

Experimental Therapy of Hepatoma with Artemisinin and Its Derivatives: *In vitro* and *In vivo* Activity, Chemosensitization, and Mechanisms of Action

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Abstract Purpose: ART and its derivatives, clinically used antimalarial agents, have recently shown antitumor activities. However, the mechanisms underlying these activities remain unclear. This study was designed to determine their antitumor efficacy and underlying mechanisms of action in human hepatoma cells.

Experimental Design: The *in vitro* cytotoxicities of ART, DHA, artemether, and artesunate were compared in human hepatoma cells, HepG2 (p53 wild-type), Huh-7 and BEL-7404 (p53 mutant), and Hep3B (p53 null), and a normal human liver cell line, 7702. Based on their activity and specificity, ART and DHA were further investigated for their *in vitro* and *in vivo* antitumor effects and their effects on the protein expression of genes associated with cell proliferation and apoptosis.

Results: ART and DHA exerted the greatest cytotoxicity to hepatoma cells but significantly lower cytotoxicity to normal liver cells. The compounds inhibited cell proliferation, induced G₁-phase arrest, decreased the levels of cyclin D1, cyclin E, cyclin-dependent kinase 2, cyclin-dependent kinase 4, and E2F1, and increased the levels of Cip1/p21 and Kip1/p27. They induced apoptosis, activated caspase-3, increased the Bax/Bcl-2 ratio and poly(ADP-ribose) polymerase, and down-regulated MDM2. In mice bearing HepG2 and Hep3B xenograft tumors, ART and DHA inhibited tumor growth and modulated tumor gene expression consistent with *in vitro* observations. DHA increased the efficacy of the chemotherapeutic agent gemcitabine.

Conclusions: ART and DHA have significant anticancer effects against human hepatoma cells, regardless of p53 status, with minimal effects on normal cells, indicating that they are promising therapeutics for human hepatoma used alone or in combination with other therapies.

Human hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide (1), and more than 80% of liver cancer cases occur in developing countries, such as

China and Africa. HCC has a long latency but is often diagnosed at late stages when tumors are of high grade and progress rapidly. These characteristics, coupled with its high likelihood of invasion, lead to a poor prognosis for patients diagnosed with the disease. Nonsurgical approaches are necessary because patients with large tumors (>5 cm in diameter) or numerous lesions (>3) typically are not suitable for hepatic resection (2). Unfortunately, the activity of single chemotherapeutic agents is limited, with a very low response rate. Moreover, aggressive combination chemotherapeutic regimens have not led to any remarkable improvement in response rates (3, 4).

In advanced HCC, cancer cells do not respond to the cytotoxic effects of most of the available chemotherapeutic agents (2). Therefore, there is a pressing need to identify alternative chemotherapeutic strategies that circumvent these limitations. Phytochemicals show promise in this area because of both their potential as chemopreventive agents and their chemotherapeutic activities against HCC in experimental studies (5, 6). Recently, gemcitabine, a novel nucleoside analogue that has a broad spectrum of antitumor activity in solid tumors, has been evaluated in clinical trials to treat HCC (7, 8). Gemcitabine monotherapy improves the results of HCC treatment, as the reported median survival time increases up to 34 weeks. Because gemcitabine is particularly promising because of its low apparent toxicity

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Translational Relevance

Hepatocellular carcinoma (HCC) is one of the leading causes for cancer-related death in China, which is often diagnosed at late stage when curative therapies are not available and is highly resistant to conventional chemotherapeutic agents. There is an urgent need for more effective agents for the clinical management of HCC, especially for patients with unresectable diseases. We are interested in developing novel natural product anticancer agents for HCC treatment. The present investigation was designed to determine the anti-HCC activities and possible mechanisms of action of artemisinin (ART) and its three analogues: dihydroartemisinin (DHA), artemether, and artesunate. ART derivatives are widely used as antimalarial agents in the clinic and have recently been shown to have antitumor activities. In this study with *in vitro* and *in vivo* HCC models, we showed that ART derivatives exerted their anticancer effects in a structure- and dose-dependent manner. Moreover, one of the leading compounds, DHA, produced strong *in vivo* antitumor effects administered alone and in combination with chemotherapeutic agent gemcitabine, with minimal host toxicity. These results indicate that DHA may be further developed as an anti-HCC agent, especially in combination therapy. Because ART derivatives are clinically used drugs, the findings may be readily translated to clinical practice.

profile, further studies in combination with other active agents are warranted (7, 8).

Artemisinin (ART), a natural product isolated from the plant *Artemisia annua* L, is widely used as an antimalarial drug (9). Various derivatives of ART, such as dihydroartemisinin (DHA), artemether (ARM), and artesunate (ARS; Fig. 1A), also have potent activities against malarial parasites (10–12). In recent years, ART derivatives have also been shown to have anticancer effects (13–18). The primary mechanism by which ART derivatives exert their anticancer activity is thought to be induction of apoptosis (19, 20), although the detailed mechanisms remain to be elucidated. Interestingly, their low host toxicity is the major incentive for developing these compounds as anticancer agents. For instance, DHA selectively inhibits the growth of Molt-4 lymphoblastoid cells but is significantly less toxic to normal human lymphocytes (21). ARS inhibits the growth of Kaposi's sarcoma, and this inhibition of cell growth correlates with the induction of apoptosis (15). However, the mechanisms by which ART and its derivatives exert specific anticancer activity remain unclear, which may limit the further development of these compounds in preclinical and clinical settings.

The present study was designed to show the *in vitro* and *in vivo* anticancer effects of various ART derivatives (ART, DHA, ARM, and ARS) on hepatoma cells with various p53 statuses (HepG2, Hep3B, BEL-7404, and Huh-7), with an emphasis on their molecular targets both *in vitro* and *in vivo*. Our results may provide a basis for future development of these compounds as anti-HCC agents used alone or in rational combination with other chemotherapeutic agents, such as gemcitabine.

Materials and Methods

Test compounds, chemicals, and reagents. ART and its derivatives, DHA, ARM, and ARS, were kind gifts of Zhejiang Yiwu Golden Fine Chemical Co. Ltd. Gemzar (gemcitabine) was purchased from Eli Lilly Co. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals used in this study were of analytic grade and purchased from Sigma-Aldrich, Inc. Tween 20 was purchased from Promega Corp., and the Annexin V-FITC Apoptosis Detection kit was purchased from BioVision, Inc. The primary antibodies against Bcl-2 (100), Bax (N-20), caspase-3 p20 (N-19), E2F1 (C-20), cyclin D1 (DCS-6), cyclin E (HE12), cyclin-dependent kinase (Cdk) 2 (M2), Cdk4 (H-22), Cip1/p21 (187), Kip1/p27 (C-19), poly(ADP-ribose) polymerase (PARP; H-250), Rb (C-15), MDM2 (SMP14), p53 (Pab1801), glyceraldehyde-3-phosphate dehydrogenase (0411), and β -actin (1-19) were from Santa Cruz Biotechnology, Inc. The secondary antibodies, horseradish peroxidase-linked anti-mouse immunoglobulin G, anti-goat immunoglobulin G, and anti-rabbit immunoglobulin G, were also purchased from Santa Cruz Biotechnology. DMEM, RPMI 1640, penicillin, streptomycin, fetal bovine serum, and trypsin/EDTA were purchased from Life Technologies. The detergent-compatible protein assay kit was purchased from Bio-Rad and the ECL Plus Western Blotting Detection System was purchased from Amersham Pharmacia Biotech.

Cell culture. Human hepatoma cell lines HepG2, Hep3B, BEL-7404, and Huh-7 and the nonneoplastic human liver cell line 7702 were gifts from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, People's Republic of China). The hepatoma cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin and maintained in an incubator with a humidified atmosphere of 5% CO₂ at 37°C. The 7702 cells were cultured with the RPMI 1640 supplemented with 10% fetal bovine serum, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin under the conditions described above.

