J. Cell. Mol. Med. Vol 13, No 7, 2009 pp. 1358-1370

Dihydroartemisinin induces apoptosis and sensitizes human ovarian cancer cells to carboplatin therapy

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Received: February 20, 2008; Accepted: April 22, 2008

Abstract

The present study was designed to determine the effects of artemisinin (ARS) and its derivatives on human ovarian cancer cells, to evaluate their potential as novel chemotherapeutic agents used alone or in combination with a conventional cancer chemotherapeutic agent, and to investigate their underlying mechanisms of action. Human ovarian cancer cells (A2780 and OVCAR-3), and immortalized non-tumourigenic human ovarian surface epithelial cells (IOSE144), were exposed to four ARS compounds for cytotoxicity testing. The *in vitro* and *in vivo* antitumour effects and possible underlying mechanisms of action of dihydroartemisinin (DHA), the most effective compound, were further determined in ovarian cancer cells. ARS compounds exerted potent cytotoxicity to human ovarian carcinoma cells, with minimal effects on non-tumourigenic ovarian surface epithelial (OSE) cells. DHA inhibited ovarian cancer cell growth when administered alone or in combination with carboplatin, presumably through the death receptor- and, mitochondrion-mediated caspase-dependent apoptotic pathway. These effects were also observed in *in vivo* ovarian A2780 and OVCAR-3 xenograft tumour models. In conclusion, ARS derivatives, particularly DHA, exhibit significant anticancer activity against ovarian cancer cells *in vitro* and *in vivo*, with minimal toxicity to non-tumourigenic human OSE cells, indicating that they may be promising therapeutic agents for ovarian cancer, either used alone or in combination with conventional chemotherapy.

Keywords: dihydroartemisinin • artemisinin • apoptosis • ovarian cancer • chemotherapy

Introduction

Ovarian cancer poses a major health problem in women worldwide and is the fourth leading cause of cancer death in women in the United States [1]. The 5-year survival rate for early-stage patients is 80–90%, but only 25% for those diagnosed at advanced stages of the disease [2]. Unfortunately, most ovarian cancer patients have advanced disease at diagnosis [2]. Although the ovarian cancer mortality rate has not changed significantly during the past few decades, the length of survival for patients has been steadily improving, largely as a result of clinical applications of newer and more effective chemotherapeutic drugs for adjuvant therapy after surgery [2, 3]. For instance, carboplatin (CBP) is one of the most important chemotherapeutic drugs used for adjuvant treatment of primary ovarian cancer and for metastatic disease [4]. Its major mechanism involves the formation of DNA adducts, resulting in G2 phase cell cycle arrest, subsequently triggering apoptosis. While effective, CBP induces side effects, including neurotoxicity and nephrotoxicity [5–7]. Therefore, it is necessary to develop novel drugs that can be used alone or in combination with conventional agents to overcome acquired drug resistance or sensitize tumours to therapy.

Artemisinin (ARS), a sesquiterpene lactone anti-malarial drug, and its analogues, including dihydroartemisinin (DHA), artesunate (ART) and artemether (ARM) (Fig. 1), have been used in the clinic for many years [8–10], and have recently been suggested to have anticancer effects [9, 11–19]. The mechanisms of action for their antitumour activities are not fully understood, but may include selective cytotoxicity of cancer cells [9, 13], induction of apoptosis [20–23], modulation of gene expression [22, 24–28], causation of cell cycle arrest [23, 29–31] and inhibition of angiogenesis [17, 23, 28, 32–34].

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doi:10.1111/j.1582-4934.2008.00360.x

The efforts of this report were directed toward examining the possibility of utilizing the ARSs alone or in combination with other drugs for ovarian cancer therapy, and to evaluate their underlying mechanisms of action. Our results demonstrated that DHA had the most potent anti-ovarian cancer activity and sensitized human ovarian cancer cells to CBP therapy. We also demonstrated that the compound exerted its effects by increasing apoptosis *via* the death receptor- and mitochondrion-mediated caspase-dependent pathways, both *in vitro* and *in vivo*.

Material and methods

Test compounds and reagents

ARS, DHA, ART and ARM were kind gifts from Yi-wu Gao-deng Fine Chemical Co., Ltd. (Zhe-jiang, China). Thiazolyl Blue Tetrazolium Bromide (M5655) (MTT), Dimethyl Sulfoxide (D5879) and CBP (C2538) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The JC-1 dye (5, 5′, 6′, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide) was from Invitrogen-Molecular Probes Co. (Eugene, OR, USA). All chemicals were of the highest analytical grade. The DC protein assay kit (500–0113) was obtained from Bio-Rad (Hercules, CA, USA), and the ECL plus system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All cell culture supplies were obtained from Invitrogen-Gibco Co. (Grand Island, NY, USA). Anti-human cleaved poly (ADP-ribose) polymerase (PARP) was obtained from BD PharMingen (San Jose, CA, USA), the anti-cytochome *c* antibody, Caspase-3 (8G10) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and anti-human β-actin (AC-74) was obtained from Sigma-Aldrich, Inc. Anti-human Fas (B-10), Fas-associated death domain (FADD) (S-18), Bcl-2 (100), Bax (N-20), Bid (N-19), procaspase-3 (N-19), procaspase-8 (8CSP03) and procaspase-9 (4i31) antibodies, together with all secondary antibodies (anti-mouse, anti-goat and anti-rabbit immunoglobulin G) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell lines and cell culture

Human ovarian IOSE144 (immortalized non-tumourigenic human ovarian surface epithelial cells) and ovarian carcinoma (A2780 and OVCAR-3) cells that were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA) were gifts from Dr. Jing Fang (Institute for Nutritional Sciences, Shanghai, China). All cells were cultured according to the ATCC instructions. The compounds (ARS, ART, ARM and DHA) were dissolved in DMSO ($<$0.1%, final concentration).

Cell viability assay

Cell growth and viability were determined *via* the MTT assay as described [35–37]. Briefly, 3×10^3 cells (per well) were seeded in 96-well plates and were treated for 48 hrs with the four compounds at serial concentrations (0, 1, 10, 25, 50, 100, 250 and 500 μM). For cell growth assays, cells were treated for 0, 24, 48 and 72 hrs with DHA alone or in combination with CBP at concentrations of 0, 5, 10, 25 and 50 μM.

After 24–72 hrs, 10 μL of MTT solution (5 mg/mL in PBS) were added to each well. Plates were incubated for an additional 2~4 hrs at 37°C, then the supernatant was carefully removed and 100 μL DMSO was added to dissolve the formazan crystals. Absorbance at 570 nm was recorded using a SpectraMax^{190} microplate reader (Molecular Devices, Sunnyvale, CA, USA) to calculate the cell survival percentages [35–37].

