Cancer Biology & Therapy
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kcbt20

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Published online: 15 May 2010.

To cite this article: Qin He, Jingxue Shi, Xiao-Ling Shen, Jie An, Hong Sun, Lu Wang, Ying-Jie Hu, Qing Sun, Lin-Chun Fu, M. Saeed Sheikh & Ying Huang (2010) Dihydroartemisinin upregulates death receptor 5 expression and cooperates with TRAIL to induce apoptosis in human prostate cancer cells, Cancer Biology & Therapy, 9:10, 819-824, DOI: 10.4161/cbt.9.10.11552

To link to this article: http://dx.doi.org/10.4161/cbt.9.10.11552

RESEARCH PAPER

Cancer Biology & Therapy 9:10, 819-824; May 15, 2010; © 2010 Landes Bioscience

Dihydroartemisinin upregulates death receptor 5 expression and cooperates with TRAIL to induce apoptosis in human prostate cancer cells

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Key words: DHA, TRAIL, prostate cancer, death receptor 5, PI3-K/Akt, ERK, combination therapy

Dihydroartemisinin (DHA) is a derivative of artemisinin and is an effective anti-malaria therapeutic used worldwide. In this paper, we report that DHA is as a potential anticancer drug for prostate cancer. Our data indicate that DHA suppresses the PI3-K/Akt and ERK cell survival pathways and triggers the induction of death receptor DR5 and activation of extrinsic and intrinsic cell death signaling. DHA-mediated DR5 induction appears to occur via increased transcriptional activity of DR5 promoter. Our data also show that, while DHA has strong cytotocixity in tumor cells, it exhibits minimal cytotoxic effects on normal prostate epithelial cells. Our studies also demonstrate that DHA worked cooperatively with death ligand TRAIL. Combination of DHA and TRAIL significantly enhanced cell killing above that noted with a single agent alone. Based on these results, we propose a novel idea of developing DHA alone and/or in combination with TRAIL for the treatment of prostate cancer.

Introduction

Prostate cancer is one of the major human malignancies. Approximately one out of six men in the United States will be diagnosed with prostate cancer during their lifetime.[1] Prostate cancer deaths account for 10% of cancer deaths in males and it is a leading cause of deaths in American men.[1] Surgical and radiation therapies are two main approaches for the treatment of localized prostate cancer; however, cancer recurrence occurs in about 30–50% of patients after surgical and/or radiation treatments.[1] Thus, more effective therapeutic approaches are urgently needed to fight against this deadly disease.

Artemisinin (ART) is a natural product originally isolated from the plant *Artemisia annua L*, an herbal drug that has been used in traditional Chinese medicine for centuries.[2,3] Dihydroartemisinin (DHA) is an derivative of ART (**Fig. 1**) and is a potent anti-malarial drug used as first-line therapeutics against malaria falciparum worldwide.[3-6] DHA is well-tolerated in human and animals with only mild adverse effects.[7] In recent years, studies have shown that ART derivatives including DHA also have profound effect against human tumors including cancers of cervix, pancreas, prostate, liver and neuroblastoma.[8-12] However, the exact molecular mechanisms by which ART and DHA exert their anticancer effect remain to be fully investigated.

In general, two apoptotic pathways control apoptosis.[13-17] The intrinsic pathway engages mitochondria while the extrinsic pathway involves membrane death receptors, including Fas, TNFR1, DR3, DR4 and DR5,[14,15,18,19] that are activated via their respective ligands. In the case of DR4 and DR5, the ligand named TRAIL (TNF-related apoptosis ligand) has emerged as an important anticancer agent that displays selective toxicity towards cancer cells including prostate cancer.[19-24] TRAIL has shown a significant promise and is currently in clinical trials.[23,25]

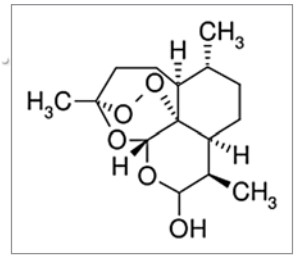

Figure 1. Chemical structure of Dihydroartemisinin (DHA).