Cell viability assay. The effects of ART derivatives on the viability of the aforementioned cells were determined using the MTT assay as previously reported (22–24). Briefly, 2,000 cells per well were plated in triplicate in 96-well plates. After a 24-h incubation, the cells were treated with varying concentrations of ART derivatives (0, 1, 5, 10, 25, 50, and 100 μ mol/L) for 48 h. The MTT assay was done as previously reported (22–24) and the resultant formazan crystals were dissolved in DMSO (100 μ L). The absorbance was then recorded at 540 nm. The effects of ART derivatives on cell viability were assessed by comparing the percent cell viability of the treated cells with the vehicle (DMSO)-treated control cells, which were arbitrarily assigned 100% viability. The experiment was repeated thrice under the same conditions.

In addition, the growth-inhibitory effects of ART and DHA and the effects in combination with gemcitabine in HepG2 and Hep3B cells were also determined using the MTT assay. Briefly, 700 cells per well were plated in 96-well culture plates. After a 24-h incubation, the cells were treated with 10 μ mol/L ART, 10 μ mol/L DHA, 10 μ g/L gemcitabine, 10 μ mol/L ART plus 10 μ g/L gemcitabine, or 10 μ mol/L DHA plus 10 μ g/L gemcitabine for various times (0, 24, 48, 72, and 96 h). The results reflect the average of three replicates.

Cell cycle analysis. Cells (2×10^5) were treated with ART and DHA (0, 1, 10, 25, and 50 μ mol/L) as described above for 48 h. The harvested cells were resuspended in 200 μ L of cold PBS, to which cold ethanol (600 μ L) was added, and the mixture was then incubated for 2 h at 4°C. After centrifugation, the pellet was washed with cold PBS, suspended in 500 μ L PBS, and incubated with 50 μ L RNase (20 μ g/mL final concentration) for 30 min. The cells were incubated with propidium iodide (50 μ g/mL final concentration) for 30 min in the dark. The cell cycle distribution was then determined using a FACSAria instrument (BD Biosciences). The experiment was done as previously reported (22–24) and repeated thrice under the same conditions.

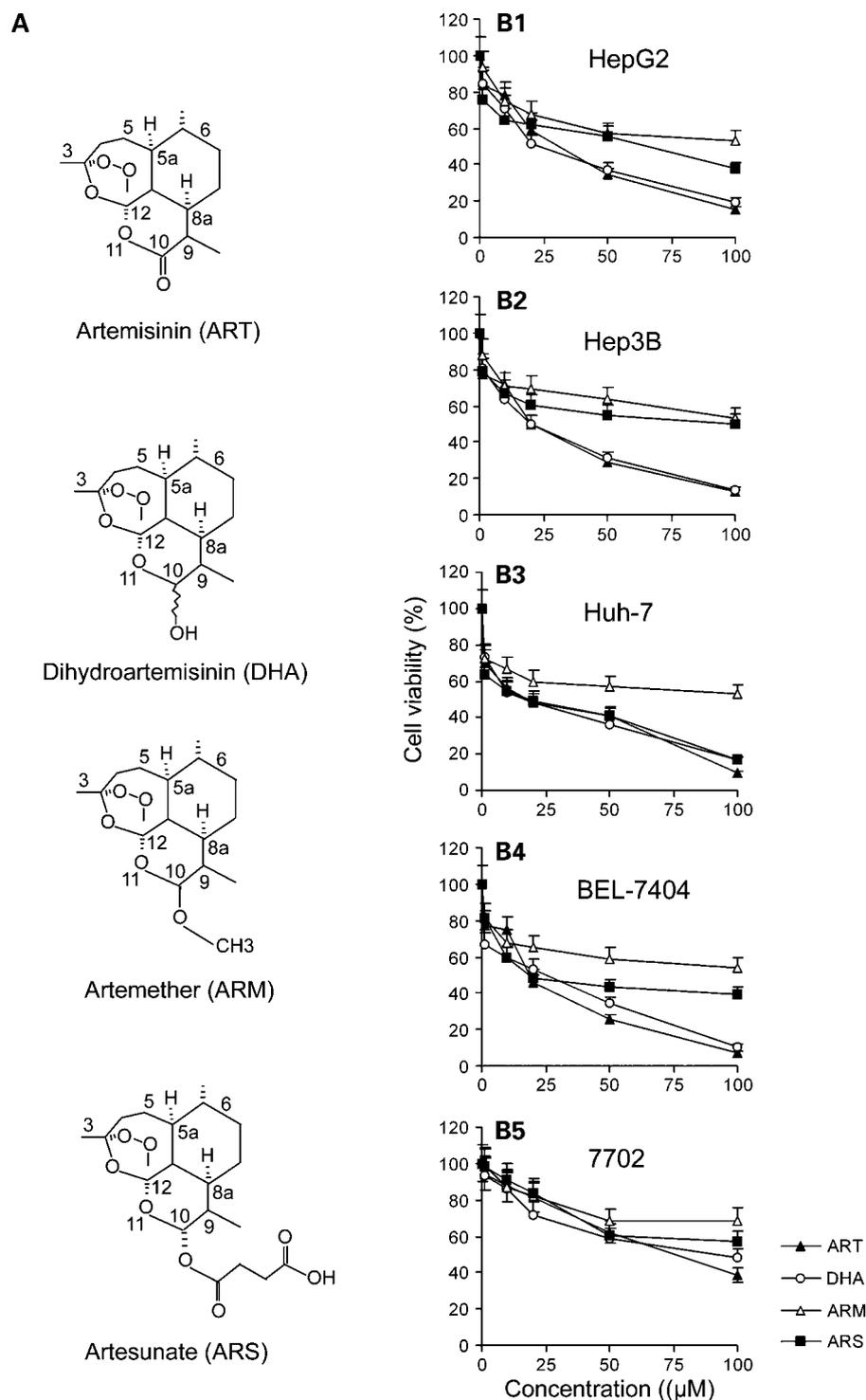


Fig. 1. A, chemical structures of the four ART compounds: ART, DHA, ARM, and ARS. Cytotoxicity of the four ART compounds to human hepatoma cells HepG2 (B1), Hep3B (B2), Huh-7 (B3), and BEL-7404 (B4) and normal human liver 7702 cells (B5). Cells were exposed to various concentrations of the compounds (0, 1, 5, 10, 25, 50, and 100 $\mu\text{mol/L}$) for 48 h followed by MTT assay. All assays were done in triplicate.

Quantification of apoptotic cells. ART- and DHA-induced apoptosis alone or in combination with gemcitabine in HepG2 and Hep3B cells was determined by flow cytometry (25–27) using the Annexin V-FITC Apoptosis Detection kit following the manufacturer's instructions. Briefly, 2×10^5 cells were treated with ART and DHA (0, 1, 10, 25, and 50 $\mu\text{mol/L}$) or 10 $\mu\text{g/L}$ gemcitabine for 48 h. The cells were then harvested, washed in PBS, and incubated with Annexin V and propidium iodide for staining in binding buffer at room temperature

for 10 min in the dark. The stained cells were analyzed using the FACSaria instrument.