Detection of apoptosis

Cell apoptosis was detected *via* an Annexin V-FITC kit purchased from BioVision Inc. (Mountain View, CA, USA) [37]. Approximately $4–6 \times 10^5$ cells were seeded in 6-cm dishes and treated with the test compounds (DHA and/or CBP) at specific concentrations for 24 hrs prior to analysis. The floating and trypsinized adherent cells were collected and prepared for detection according to the manufacturer's instructions. Samples were analysed with a FACSAria™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA after incubation in the dark at room temperature for 5 min. Cells positive for early apoptosis (Annexin V-FITC stained only, see Q_4 in figure) and for late apoptosis (Annexin V-FITC and PI stained, see Q_2 in figure) were combined.

Mitochondrial membrane potential ($\Delta\psi m$) quantitation

The effect of DHA on mitochondrial membrane potential was assessed using JC-1 dye. JC-1 is a lipophilic, dual-emission fluorescent dye capable of selectively entering mitochondria. Due to the reversible formation of aggregates upon membrane polarization, JC-1 reversibly changes colour from green to red at a specific excitation wavelength when membrane potentials increase. It produces red fluorescence (Ex = 550 nm; Em = 600 nm) within the mitochondria (as JC-1-aggregates) proportional to the $\Delta\Psi m$. When the $\Delta\Psi m$ dissipates, JC-1 dye leaks into the cytoplasm (turns into JC-1-monomers) and emits green fluorescence (Ex = 485 nm; Em = 535 nm). JC-1 is used qualitatively to evaluate the $\Delta\Psi m$ change according to the pure fluorescence intensity shift between green and red.

To quantify the effect of DHA on mitochondrial membrane potential, A2780 and OVCAR-3 cells were seeded in 24-well plates (~$2–4 \times 10^4$ cells per well), followed by a 24-hr exposure to DHA at serial concentrations (0, 5, 10, 25, 50 μM). Media were removed, and cells were then incubated with RPMI 1640 containing 10 μg/mL of JC-1 dye at 37°C in the dark for 15 min. Cells were trypsinized and washed with PBS after the removal of JC-1 dye. Aliquots of 100 μL cell suspensions from the different treatments were transferred to black 96-well plates. Pure red and green fluorescence intensity was measured *via* a fluorescence plate reader (Flexstation II 384, Molecular Devices). Ratios of red/green fluorescence intensity (% of control) were calculated.

Protein immunoblotting analysis

Immunoblotting was accomplished as described previously [37–40]. Cells were cultured and exposed to various concentrations of DHA. After 24 hrs, cells were collected and lysed with RIPA buffer (#9806, Cell Signaling). After centrifugation at 13,000 rpm for 15 min. at 4°C, the supernatant was removed and kept for analysis. Total cellular protein concentrations were assessed using a Bio-Rad protein assay kit. Aliquots containing identical amounts of protein were fractionated by SDS-PAGE, then transferred to methanol-pre-activated-PVDF membranes (Millipore,

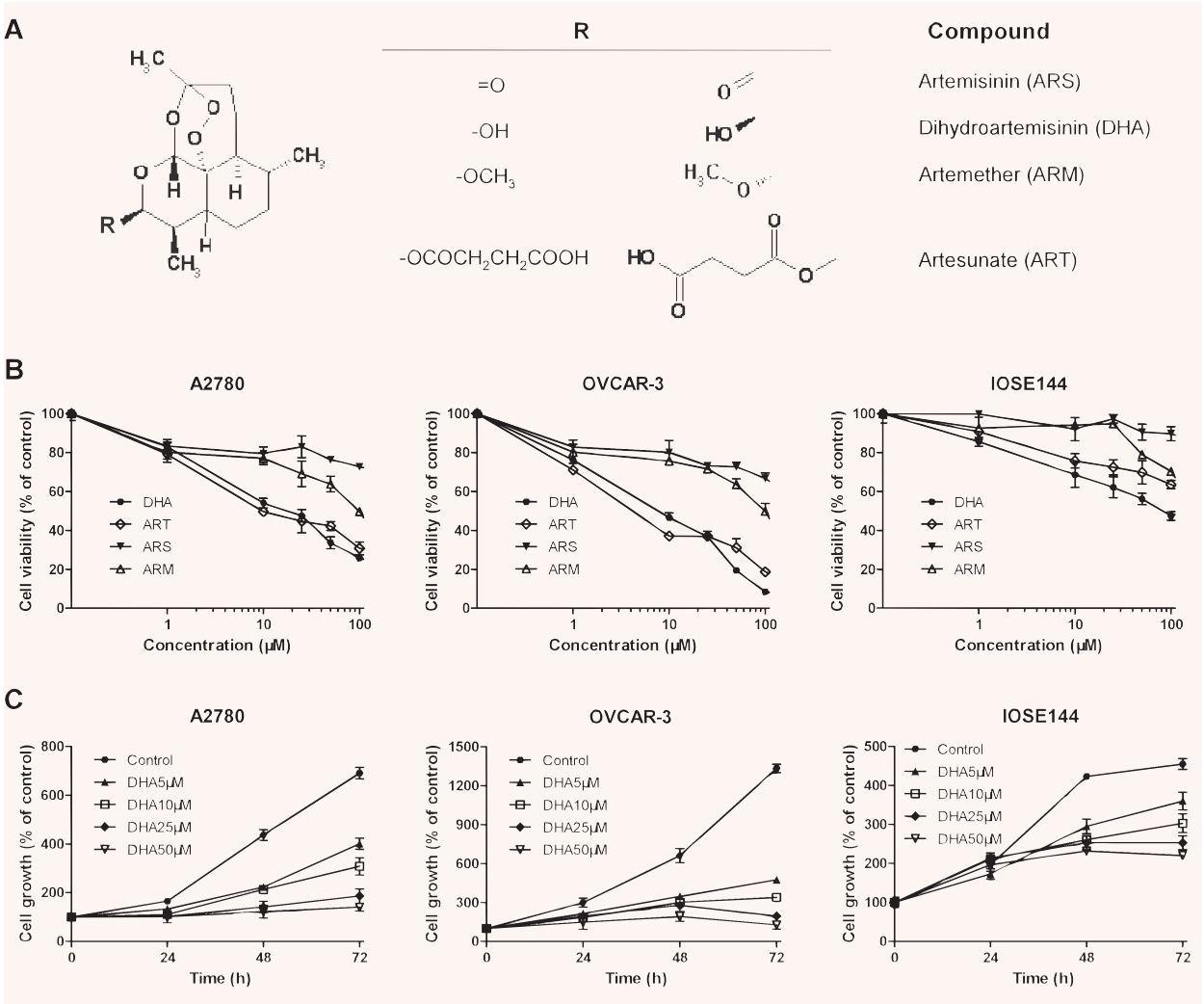

Fig. 1 DHA selectively decreases cell viability and inhibits the growth of human ovarian carcinoma cells, but not non-tumourigenic ovarian surface epithelial cells. (**A**) Chemical structures of the four artemisinin (ARS) compounds; (**B**) Viability of human ovarian carcinoma cells (ovarian carcinoma A2780 and OVCAR-3) and non-tumourigenic OSE cells (IOSE144) after 48 hrs exposure to the ARS compounds as determined by MTT assay; C, Cell growth inhibition after 0, 24, 48 and 72 hrs exposure of A2780 and OVCAR-3 cells to DHA. Values are representative of at least three independent experiments with similar results, and are presented as the percentage of cell inhibition where vehicle-treated cells were regarded as 100% viable/0% growth inhibition.