In this study, we have studied the anticancer effect of DHA in prostate cancer cells. We demonstrate that DHA induces prostate cancer cell death via induction of death receptor-5 (DR5). We also show that DR5 ligand TRAIL strongly augments DHA-induced cell death in prostate cancer cells. Overall, our results indicate that DHA either alone or in combination with TRAIL has potential to be a novel therapeutic for the treatment of prostate cancer.

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Submitted: 01/15/10; Revised: 02/10/10; Accepted: 02/18/10
Previously published online: www.landesbioscience.com/journals/cbt/article/11552

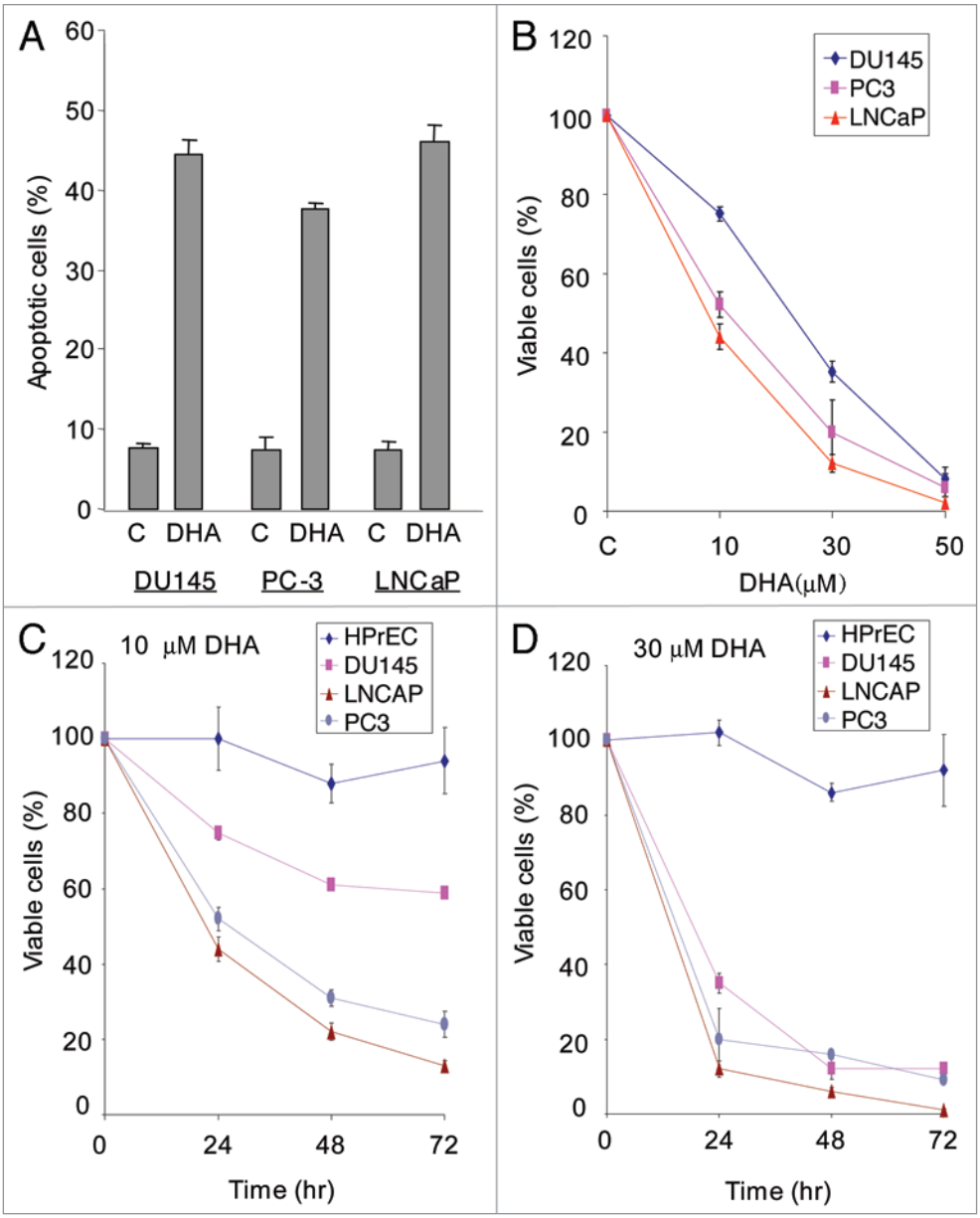

Figure 2. DHA inhibits cell growth in human prostate cancer cells, but not in human normal prostate epithelial cells. (A) DHA induces apoptosis in prostate cancer cells. Cells were treated with DHA (50 μM) for 24 h. Apoptotic cells were evaluated as we previously described.[27] (B–D) DHA-mediated prostate cancer cell suppression is in a time and dose-dependent manners. LNCaP, DU145 and PC3 prostate cancer cells and HPrEC normal prostate epithelial cells were treated with indicated concentrations of DHA for 24 h (B) or the indicated times (C and D) and cell viability was evaluated by MTT assays as we previously described.[26] Values represent the means ± S.E.M. of three independent experiments.

Results