Western blot analysis. Whole-cell lysates were generated with radioimmunoprecipitation assay lysis buffer, and after centrifugation, the supernatant fraction was collected for immunoblotting (23–28). Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk in blocking buffer [20 mmol/L TBS (pH 7.5) containing 0.1% Tween 20], the

Table 1. Growth-inhibitory activity of ART compounds

Cell line	Inhibitory concentration ($\mu\text{mol/L}$)	ART	DHA	ARM	ARS
7702	IC ₂₀	3.6	3.8	7.9	7.3
	IC ₅₀	60.9	167.7	492	>500
	IC ₈₀	>500	>500	>500	>500
HepG2 (p53 wild-type)	IC ₂₀	1.3	1.2	2.6	1.3
	IC ₅₀	13.98	13.35	54.8	20.5
	IC ₈₀	145.1	145.8	>500	338.2
Hep3B (p53 null)	IC ₂₀	0.97	0.96	2.4	1.5
	IC ₅₀	10.4	10.3	51.5	39.4
	IC ₈₀	113.3	110.7	>500	>500
Huh-7 (p53 mutant)	IC ₂₀	0.7	0.7	1.2	0.6
	IC ₅₀	8.9	9.6	31.4	9.22
	IC ₈₀	115.4	130.9	>500	146.1
BEL-7404 (p53 mutant)	IC ₂₀	0.9	0.7	2.4	1.0
	IC ₅₀	9.9	9.3	31.78	15.0
	IC ₈₀	107.1	129.7	>500	215.4

membrane was incubated with the desired primary antibody for 2 h at room temperature and then incubated with appropriate peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the ECL Plus Western Blotting Detection System. The level of β -actin for each sample was used as loading control. Tumor tissues were collected at the termination of the experiment and homogenized using a homogenizer in ice-cold lysis buffer. Supernatants were collected and used to examine the expression of different proteins by Western blot analysis.

Hepatoma xenograft models. Female athymic nude mice (*nu/nu*; 4-6 wk of age) were obtained from Shanghai Slac Laboratory Animal Co. Ltd. All animals were fed with commercial diet and water *ad libitum*. The human HCC xenograft models in mice were established using the method described previously (25, 28). HepG2 and Hep3B cells were

resuspended in serum-free DMEM with Matrigel basement membrane matrix at a 5:1 ratio. The cell suspension was then injected (7×10^6 cells; total volume, 0.2 mL) into the left inguinal area of the BALB/c nude mice. The animals were monitored for activity and physical condition everyday, and the determination of body weight and measurement of tumor mass were done every 3 d. Tumor mass was determined by caliper measurement in two perpendicular diameters of the implant and calculated using the formula $1/2a \times b^2$, where *a* stands for the long diameter and *b* is the short diameter (24–28). The animal use and care protocol was approved by the Institutional Review Board of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

In vivo chemotherapy. Nude mice bearing HepG2 and Hep3B xenografts, randomly divided into various treatment and control groups (five mice per group), were treated orally with either ART or

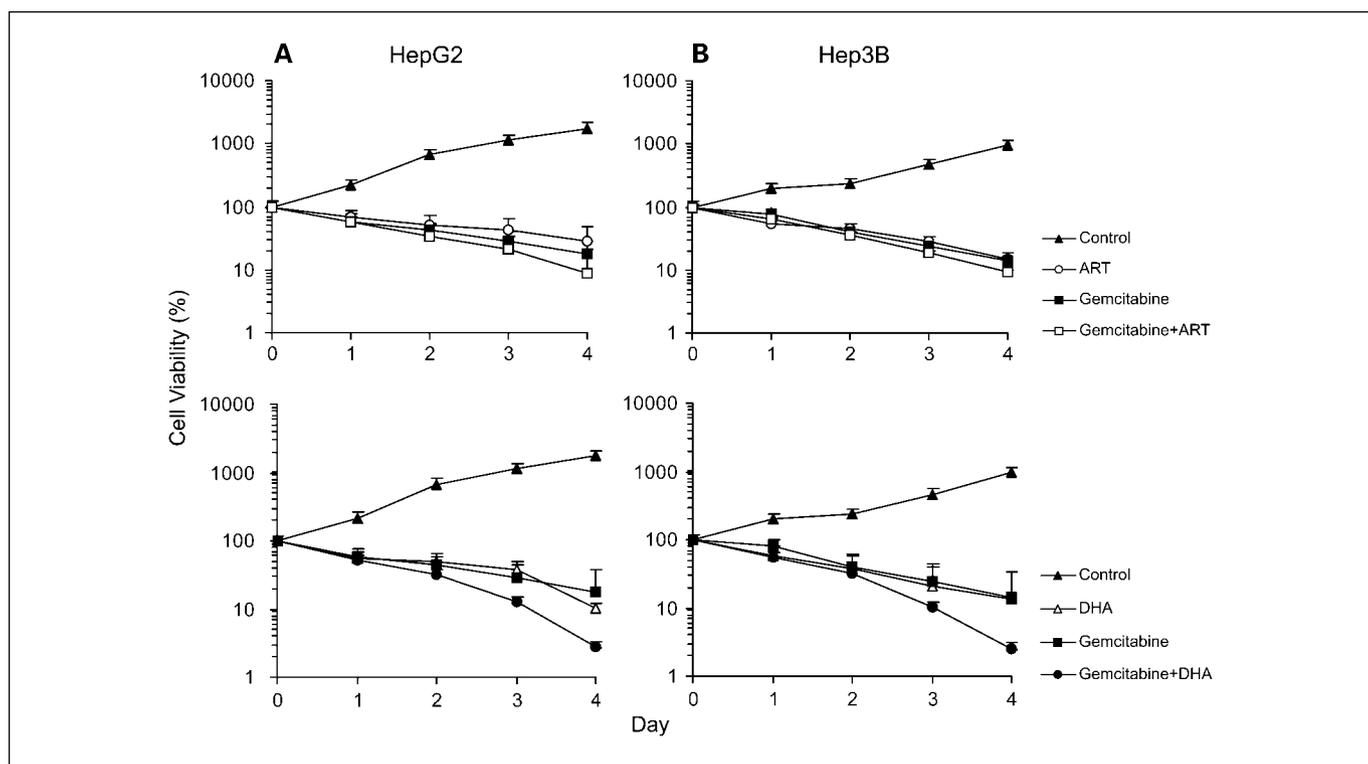


Fig. 2. The inhibitory effects of ART compounds on the growth of human hepatoma cells. HepG2 (A) and Hep3B (B) cells were exposed to 10 $\mu\text{mol/L}$ ART and DHA alone or in combination with 10 $\mu\text{g/L}$ gemcitabine for various durations (0, 24, 48, 72, and 96 h) followed by the MTT assay. All assays were done in triplicate.

DHA suspended in 5% sesame oil + 95% saline, at a dose of 50 or 100 mg/kg/d, or a combination of ART or DHA with gemcitabine or with saline (as controls). In the mice receiving combination therapy, 80 mg/kg gemcitabine, representing one fifth of the reported most tolerated dose in mice (29, 30), was administered i.p. on days 7, 11, and 15 to avoid possible side effects and to illustrate potential chemosensitization effects in this combination regimen (30).