Bedford, MA, USA). Membranes were blocked and sequentially incubated with primary and secondary antibody [37–40], then bands of the proteins of interest were visualized using the ECL plus system from Amersham Pharmacia Biotech.

Mouse xenograft model of ovarian cancer and treatment protocols

Four to six week old female athymic nude mice (BALB/c, nu/nu) were purchased from Shanghai Experimental Animal Center (Shanghai, China). Animal studies were approved by the Institute for Nutritional Sciences. The tumour xenograft model was established as reported [40, 41]. Briefly, A2780 and OVCAR-3 cells were harvested and resuspended in serum-free RPMI 1640 medium containing 20% (v/v) Matrigel (BD Biosciences, Bedford, MA, USA). Aliquots of cells (~5 $\times$ 10^6 cells/0.2 ml) were injected subcutaneously into the left inguinal area of the mice. The tumour growth and body weight of the mice were monitored every other day. Tumour mass was determined as described previously [40, 41]. Mice bearing palpable tumours (~70 mg) were randomly divided into treatment and control groups $n = 5$ mice/group). CBP was dissolved in saline, and DHA was dissolved in cremophor EL : ethanol : saline (5:5:90, v/v/v). DHA was administered *via* i.p. injection at doses of 10 and 25 mg/kg/5 days/week for

Table 1 Growth inhibitory activity of the four artemisinin compounds on ovarian epithelial cells

Cell line	Inhibitory concentration*	Concentration (μM)			
		DHA	ART	ARS	ARM
A2780	IC20	0.83	0.73	7.68	2.08
	IC50	16.45	17.60	>500	53.90
	IC80	327.63	426.16	>500	>500
OVCAR-3	IC20	0.56	0.46	3.93	2.08
	IC50	6.58	6.86	342.51	38.79
	IC80	77.38	103.21	>500	>500
IOSE144	IC20	1.84	5.60	>500	97.95
(Immortalized non-tumourigenic OSE cells)	IC50	106.03	>500	>500	>500
	IC80	>500	>500	>500	>500

*IC_{20}, IC_{50}, and IC_{80} are the concentrations of drug that inhibit growth by 20%, 50% and 80%, respectively, relative to the control.

3 weeks) alone or combined with CBP (at a single dose of 120 mg/kg, once on day 0). The control group received saline only. Mice were killed on day 18. Tumours were carefully excised, trimmed of extraneous fat or connective tissue, and homogenized in RIPA buffer (100 mg tumour tissue/1 ml RIPA) and prepared for immunoblotting analysis as described above.

Statistical analysis

All values in the present study are reported as mean ± S.D. from at least three independent experiments. One-way ANOVA was used to test statistical differences for single group analysis, followed by Tukey's multiple comparisons. Two-way ANOVA was used for grouped analysis of statistical differences followed by Bonferroni post-tests.

Results

DHA has the most potent *in vitro* cytotoxicity in human ovarian cancer cells

Among the four compounds tested (DHA, ART, ARS, ARM), exposure of ovarian cancer cells to DHA and ART led to the greatest decreases in cell viability (Fig. 1B, $P < 0.05$). DHA had the lowest IC_{20}, IC_{50} and IC_{80} values, and produced the most significant effects on cell survival, inhibiting viability by 24% (1 μM) to 95% (500 μM) (Fig. 1B and Table 1, $P < 0.05$). DHA significantly inhibited the growth of A2780 and OVCAR-3 cells, although the OVCAR-3 cells appeared to be more sensitive (Fig. 1C, $P < 0.05$). Immortalized non-tumourigenic ovarian surface epithelial IOSE144 cells were less sensitive to the inhibitory effects of the four drugs than the ovarian carcinomacells (Fig. 1B and 1C; Table 1, $P < 0.05$), indicating that the ARSs may have selective activity against cancer cells. Taken together, these data suggest that DHA is the most effective ARS, and that it can inhibit the growth of ovarian cancer cells while exerting less potent effects on non-tumourigenic ovarian surface epithelial (OSE) cells.

DHA induces apoptosis in human ovarian cancer cells

To examine the mechanism responsible for decreasing the viability of the ovarian cancer cells, we investigated whether DHA had an effect on apoptosis. As demonstrated in Fig. 2A, DHA strongly induced apoptosis in both A2780 and OVCAR-3 cells in a dose-dependent manner. At a concentration of 10 μM, DHA increased apoptosis by about fivefold in A2780 cells, and increased apoptosis by more than eightfold in the more sensitive OVCAR-3 cells (Fig. 2B, $P < 0.01$). These effects were even more pronounced at 25μM, when apoptosis was increased by more than eightfold in A2780 cells and 18-fold in OVCAR-3 cells (Fig. 2B, $P < 0.01$). When the cells were exposed to 50 μM DHA, they demonstrated a 17-fold (A2780) and 22-fold (OVCAR-3) increase in apoptosis (Fig. 2B, $P < 0.01$).

To confirm the effects on apoptosis, we evaluated the expression of apoptosis-related proteins, including PARP, Bax, Bcl-2 and Bid (see Fig. 3). We observed a dose-dependent increase in cleaved-PARP and Bax, while there was a dose-dependent decrease in Bcl-2 and Bid. Moreover, we investigated the expression of pro-caspases-3, 9 and 8, and observed a dose-dependent cleavage, indicative of