We investigated the effects of DHA on several prostate cancer cell lines. **Figure 2A** shows that DHA induced apoptosis in three different prostate cancer cells lines including LNCaP, PC-3 and DU145 cells as was determined by morphological assay. DHA effect on cell viability was also determined by MTT assay and as shown in **Figure 2B–D**, DHA reduced cell viability in a time and dose-dependent manner and in both androgen-dependent (LNCaP) and-independent (DU145 and PC-3) cells. We noted that while DHA strongly affected prostate cancer cell viability, it had only minimal affect on HPrEC normal prostatic epithelial cells (**Fig. 2C and D**). We also investigated the effect of DHA on caspase-activation in these cells and as shown in **Figure 3**, DHA induced caspases 3, 8 and 9 activation in all three prostate cancer cell lines indicating that DHA induced cell death involves caspase cascade and appears to occur via extrinsic (caspase 8 activation) and intrinsic (caspase 9) pathways.

It is known that tumor suppressor PTEN is frequently inactivated in human prostate tumors[29-32] which leads to constitutive PI3-K/Akt activation that is believed to promote cell proliferation and cell survival. PC-3 and LNCaP cells are considered

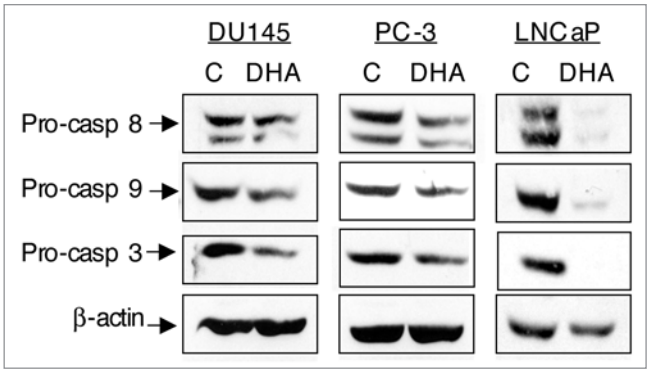

Figure 3. DHA activates caspases 8, 9 and 3. Cells were either left untreated (C) or treated with DHA (50 μM) for 24 hr. Cell lysates were analyzed by immunoblotting using pro-caspases 8, 9 and 3 antibodies.