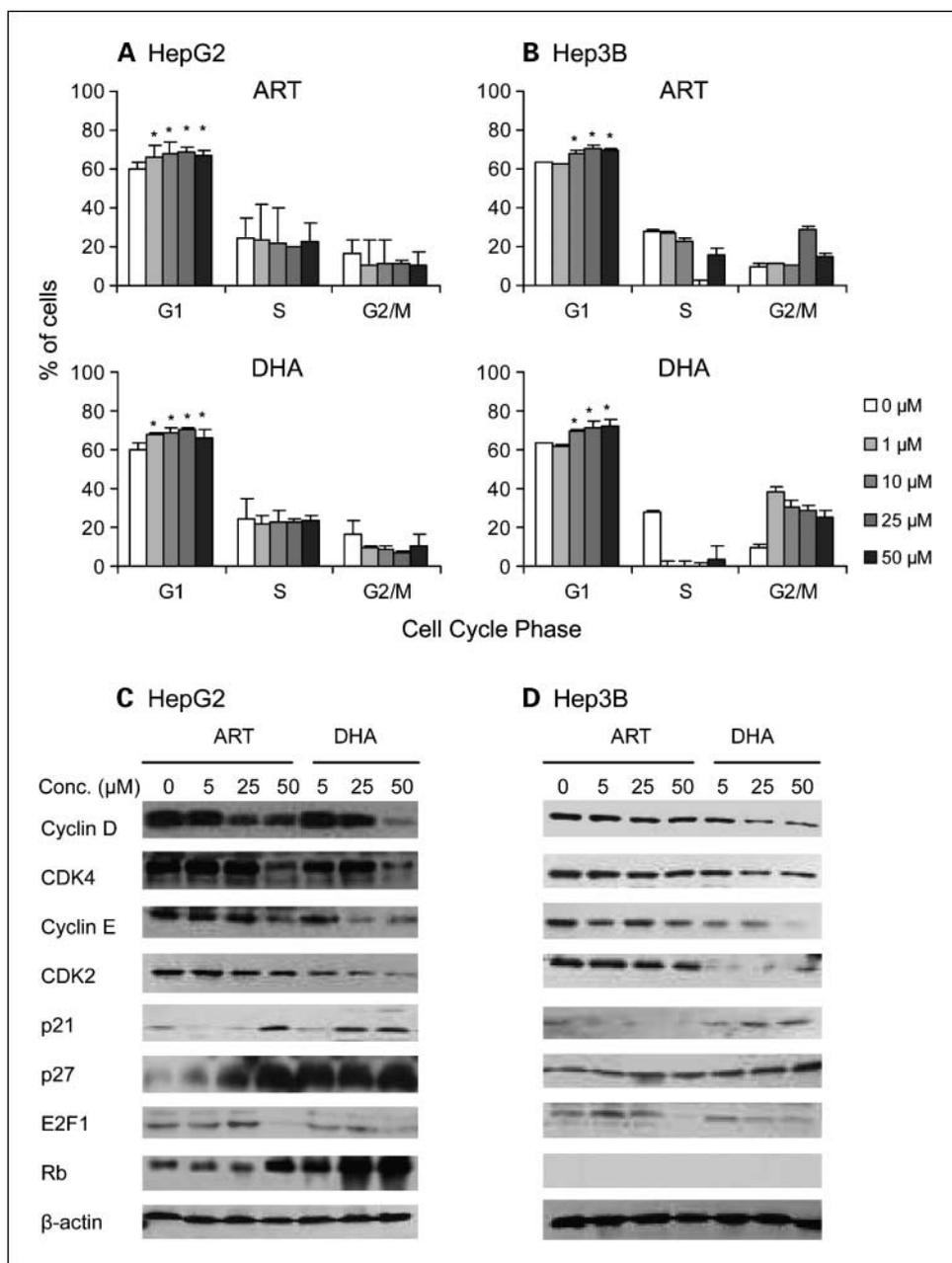
Statistical analysis. The experimental data are expressed as mean and SD, and the statistical significance of differences between control and treated groups was determined by the paired *t* test or ANOVA.

Results

ART and its derivatives selectively inhibit cell growth in human hepatoma cells. We determined the cytotoxicity of ART and its derivatives (Fig. 1A) against HepG2, Hep3B, BEL-7404,

and Huh-7 hepatoma cells as well as 7702 normal human liver cells. The treatment of HepG2 cells with ART or DHA (1-100 $\mu\text{mol/L}$) resulted in a significant reduction in cell viability as assessed by the MTT assay, with the percentage of viable cells ranging from 84.7% to 15.5% ($P < 0.01$) after a 48-h exposure (Fig. 1B1). Similar effects were obtained with Hep3B, Huh-7, and BEL-7404 hepatoma cells ($P < 0.01$; Fig. 1B2-B4). The concentrations that reduced growth by 20%, 50%, and 80% (IC_{20} , IC_{50} , and IC_{80}) are summarized in Table 1. A comparison of the IC_{50} values indicated that ART and DHA were the most active compounds, followed by ARS and then ARM (Fig. 1B1-B4; Table 1). The overall mean IC_{50} values in the four hepatoma cell lines were 10.8 $\mu\text{mol/L}$ (ART), 10.6 $\mu\text{mol/L}$ (DHA), 21.0 $\mu\text{mol/L}$ (ARS), and 42.3 $\mu\text{mol/L}$ (ARM), respectively. In contrast, the sensitivity of the 7702 cells

Fig. 3. Effects of ART and DHA on cell cycle progression of human hepatoma cells. HepG2 (A) and Hep3B (B) cells were exposed to various concentrations of the compounds (0, 1, 10, 25, and 50 $\mu\text{mol/L}$) for 48 h followed by cell cycle distribution assay. All assays were done in triplicate. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control. The effects of the compounds on the expression of cell cycle-related proteins were determined by Western blot analyses after HepG2 (C) and Hep3B (D) cells were exposed to various concentrations (5, 25, and 50 $\mu\text{mol/L}$) of the compounds for 48 h.



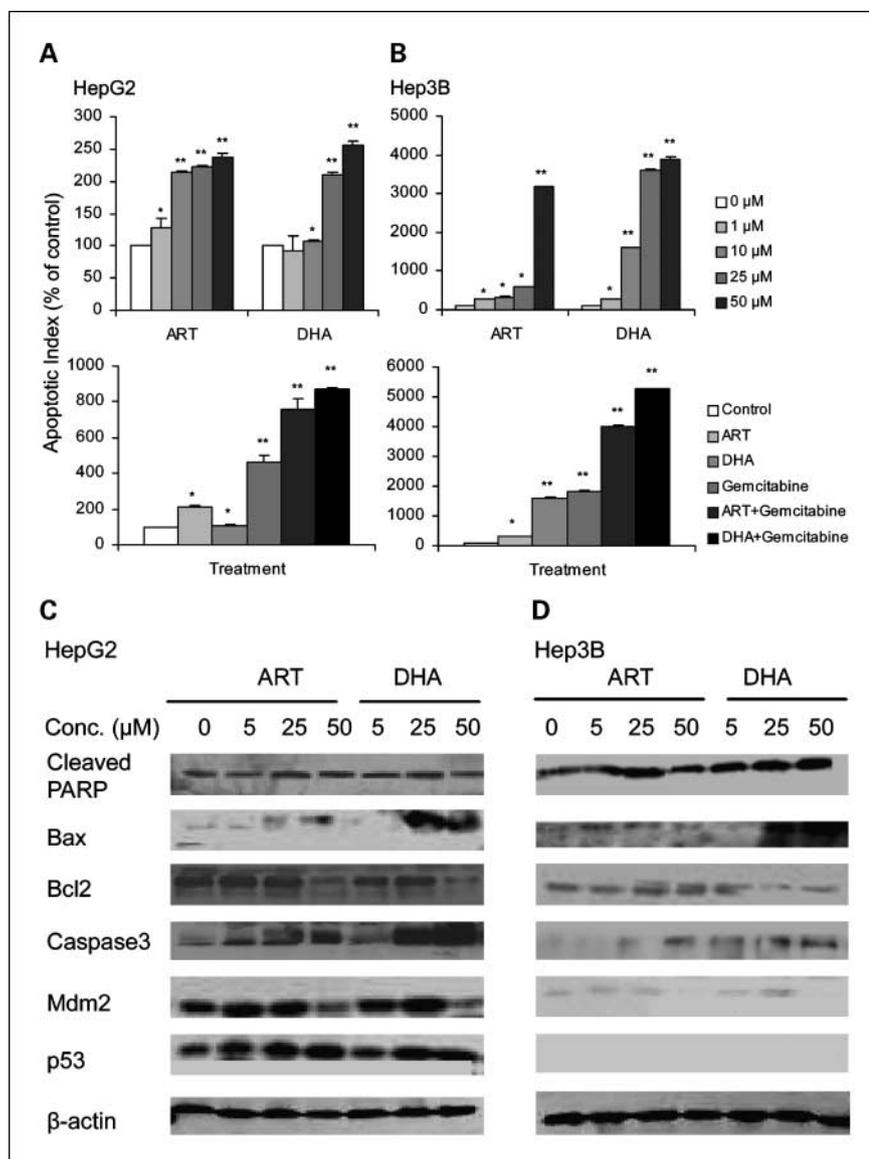


Fig. 4. Induction of apoptosis in human hepatoma cells HepG2 (A) and Hep3B (B). Cells were exposed to various concentrations of ART or DHA alone or in combination with gemcitabine for 48 h followed by apoptosis assay. All assays were done in triplicate. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control. HepG2 (C) and Hep3B (D) cells were exposed to various concentrations of the compounds for 48 h, and the target proteins were detected by Western blot analyses.