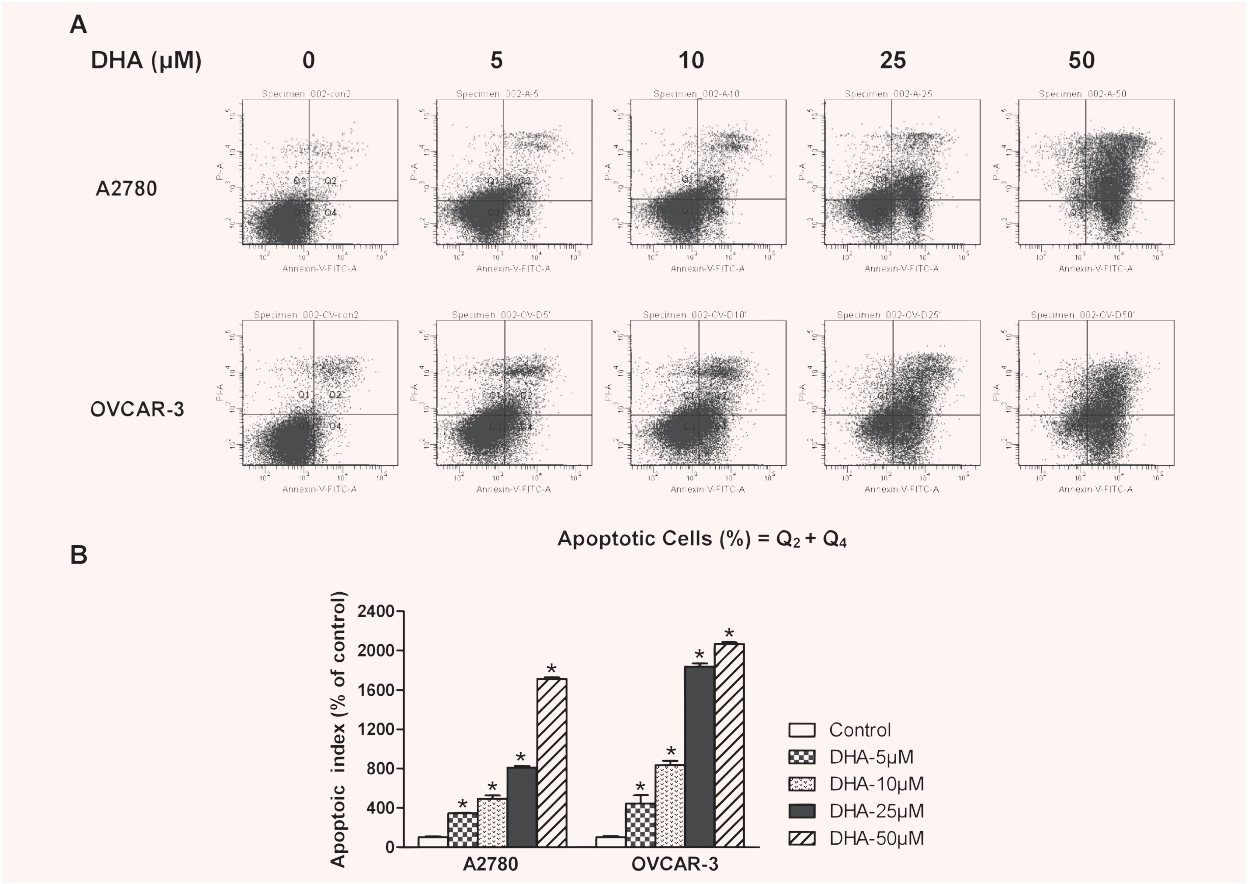

Fig. 2 DHA induces dose-dependent apoptosis in human ovarian carcinoma cells. (**A**) Apoptosis in A2780 and OVCAR-3 cells treated with DHA (0, 5, 10, 25, 50 μM) for 24 hrs; (**B**) Data summary and analysis (*, $P < 0.001$ *versus* the control, respectively). Data are representative of values from at least three independent experiments with similar results. The percentage of cellular apoptosis in control cells was regarded as 100%.

caspase activation. We also observed a dose-dependent up-regulation of Fas and its downstream adaptor protein, FADD, which can activate caspase-8, the main enzyme responsible for truncation of Bid. These data indicate that the activation of caspases plays a major role in the apoptosis induced by DHA, and that this apparently occurs *via* activation of the death receptor pathway.

DHA-induced apoptosis is associated with disruption of the mitochondrial membrane and release of cytochome c

After exposure to different concentrations of DHA, we evaluated the integrity and potential of the mitochondrial membrane in the A2780 (Fig. 4A) and OVCAR-3 (Fig. 4B) cells. These figures demonstrate that exposure to DHA for 24 hrs resulted in a dose-dependent dissipation in potential (from 5 to 50 μM) in both A2780 and OVCAR-3 cells, which was indicated by decreased ratios of red/green fluorescence intensity (Fig. 4A and B, $P < 0.05$). We also examined mitochondrial cytochrome *c* levels after exposure to the compound, and found a dose-dependent reduction in mitochondrial cytochrome *c* (Fig. 4C), which is in consistent with the activation of Bid by caspase-8 (Fig. 3). This suggests that DHA caused the release of cytochrome *c* from the mitochondria to the cytosol. Together, these results provide evidence that the DHA-induced apoptosis likely occurs through the mitochondrial pathway.

DHA increases the effectiveness of CBP in ovarian cancer cells through an increase in apoptosis

Since combination therapy is a major clinical approach to treatment, we investigated the effects of combining DHA with CBP, an agent commonly used to treat ovarian cancer [4]. As shown in Fig. 5A and B, CBP dramatically decreased the viability of ovarian cancer cells when used in combination with DHA. In fact, when

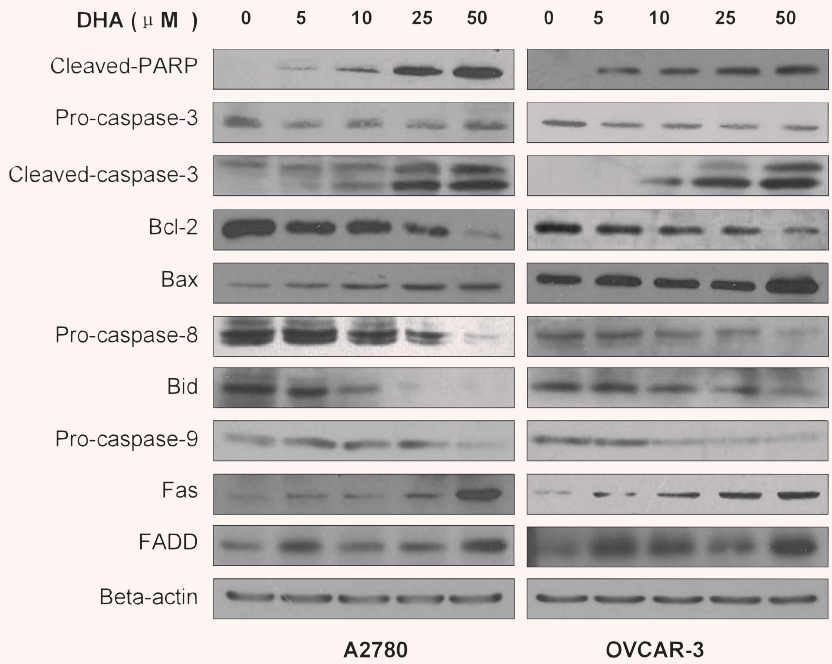

Fig. 3 Western blot analysis of protein expression levels indicating the effects of DHA on A2780 and OVCAR-3 ovarian cancer cell lines after 24 hrs exposure to specific doses of DHA.

cells were exposed to 1 μM CBP and 1 μM DHA, there was a 69% decrease in the viability of A2780 cells, and a 72% decrease in the viability of OVCAR-3 cells. Conversely, IOSE144 cells were much less sensitive to the treatment, with only a 28% decrease in viability when the two compounds were combined at 1 μM each (Fig. 5A, $P < 0.05$).

