity. Future studies may attempt disrupting p53 combined with DHA and radiation for the p53-positive cells. Cells lacking wild-type p53 gene are not anticipated to exhibit a G1 checkpoint and would therefore depend more on the G2 checkpoint, arresting in G2 phase following DNA damage to maintain cell viability by providing time for DNA repair prior to mitotic entry. Therefore, the abrogation of the G2 checkpoint might preferentially kill p53-inactive cancer cells by removing the only checkpoint that prevents these cells from premature mitotic entry in the presence of DNA damage.

In summary, our results show that DHA treatment enhanced the radiosensitivity of HeLa cells by suppressing Wee1 kinase and increasing Cyclin B1 protein levels after x-ray irradiation. Dihydroartemisinin is a potent abrogator of the G2 checkpoint control in cancer cells with defective p53 function. Dihydroartemisinin can relieve the G2/M arrest induced by irradiation and thus be used as an effective radio sensitizer, which probably drives more irradiation-damaged cells into mitosis. Dihydroartemisinin might be capable of enhancing the effectiveness of DNA-damaging agents in the treatment of tumors with cells lacking normal p53 function. Clinical application of this anti-malarial drug can be expanded to complement the radiation therapy of cancer.

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