to the cytotoxic effects of ART and DHA was much lower, with IC_{50} values ranging from 60.9 to >500 $\mu\text{mol/L}$ (Fig. 1B5; Table 1), representing a 6- to 16-fold difference in cytotoxicity. These data suggest that ART and its derivatives are cytotoxic to human hepatoma cells, with almost equal efficacy against cancer cells with various p53 statuses, including p53 wild-type, p53 mutant, and p53 null cells, but that these compounds are less cytotoxic to normal human liver cells (Fig. 1B1-B5).

ART and DHA sensitize hepatoma cells to gemcitabine in vitro. The possible chemosensitization effects of ART and DHA were first determined *in vitro* using the MTT assay. As illustrated in Fig. 2A, exposure of HepG2 cells to the two compounds, especially DHA, resulted in significant growth inhibition. When compared with vehicle-treated cells, HepG2 cells exposed to ART and DHA alone showed growth inhibition at as early as 24 h, with 69% and 74% growth inhibition ($P < 0.05$), and with 92% and 93% growth inhibition ($P < 0.05$) at 48 h, and 96% and 97% ($P < 0.05$) at 72 h.

Hep3B cells exhibited an almost identical reduction in viability under these conditions (Fig. 2B).

As shown in Fig. 2, the combination of ART with gemcitabine led to a slight increase in the inhibition of proliferation of hepatoma cells compared with the single agents alone. In both HepG2 and Hep3B cells, the combination of gemcitabine and DHA led to a statistically significant decrease in cell survival ($P < 0.05$; Fig. 2A and B, bottom). The increase in inhibition of proliferation by DHA plus gemcitabine compared with gemcitabine alone was 1.2-fold.

ART and DHA induce G_1 -phase cell cycle arrest in human hepatoma cells. As we observed a significant growth-inhibitory effect of ART and DHA on hepatoma cells, we investigated whether ART and DHA had any inhibitory effect on cell cycle progression. Treatment of HepG2 cells with ART resulted in a higher number of cells in the G_1 phase at the concentrations used [10 $\mu\text{mol/L}$ (67.41%), 25 $\mu\text{mol/L}$ (70.72%), and 50 $\mu\text{mol/L}$ (69.21%)], respectively, compared

with untreated control cells (63.05%; Fig. 3A, *top*). Similar, but slightly more pronounced, results were obtained when the effect of DHA on HepG2 cells was tested, with even the 10 $\mu\text{mol/L}$ concentration significantly increasing the number of cells in the G_1 phase (69.36%, $P < 0.01$), and the higher concentrations leading to greater G_1 arrest [25 $\mu\text{mol/L}$ (70.91%, $P < 0.01$) and 50 $\mu\text{mol/L}$ (72.03%, $P < 0.01$); Fig. 3A, *bottom*].

G_1 -phase arrest was also observed when the effects of ART and DHA on cell cycle progression of Hep3B were analyzed ($P < 0.05$; Fig. 3B). The lowest concentration of 1 $\mu\text{mol/L}$ led to a modest increase in the number of cells in the G_1 phase (66.11%, 68.11%), and higher concentrations of the compounds led to greater cell cycle arrest [10 $\mu\text{mol/L}$ (67.48%, 69.03%), 25 $\mu\text{mol/L}$ (68.70%, 70.50%), and 50 $\mu\text{mol/L}$ (62.99%, 62.99%), respectively]. DHA showed stronger inhibitory effects on cell cycle progression. These data suggest that inhibition of cell proliferation in both p53 wild-type and p53 null hepatoma cells by ART and DHA is associated with the induction of G_1 arrest.

ART and DHA down-regulate cyclins and Cdks and up-regulate Cip1/p21 and Kip1/p27 in human hepatoma cells. Because Cdks, Cdk inhibitors, and cyclins play essential roles in the regulation of cell cycle progression (31, 32), we examined the effects of ART and DHA on the expression of these proteins. As shown in Fig. 3C and D, the effects of DHA were dose dependent and were stronger than those in cells exposed to ART. Treatment with DHA resulted in a marked reduction in the expression of cyclin D1, cyclin E, Cdk2, and Cdk4 in a dose-dependent manner in HepG2 and Hep3B cells. Analysis of the expression of Kip1/p27, Cip1/p21, and E2F1 indicated that DHA caused dose-dependent increases in Kip1/p27 and Cip1/p21 expression and decreased E2F1 expression in HepG2 and Hep3B cells (Fig. 3C and D). The expression of Rb was also induced by ART and DHA in HepG2 cells (Fig. 3C). These observations suggest that the increases in the levels of Cdk inhibitors may play an important role in the induction of G_1 arrest in p53 wild-type and p53 null human hepatoma cells, possibly through their inhibition of Cdk kinase activity.

ART and DHA induce apoptosis in human hepatoma cells. To determine whether the ART- and DHA-induced growth inhibition in hepatoma cells was associated with the induction of apoptosis, HepG2 and Hep3B cells were treated with ART and DHA as described above, and the numbers of apoptotic cells were assessed. Exposure of HepG2 cells to ART for 48 h resulted in a significant dose-dependent increase in apoptotic cells: 0 $\mu\text{mol/L}$ (7%), 1 $\mu\text{mol/L}$ (9%, $P < 0.05$), 10 $\mu\text{mol/L}$ (14.95%, $P < 0.01$), 25 $\mu\text{mol/L}$ (15.45%, $P < 0.01$), and 50 $\mu\text{mol/L}$ (16.45%, $P < 0.01$; Fig. 4A, *top*). Similar results were obtained when the HepG2 cells were exposed to DHA (Fig. 4A, *top*).

Gemcitabine is a known inducer of apoptosis in human cancers, including HCC cells (33), and combination with ART or DHA seemed to further increase apoptosis in HepG2 cells (Fig. 4A, *bottom*). Exposure of Hep3B cells to ART and DHA also resulted in a significant dose-dependent induction of apoptosis, and the effect of DHA was stronger than that of ART: 0 $\mu\text{mol/L}$ (0.8%), 1 $\mu\text{mol/L}$ (2.25% and 2.15%), 10 $\mu\text{mol/L}$ (2.65%, $P < 0.05$; 12.9%, $P < 0.01$), 25 $\mu\text{mol/L}$ (4.8%, $P < 0.05$; 28.75%, $P < 0.01$), and 50 $\mu\text{mol/L}$ (25.3%, 31.05%, $P < 0.01$; Fig. 4B, *top*), again indicating that ART and DHA are effective

against p53 wild-type and null hepatoma cells. Although apoptosis was induced by all three of the agents alone, it was further increased by the two combinations, especially the combination of DHA and gemcitabine, which improved the efficacy by 2-fold (Fig. 4B, *bottom*), suggesting that the chemosensitizing capacities of ART and DHA may be associated with induction of apoptosis in the hepatoma cells.

ART and DHA induce changes in the expression of apoptosis-related proteins in HepG2 and Hep3B cells. The proteins of the Bcl-2 family play critical roles in the regulation of apoptosis (31, 34). Because we observed that both ART and DHA induced apoptosis in hepatoma cells, we further determined the levels of Bcl-2 and Bax in cells treated with ART and DHA. HepG2 cells exposed to ART or DHA showed a dose-dependent reduction in the level of Bcl-2 protein, with a concomitant increase in the level of Bax, compared with the control cells (Fig. 4C), although DHA exhibited a greater effect on the level of Bax protein than ART.