We also evaluated the effects of the combination on the apoptosis of ovarian cancer cells. In accord with the previous results, we observed that exposure to a combination of DHA and CBP (24 hrs) appeared to induce a synergistic increase in apoptosis in OVCAR-3 cells (Fig. 5C). The rate of apoptosis in cells treated with 1 μM DHA in combination with 500 μM CBP (1155%) was significantly higher than the rate of apoptosis in the cells exposed to 1 μM DHA (266%) or 500 μM CBP (528%) alone, and the rate of apoptosis exceeded the additive effects of the compounds, indicating a potential synergistic effect. However, exposure of the A2780 cells to combination treatment led to an additive effect, rather than synergistic effect. This may be due to their lower sensitivity to the DHA compound (Fig. 5D, $P < 0.05$). A longer exposure to the compound may produce more dramatic effects on apoptosis.

DHA inhibits tumour growth, induces apoptosis and improves CBP therapy *in vivo*

We established A2780 and OVCAR-3 tumour xenograft models to determine whether DHA can exert antitumour effects *in vivo*. DHA was administered 5 days a week to mice in the treatment groups. CBP was given once on day 0 at a single dose of 120 mg/kg. Therapeutic effects were evaluated by examining tumour growth. As shown in Fig. 6A1 and B1, DHA (at doses of 10 and 25 mg/kg) resulted in 24% and 41% tumour growth inhibition (compared to control mice treated with saline) in the A2780 xenograft tumour model (Fig. 6A1, $P < 0.05$), and 14% and 37% tumour growth inhibition in the OVCAR-3 model (Fig. 6B1, $P < 0.05$). In the CBP-only group, tumour growth was inhibited by 56% (A2780) and 46% (OVCAR-3) (Fig. 6A1 and 6B1, $P < 0.05$). Combining the two compounds (25 mg/kg DHA) led to 70% tumour growth inhibition in both the A2780 and OVCAR-3 models ($P < 0.05$). Moreover, based on observation of body weight (Fig. 6A2 and 6B2), only mice receiving CBP treatment experienced slight weight loss. No other host toxicities were observed.

As further validation of the mechanism by which DHA exerts its effects, we assessed the in vivo expression of some of the key apoptosis-related proteins mentioned above (Fig. 6C). Consistent with the *in vitro* findings, we observed a dose-dependent decease in the Bcl-2/Bax ratio and a decrease in pro-caspase-8, confirming that DHA appears to exert its effect at least partly by causing apoptosis through the death receptor- and mitochondrion-mediated pathway.

Discussion

To our knowledge, this is the first published report systematically demonstrating that DHA exhibits significant *in vitro* and *in vivo* anticancer activities by inducing apoptosis through the death

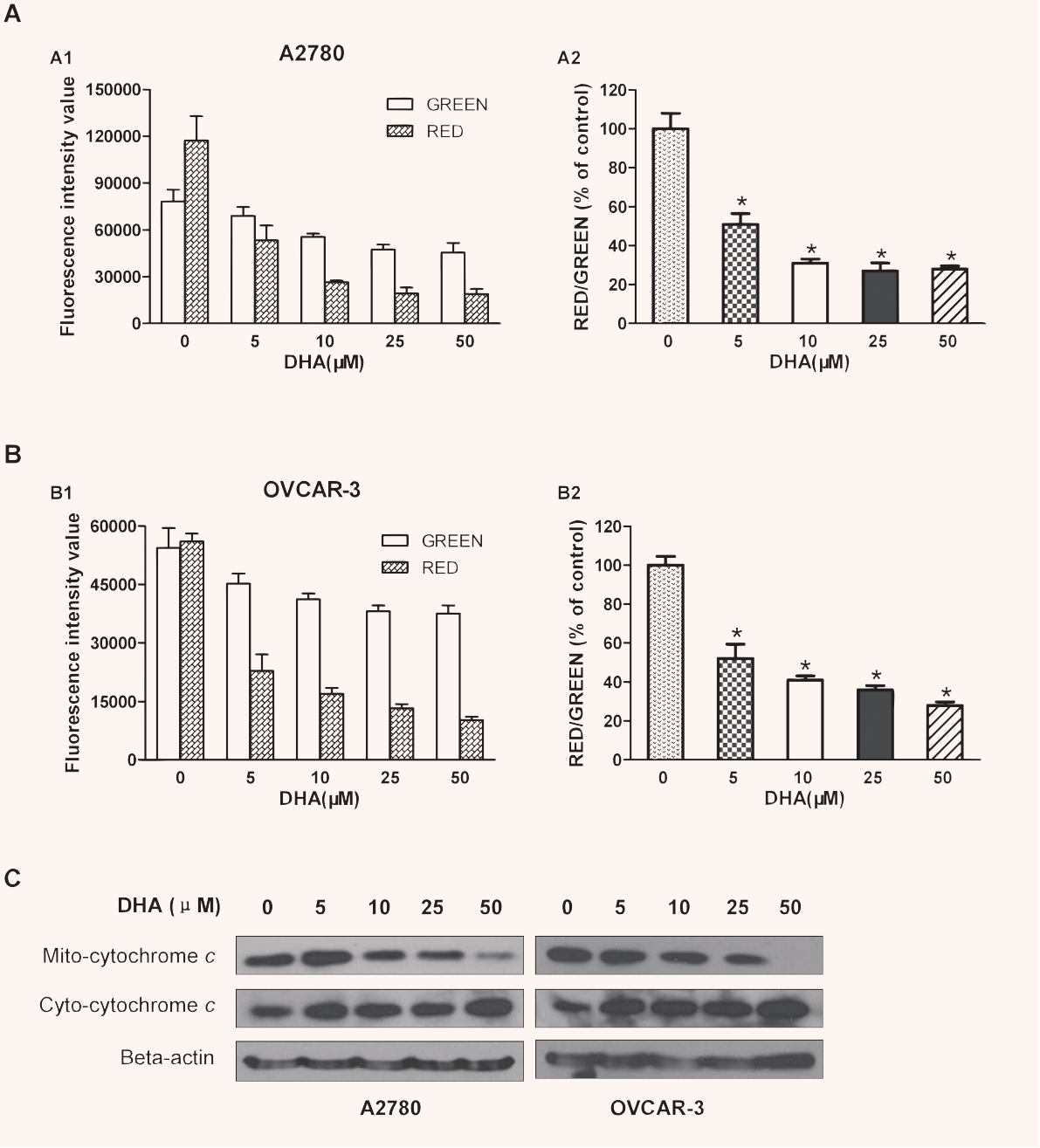

Fig. 4 DHA causes disruption of the mitochondrial membrane potential and cytochrome c release. (**A**) and (**B**) Fluorescence (red and green) intensity values emitted by JC-1 fluorescent dye at specific excitation wave-lengths (detailed information is described in the Materials and methods) and the corresponding ratio of red/green (% of the control) after exposure to different concentrations of DHA for 24 hrs. Values are representative of at least three independent experiments with similar results (*, $P < 0.001$ *versus* the control). (**C**) Western-blot analysis of the effects of 24 hrs DHA exposure (0, 5, 10, 25, 50 μM) on cytochrome *c* release from the mitochondria to the cytosol (mito-, mitochondrial; cyto-, cytoplasmic).