To define how the apoptotic pathway was activated by ART and DHA, we further determined their effects on the activation of caspase-3 and PARP. Exposure of HepG2 cells to ART and DHA resulted in a dose-dependent increase in the cleavage of caspase-3 and PARP and ART was less effective than DHA (Fig. 4C). This indicates that the mitochondrial apoptotic pathway is activated preferentially by the compounds.

Caspase-3, an executioner caspase activated by caspase-9, cleaves a broad spectrum of cellular target proteins, including nuclear PARP, leading to a cell death cascade (34). One of the critical mediators of the mitochondrial apoptotic pathway is p53 (25, 31, 35). Treatment of HepG2 cells with ART and DHA resulted in a dose-dependent increase in p53 and a decrease in MDM2 (Fig. 4C), suggesting that ART and DHA may induce apoptosis by increasing the level of p53 in HepG2 cells. However, a p53-independent mechanism for an increase in the ratio of Bax/Bcl-2, activation of caspase-3 and the mitochondrial apoptotic pathway, as well as inhibition of MDM2 was also observed in p53 null Hep3B cells (Fig. 4D), suggesting that the ART- and DHA-induced caspase-3 activation can be both p53 dependent and independent.

ART and DHA inhibit tumor growth and have chemosensitization effects in vivo. The *in vivo* antitumor activities of ART and DHA were studied in mouse HepG2 and Hep3B xenograft models. When mean tumor mass reached 100 ± 40 mg, animals were treated with ART or DHA at oral doses of 50 and 100 mg/kg/d. In the HepG2 xenograft model, both ART and DHA alone showed a dose-dependent inhibitory effect on tumor growth (30.0% and 39.4% tumor growth inhibition for ART; 36.1% and 60.6% for DHA; $P < 0.01$; Fig. 5A1 and A2). Consistent with the *in vitro* findings, DHA showed greater therapeutic effects *in vivo* compared with ART.

Because we had observed an increase in anticancer activity following combination treatment with the ART compounds and gemcitabine *in vitro*, the effects of ART and DHA in combination with gemcitabine were investigated *in vivo*. As illustrated in Fig. 5A3, gemcitabine alone decreased tumor growth (34.9% tumor growth inhibition). A simple additivity was observed for the combination of ART with gemcitabine (62.3% tumor growth inhibition). However, combining DHA with gemcitabine significantly increased the anticancer effect (78.4% tumor growth inhibition; $P < 0.01$; Fig. 5A4), indicating that the combination of DHA and gemcitabine was

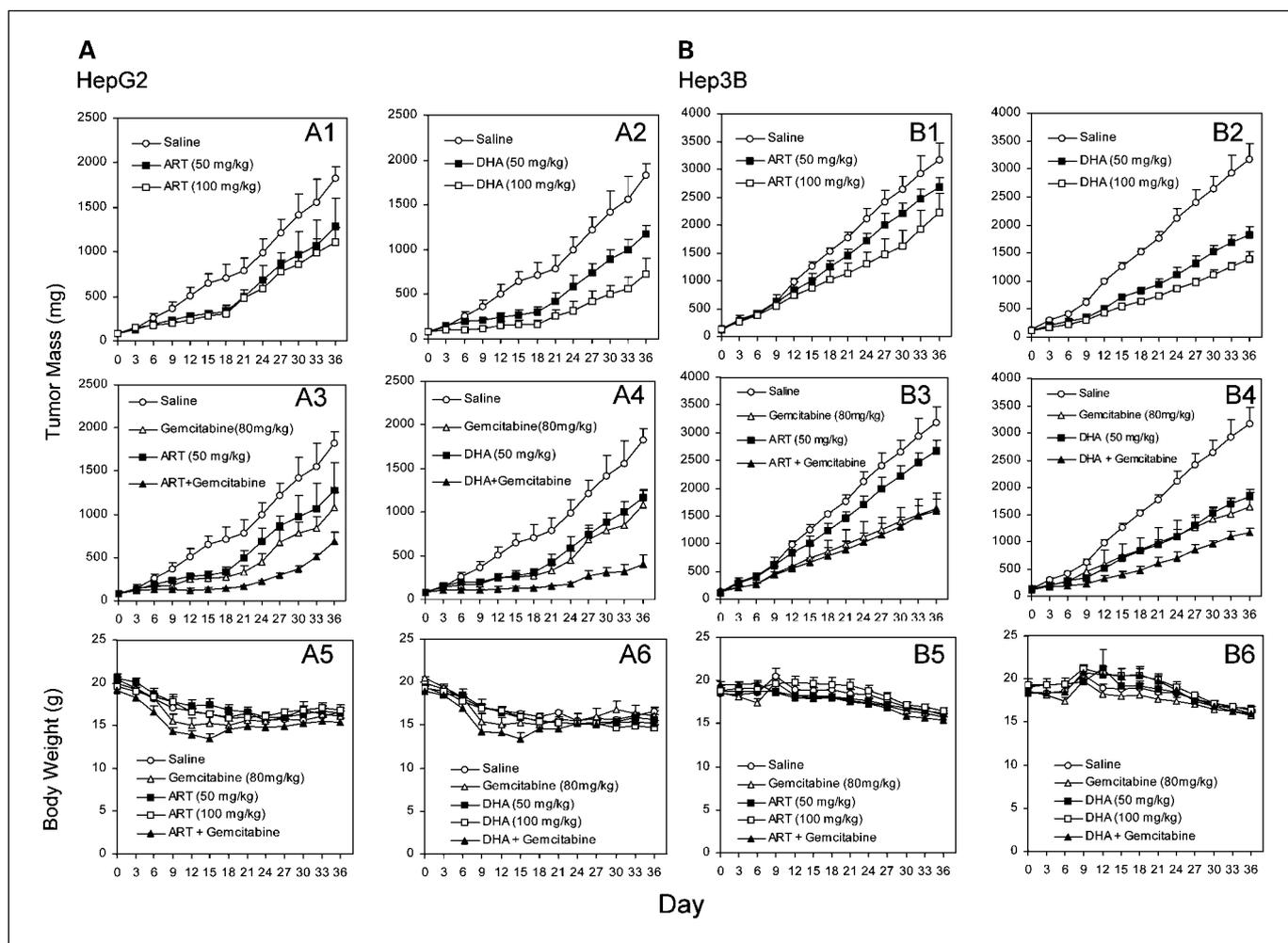


Fig. 5. *In vivo* antitumor activity and effects on body weight of ART and DHA administered alone or in combination with gemcitabine to nude mice bearing HepG2 (A) and Hep3B (B) xenograft tumors. ART and DHA were given orally at doses of 50 and 100 mg/kg/d, 5 d/wk for 4 wk (A1, A2, B1, and B2), or in combination with gemcitabine (A3, A4, B3, and B4). Gemcitabine (80 mg/kg) was given on days 7, 11, and 15 by i.p. injection. Tumor mass was determined by caliper measurement in two perpendicular diameters of the implant every 3 d. The toxic effects of administration of ART and DHA alone or in combination with gemcitabine on nude mice were determined by recording the body weight of each mouse every 3 d throughout the experiment (A5, A6, B5, and B6).

more effective than ART with gemcitabine. Moreover, based on observations of body weight, neither ART nor DHA caused any observable toxic effects when administered alone or in combination with gemcitabine (Fig. 5A5 and A6).

Similarly, ART showed a slight inhibitory effect on tumor growth in the Hep3B xenograft model (Fig. 5B1), and DHA showed greater, dose-dependent therapeutic effects compared with ART ($P < 0.01$; Fig. 5B2). As illustrated in Fig. 5B3, the combination of ART with gemcitabine showed no statistically significant increase in the inhibition of tumor growth. However, there was a further increase in the antitumor effects when the animals were treated with the combination of DHA and gemcitabine ($P < 0.01$; Fig. 5B4). Neither compound caused any observable toxic effects in this model (Fig. 5B5 and B6).

ART and DHA modulate the expression of proteins associated with apoptosis and cell cycle regulation in vivo. To determine whether the changes in expression of proliferation- and apoptosis-related proteins induced by ART and DHA *in vitro* also occurred *in vivo*, protein expression profiles of HepG2

xenograft tissue samples from animals treated with ART and DHA were determined, showing a decrease in G₁-specific Cdk, cyclin D1, cyclin E, Cdk2, Cdk4, and E2F1 in a dose-dependent manner and an increase in p21 and p27 (Fig. 5C). There were also increases in activated caspase-3, cleaved PARP, Rb, p53, and the ratio of Bax/Bcl-2 and a decrease in MDM2 (Fig. 5C), suggesting that the *in vivo* antitumor activities of ART and DHA are associated with their capacity to induce G₁-phase arrest and apoptosis. Similar protein expression profiles were observed in tumors from the p53 null Hep3B xenograft model (Fig. 5D). Taken together, these data suggest that DHA is effective for suppressing the growth of HepG2 and Hep3B xenograft tumors in nude mice and that the compound can be used in combination with gemcitabine to improve the antitumor effect of treatment.

Discussion

The overall response rate to systemic chemotherapy for the treatment of HCC is generally less than 10%, owing to drug

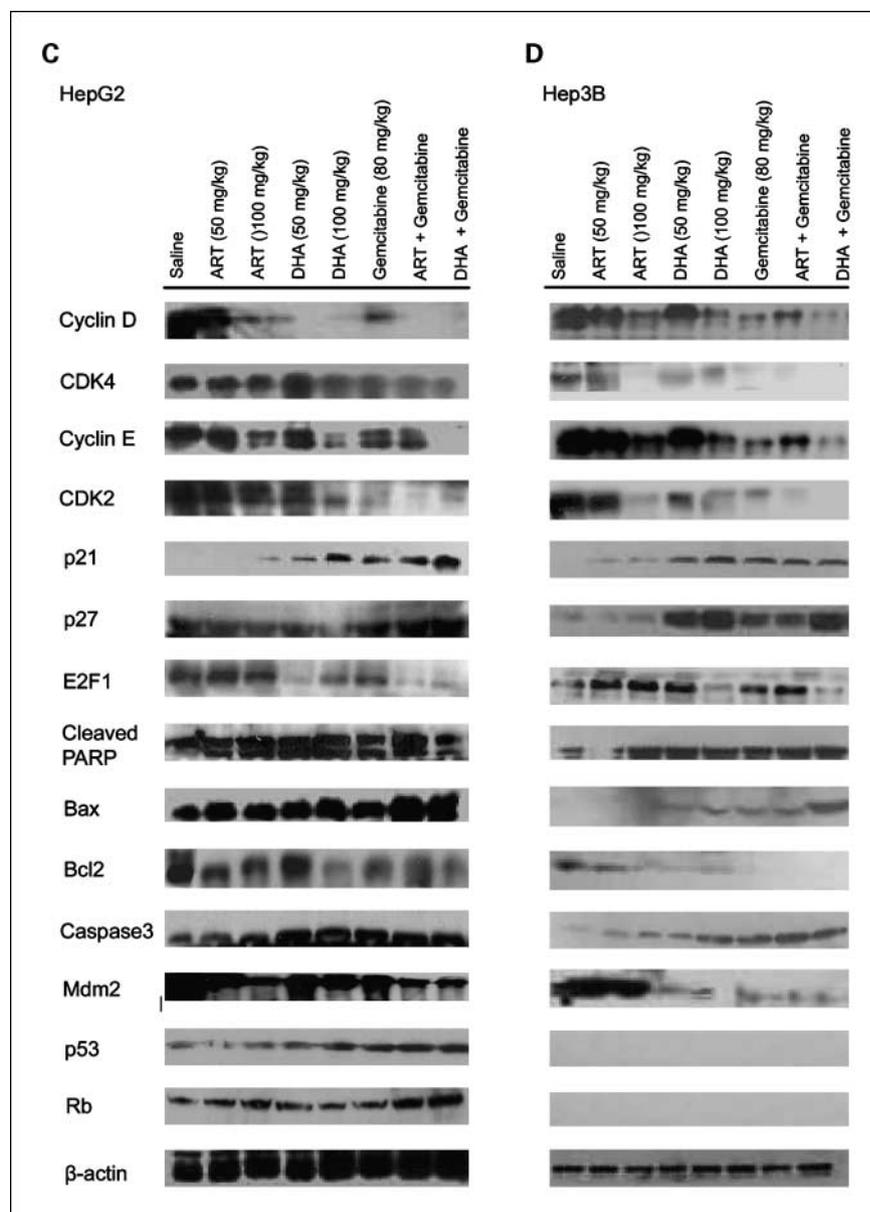


Fig. 5 Continued. C and D, at the end of the treatment, tumor xenografts were removed, and proteins in the tumor homogenate were analyzed by Western blotting.

resistance and the advanced stage of the disease (36, 37). This study represents an effort in searching for natural product anti-HCC agents. ART and its derivatives inhibit the growth of several types of cancer cells (13–18), including drug-resistant cell lines (37), suggesting that ART could become the basis of a new class of potent anticancer drugs.

Although the four test ART compounds in the present study share a common core structure, they have remarkably different effects on cancer cells. The effects of the different ART derivatives on human hepatoma cells, especially in comparison with their effects on normal human liver cells, have not been reported previously. In our present investigation, we showed that among the four ART derivatives, ART and DHA significantly reduced the viability of human hepatoma cells, with significantly lower toxicity toward nonneoplastic liver cells, which suggests that these compounds are specific and effective agents against human hepatoma cells.

Obstruction of cell cycle progression in cancer cells is considered one of the most effective strategies for the control of tumor growth (20). It has been reported that ARM suppresses concanavalin A-induced or alloantigen-induced splenocyte proliferation and inhibits cell cycle progression through the G_0 - G_1 transition in T cells (38). There have been very few reports on the effects of ART derivatives on cell cycle regulation. We have shown that treatment of both p53 wild-type (HepG2) and p53 null (Hep3B) cells with DHA resulted in significant G_1 -phase arrest, indicating that one of the mechanisms by which these compounds act is via inhibition of cell cycle progression. Our observation of significant decreases in cyclin D1, cyclin E, Cdk2, Cdk4, and E2F1 may explain the observed disruption of cell cycle progression and provides a mechanism by which ART and DHA induce G_1 -phase arrest. That is, that the cell cycle arrest is mediated through the up-regulation of Cip1/p21 and Kip1/p27 proteins (Fig. 3C and