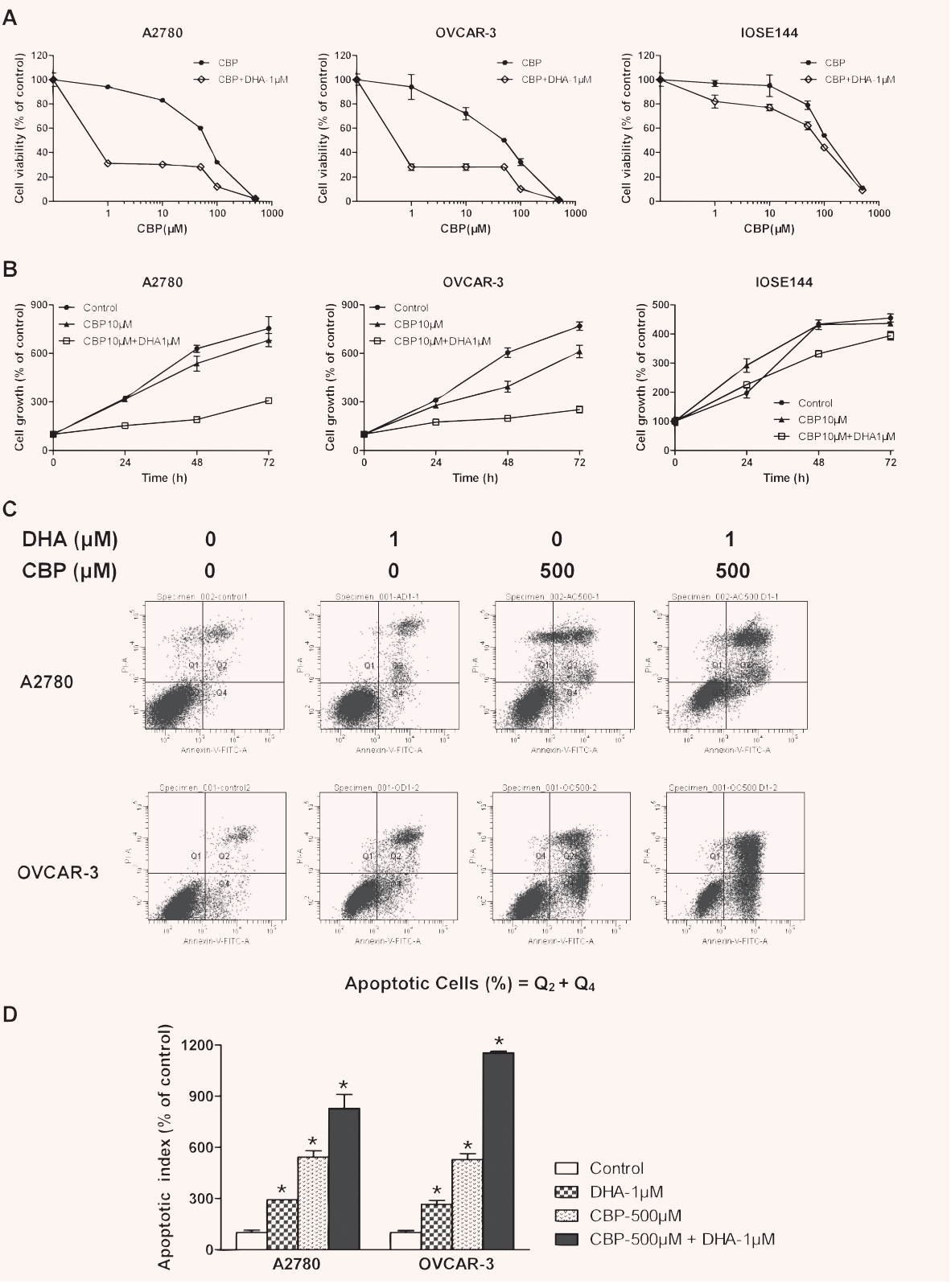

Fig. 5 DHA significantly decreases cell viability and inhibits cell growth in human ovarian carcinoma cells by increasing apoptosis, both alone and in combination with carboplatin (CBP). (**A**) Viability of ovarian epithelial cells after 48 hrs exposure to CBP (0, 1, 10, 50,100, 500,1000 μM) in the presence or absence of 1 μM DHA; (**B**) Cell growth inhibition following exposure to CBP (10 μM) with 1 μM DHA for 0, 24, 48 or 72 hrs; (**C**) Apoptosis of ovarian cancer cells after exposure to 500 μM CBP with or without 1 μM DHA for 24 hrs, and the corresponding data summary and analysis (*, $P < 0.001$ *versus* the control, respectively).

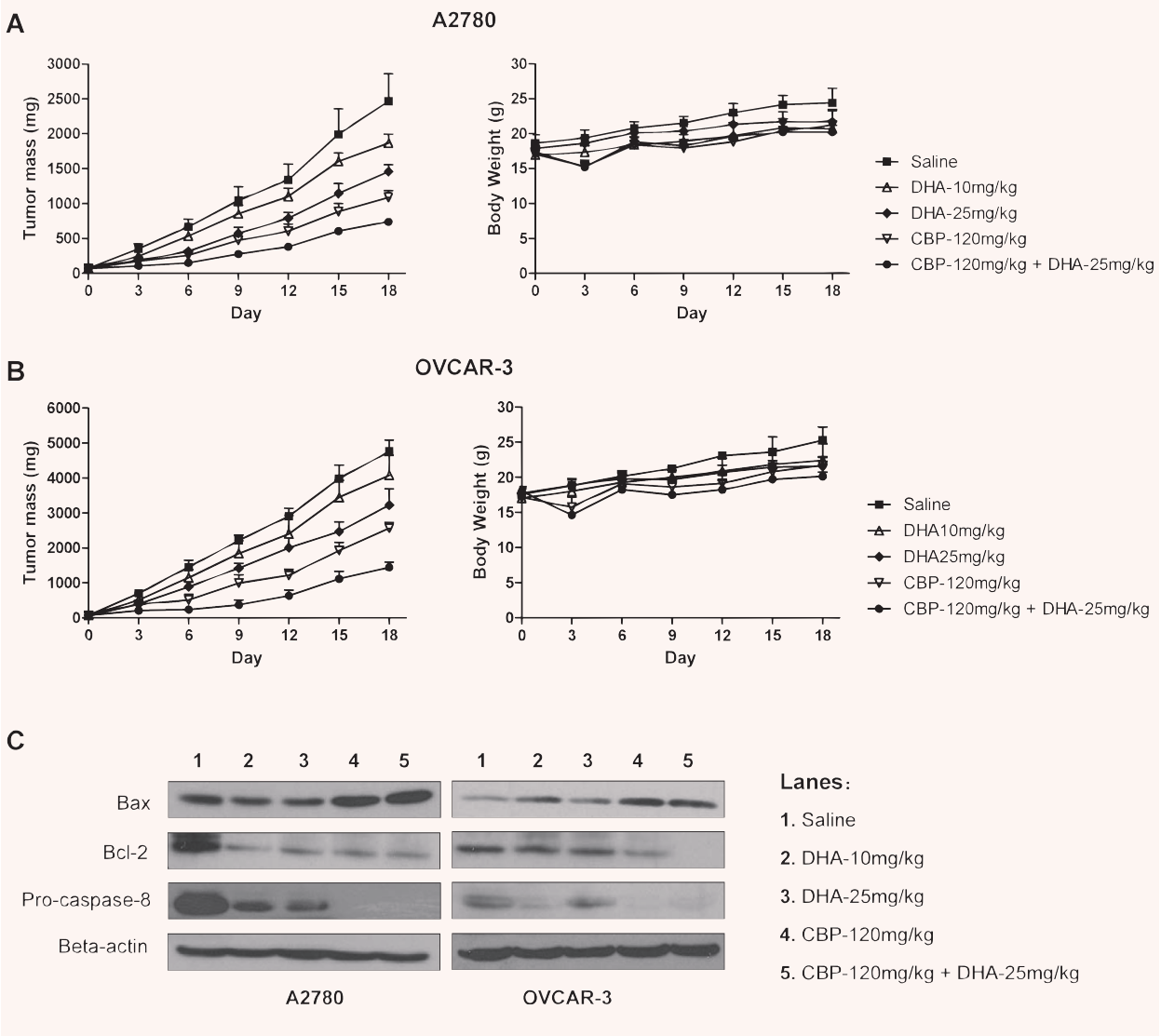

Fig. 6 DHA significantly inhibits tumour growth and induces apoptosis alone or in combination with carboplatin (CBP) in mice bearing A2780 and OVCAR-3 xenograft tumours. (**A**) and (**B**) Inhibition of tumour growth in mice bearing A2780 or OVCAR-3 xenograft tumours, and the corresponding body weight changes during the treatments; (**C**) Western-blot analysis of proteins involved in the apoptotic pathway.

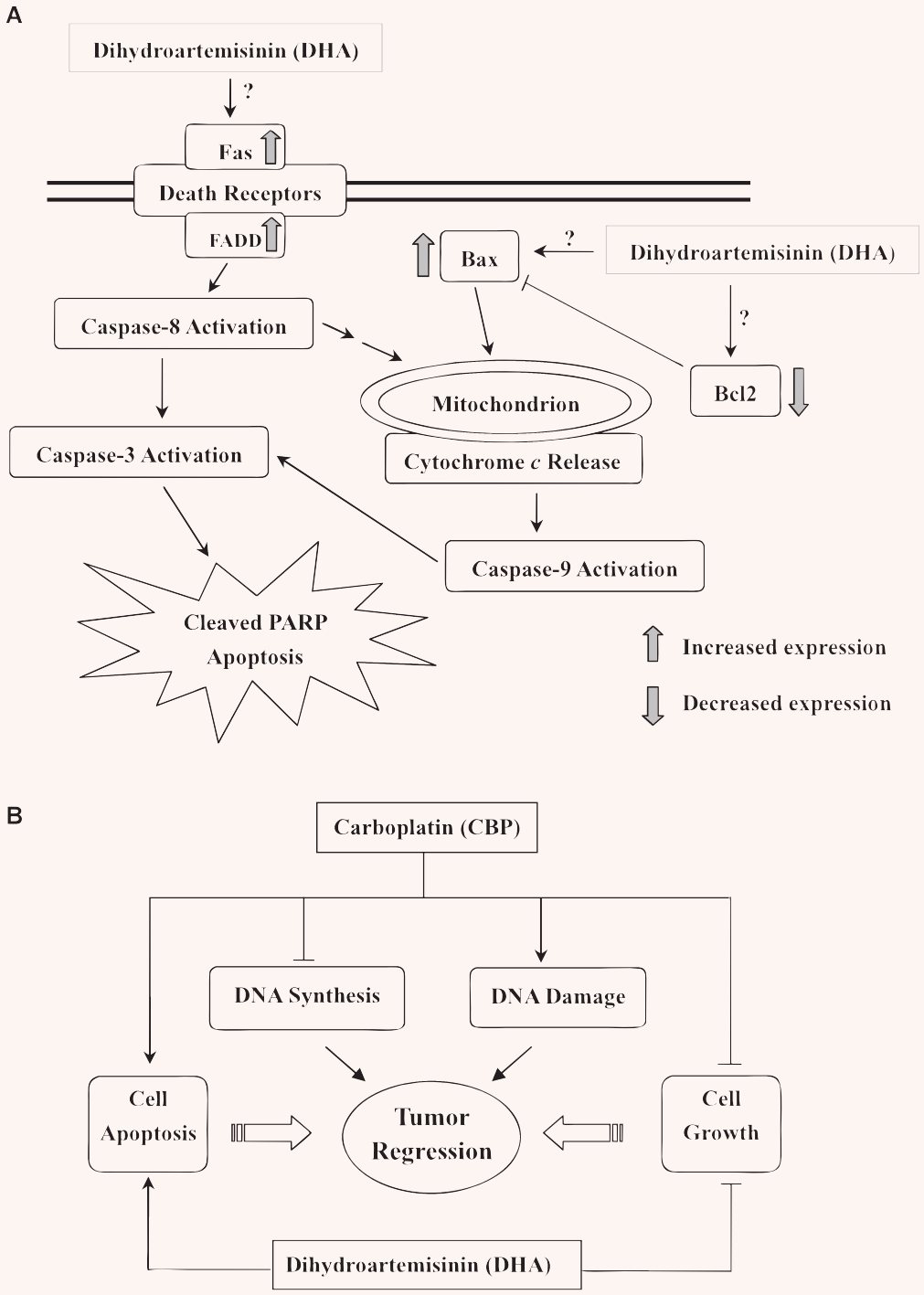

Fig. 7 Cartoons of the proposed mechanisms of action of DHA alone and in combination with CBP: (**A**) The 'death receptor- and mitochondrion-mediated caspase-dependent apoptotic pathway' demonstrates how DHA may exert anticancer effects in ovarian cancer cells; (**B**) Mechanism by which DHA enhances the therapeutic effects of carboplatin.