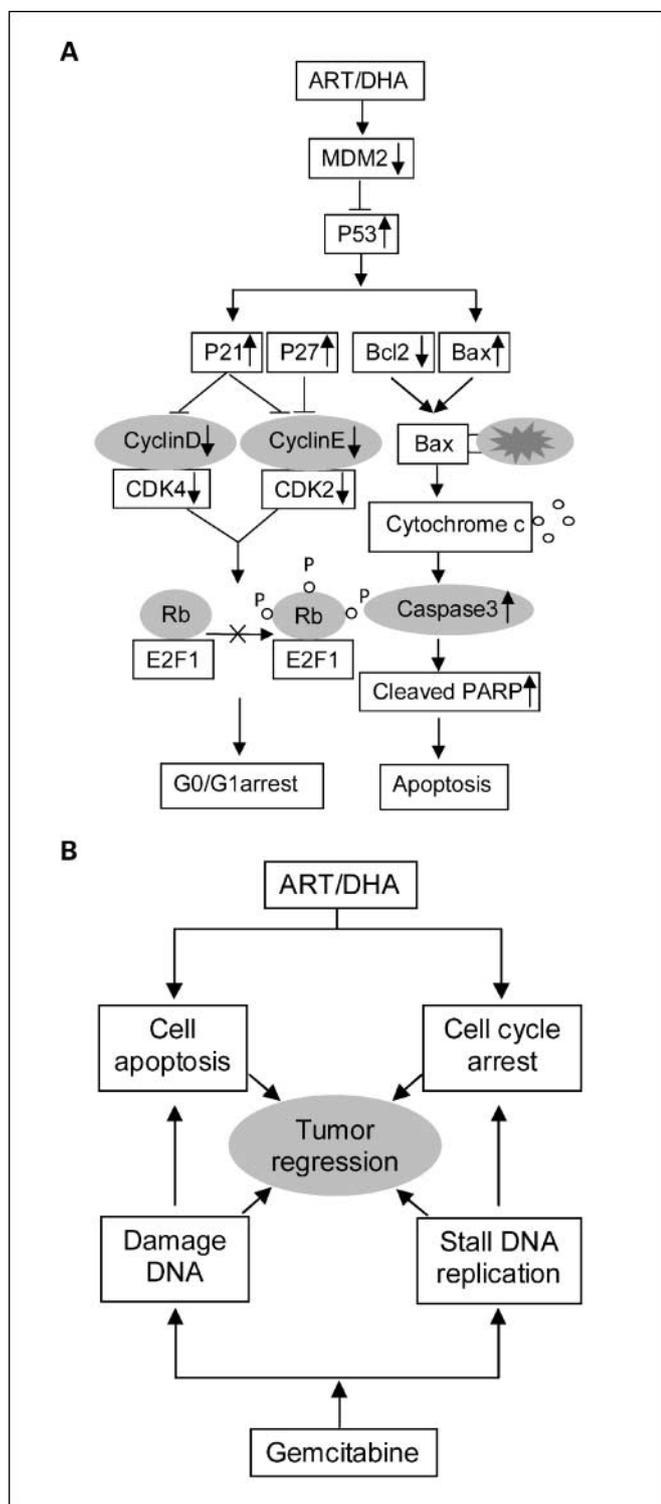


Fig. 6. A, the proposed mechanism(s) by which ART and/or DHA exert their effects via various proliferation- and apoptosis-related proteins. B, the proposed mechanism(s) by which ART and/or DHA enhance the therapeutic effects of gemcitabine.

D), which enhances the formation of complexes with the G₁-S Cdk and cyclins, thereby inhibiting their activity (26, 27, 31, 39, 40). The nearly identical effects of the compounds on

HepG2 and Hep3B cells suggest that ART and DHA can regulate cell cycle progression in both a p53-dependent and p53-independent manner.

Although ART and its derivatives are also known to induce apoptosis in cancer cells (19, 20, 41), the underlying mechanisms are not fully understood. It is important to elucidate the mechanisms by which ART and DHA induce apoptosis in hepatoma cells to optimize their activity, particularly in combination with other agents. In the present study, we found that ART and DHA induced apoptosis in both p53 wild-type (HepG2) and p53 null (Hep3B) cells and that this effect was increased when the compounds were combined with gemcitabine. We investigated the contribution of Bcl-2 family proteins to ART- and DHA-induced apoptosis and found an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 in p53 wild-type HepG2 and p53 null Hep3B cells.

An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome *c* from the mitochondria into the cytosol and the cytosolic cytochrome *c* then binds to Apaf-1, leading to the activation of caspase-3 and PARP (31, 42, 43). We found that treatment with ART and DHA resulted in a dose-dependent activation of caspase-3 and cleavage of PARP, supporting the role of caspase-3 in the ART- and DHA-induced apoptosis in human hepatoma cells, regardless of their p53 genotypes. Additionally, our findings showed a dose-dependent decrease in the expression of MDM2, which has been shown to decrease p53 protein level by targeting p53 for ubiquitin-mediated degradation (44). In addition, MDM2 is a negative regulator of p21 (26) and a positive regulator of E2F1 (27). MDM2 has been suggested as a molecular target for cancer prevention and therapy (22, 23, 25–27, 31) and can be inhibited by HIPK2 in both p53-dependent and p53-independent manners (45). Because the effects on the apoptosis of ART and DHA were observed in both HepG2 and Hep3B cells, it seems that both p53-dependent and p53-independent mechanisms may underlie the activation of caspase-3 and the apoptotic pathway (46, 47). These results are consistent with the concept that induction of apoptosis can be both p53 dependent (48) and p53 independent (49). Moreover, Hep3B cells lack both endogenous p53 and Rb, indicating that the induction of apoptosis may be Rb independent as well. This characteristic would allow broad therapeutic application of ART and DHA for HCC as well as other cancers.

ART and its derivatives are well-tolerated antimalarial drugs and have antitumor activity that may form the basis of novel antitumor combination therapies. However, ART derivatives have never been tested in combination with chemotherapy in HCC. In the present study, ART and DHA were tested alone or in combination with gemcitabine *in vitro*. We showed that the cell growth was significantly inhibited in HepG2 and Hep3B cells exposed to a combination of DHA and gemcitabine, showing the benefits of combination treatment. Because DHA and gemcitabine are clinically used drugs, the findings in this investigation can be translated relatively rapidly to clinical practice.

Dell'Eva et al. (15) have shown that ARS reduces the growth of Kaposi's sarcoma xenograft tumors *in vivo*. In the present study, the *in vivo* therapeutic effects of DHA, administered alone or in combination with gemcitabine in the HepG2 and Hep3B xenograft models, were shown to be dose dependent

and greater than the effects of ART. Perhaps the most striking finding in the present study is that DHA significantly increased the antitumor activities of gemcitabine *in vivo*. As yet, there have been no major toxicities noted in animals treated with ART or DHA alone or in combination with gemcitabine.

In the current study, we elucidated that ART and DHA induce apoptosis of tumor cells through the caspase-dependent mitochondrial pathway, and G₁-phase arrest by regulating G₁-checkpoint proteins *in vivo*. To our knowledge, there has been limited *in vivo* evidence for ART-induced apoptosis, and the present study may be the first of its kind comparing changes in proliferation- and apoptosis-related proteins induced by ART and its analogues *in vitro* and *in vivo*. The effects of ART and DHA on various proliferation- and apoptosis-related proteins and the potential mechanism(s) of action of the compounds are summarized in Fig. 6A. The most striking findings are the consistent results from the *in vitro* and *in vivo* Western blotting analyses.

It has been reported that DNA damage and stalling replication signaling pathways can both be attributed to exposure to gemcitabine. Double-strand breaks activate ataxia-telangiectasia mutated kinase, checkpoint kinase 2, ataxia-telangiectasia mutated and Rad3-related kinase, and

checkpoint kinase 1, four protein kinases that regulate apoptosis, cell cycle arrest, and DNA repair (50). Our demonstration that the combination of DHA with gemcitabine enhanced the induction of apoptosis suggests that combination therapy can improve the antitumor activity of gemcitabine and help define the mechanism by which this occurs (Fig. 6B).

Of the compounds tested, DHA showed the most potent cytotoxic, antiproliferative, proapoptotic, and cell cycle regulatory effects. Moreover, it produced strong antitumor effects against an *in vivo* model of HCC both alone and in combination with a chemotherapeutic agent. These results indicate that DHA may be an appropriate candidate for further development as an anti-HCC agent, either alone or in combination with conventional therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Experimental Therapy of Hepatoma with Artemisinin and Its Derivatives: *In vitro* and *In vivo* Activity, Chemosensitization, and Mechanisms of Action

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