receptor- and mitochondrion-mediated, caspase-dependent apoptotic pathway. Although many reports have suggested that DHA has antitumour effects in various tumour types [9, 12, 14, 15, 42, 43], limited studies have examined the effects on ovarian cancer [27, 44]. DHA-induced apoptosis was reported to occur through the caspase cascade [21] or *via* modulation of the Bcl-2 family [44]. DHA has also been shown to regulate angiogenesis-related genes [17, 22, 27, 28, 33] and cause cell cycle arrest [44]. However, all of these previous studies were accomplished solely *in vitro*, and the underlying mechanisms, especially *in vivo* in ovarian cancer, have not been explored previously.

Cellular apoptosis can be triggered by the death-receptor-induced extrinsic pathway or the mitochondria-apoptosome mediated intrinsic pathway [45–47]. These two major apoptotic pathways then lead to caspase activation and cleavage of specific cellular substrates (such as PARP). To confirm the effects on apoptosis, we evaluated the expression of several apoptotic substrates, and observed a dose-dependent cleavage of PARP corresponding to previous findings [21]. Unexpectedly, we also found that there is a dose-dependent up-regulation of Fas; which we believe is the first published indication that DHA-induced apoptosis occurs through the death receptor pathway.

The death receptor apoptotic pathway includes receptors (such as Fas) and their ligands, and downstream molecules such as the Bcl-2 family and the caspases [45]. Members of the Bcl-2 family are known regulators of apoptosis [45, 46], with Bcl-2, Bax and Bid being the best characterized and most critical for cell survival/apoptosis [48]. The ratio of Bax/Bcl-2 is critical for cell survival, and any increase in Bax shifts the process toward apoptosis. In contrast, the death-promoting protein Bid is generally thought to be cleaved primarily by caspase-8 to generate truncated Bid (tBid), which can insert into the mitochondrial membrane, together with other pro-apoptotic members of the Bcl-2 family, to induce cytochrome c release [48].

In the present study, we demonstrated that DHA-induced apoptosis is accompanied by a decrease in Bcl-2 and a concomitant increase in Bax and Bid truncation. We found that DHA can induce the expression of Fas, which may lead to the recruitment and aggregation of the adapter protein FADD, in turn resulting in the activation of caspase-8 and downstream caspases. In support of this, we observed a significant dose-dependent activation of several key caspases, including the initiator caspases-8 and -9, as well as the effector caspase-3, after exposure of cancer cells to DHA. We propose that DHA induces apoptosis by triggering the Fas-receptor and/or regulating the expression of Bcl-2 and Bax, both of which can lead to the activation of caspase-8 and subsequent truncation of Bid. This leads to the release of cytochrome c from the mitochondria to trigger the activation of downstream initiator caspase-9, resulting in activation of the effector caspase-3, thereby inducing apoptosis. In our study, we have demonstrated that both dissipation of the mitochondrial membrane potential and the release of cytochrome *c* occur in a dose-dependent manner in ovarian cancer cells exposed to DHA. Given these findings, it appears that the DHA-induced apoptotic signal is passed from caspase-8 and Bcl-2 family members (such as Bcl-2, Bax and Bid) to the mitochondria to induce mitochondrial membrane disruption and cytochrome *c* release.

Ovarian cancer is the most lethal malignant gynaecological disease worldwide [1, 2]. The acquired drug resistance of tumour cells and cumulative side effects of the cytotoxic agent present serious clinical obstacles [7]. Resistance and evasion of apoptosis are critical factors that contribute to carcinogenesis and drug resistance [46, 47]. Therefore, it is necessary to explore new agents that can act *via* novel mechanisms of action to enhance the effects of therapy or sensitize cancer cells to apoptosis. As such, we focused the present study on examining the anticancer activities of DHA, both alone and in combination with a current clinically relevant anticancer drug, CBP. DHA potently enhanced the inhibitory effects of CBP on ovarian cancer cells (but not non-tumourigenic OSE cells) at a concentration of 1 μM. The combined treatment of DHA and CBP led to a significant increase in apoptosis, compared to that produced by either compound alone. These findings indicated that DHA can inhibit the growth of ovarian cancer cells by increasing their apoptosis, especially in combination with CBP. To accomplish a more thorough evaluation of the therapeutic potential of DHA, we examined the effects of DHA alone or in combination with CBP in mouse xenograft models of ovarian carcinoma. We believe that ours is the first study to demonstrate that DHA can remarkably inhibit the *in vitro* and *in vivo* growth of ovarian carcinomas both alone and in combination with CBP, and that it can enhance the therapeutic effect of CBP by increasing apoptosis. Moreover, we have also examined the mechanism through which DHA induces apoptosis both *in vitro* and *in vivo*; and demonstrated that apoptosis induced by DHA likely occurs both as a result of alterations in the Bax/Bcl-2 expression ratio, and *via* activation of caspase-8.

In conclusion, our study demonstrates that DHA potently inhibits the growth of ovarian cancer cells and *in vivo* tumours, and enhances the therapeutic effects of CBP both *in vitro* and *in vivo* by increasing apoptosis. To the best of our knowledge, these results are the first report that implicates the death receptor- and mitochondrion-mediated, caspase-dependent, apoptotic pathway in the activity of DHA (Fig. 7A), and indicates the possible mechanism by which DHA enhances the therapeutic effects of CBP (Fig. 7B). This work, together with the previously reported findings, will help improve our understanding about the mechanisms of action for aretemisinin compounds as anticancer agents. Moreover, our study provides a basis for future clinical studies of ARS in patients with ovarian cancer, used alone or in combination with conventional anticancer drugs. A mechanism-based adjuvant therapy with aretemisinin compounds may significantly improve clinical efficacy.

Acknowledgements

This work was supported in part by a grant (06DZ19021) from the Science and Technology Commission of Shanghai Municipality, Pujiang Talent Program (06PJ14107), a grant (2007CB947100) from the Ministry of

Science and Technology of China, and the Knowledge Innovation Program of the Chinese Academy of Sciences, Food Safety Research Center and Key Laboratory of Nutrition and Metabolism. R.Z. was supported in part by NIH/NCI grants R01 CA112029 and R01 CA121211. We thank Ms. Li-hua Chen for helpful discussions. We thank Dr. Elizabeth R. Rayburn for assistance in editing this manuscript.

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