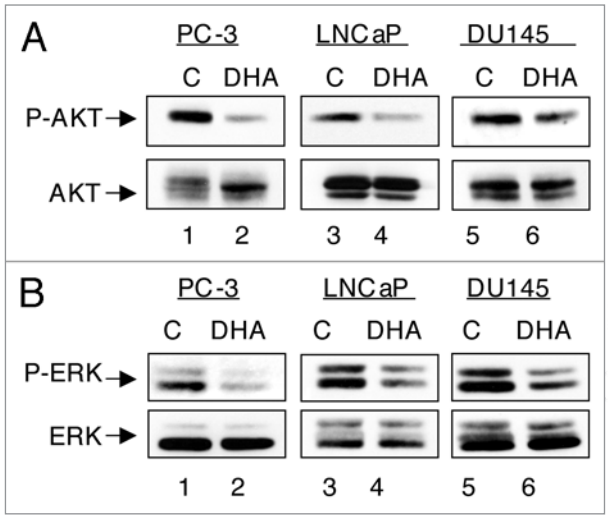

Figure 4. DHA suppresses the PI3-3/Akt and ERK proliferation/survival signaling pathways. (A) DHA inhibits Akt phosphorylation. Prostate cancer cells were either left untreated (C) or treated with DHA (30 μM for DU145 & PC3 and 50 μM for LNCap cells) for 24 h and Akt phosphorylation was analyzed by immunoblotting using Akt phosphorylation specific antibodies (Ser473) (upper). Lower panels show the levels of pan-Akt as loading controls (B) DHA inhibits phosphorylation of ERK. Cells were either left untreated (C) or treated with DHA (30 μM) for 24 h and ERK phosphorylation was detected by immunoblotting using phosphor-specific ERK antibodies (upper). Lower panels show the levels of pan-ERK as loading controls.

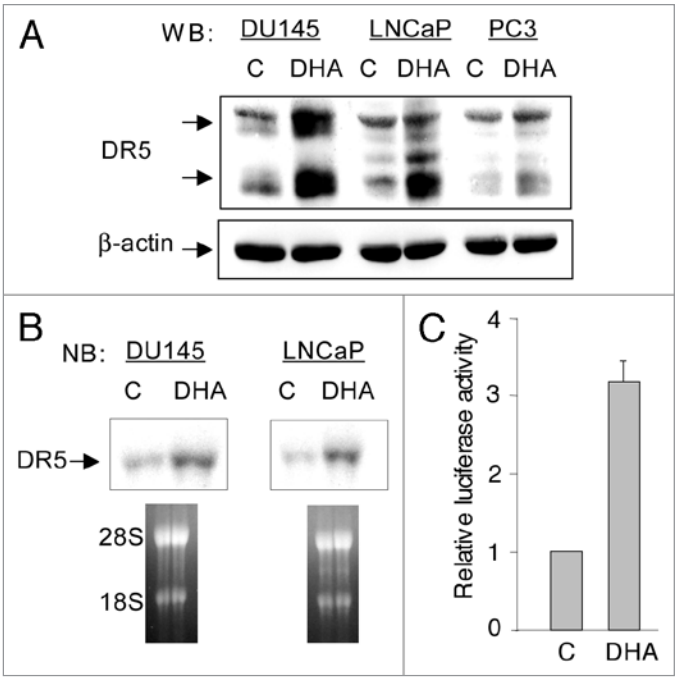

Figure 5. DHA elevates DR5 expression in prostate cancer cells. (A) DHA upregulates DR5 protein levels. Cells were treated with DHA (30 μM) for 24 h. DR5 protein was analyzed by western blotting using DR5 specific antibodies. The bottom panel shows the β-actin loading control. (B) DHA induces DR5 mRNA expression. Cells were treated with DHA (50 μM) for 24 h. DR5 mRNA expression was detected by northern blotting using a^{32} P-labeled full-length DR5 cDNA as a probe. (C) DHA activates DR5 promoter. DU145 cells were transiently transfected overnight with DR5 promoter luciferase reporter construct, then either left untreated (C) or treated with DHA (50 μM) for 24 h. Cell lysates were analyzed for promoter activity as we previously described.[34]

PTEN-negative and exhibit constitutively higher active Akt whereas DU145 cells are PTEN-positive.[33] Because DHA induced apoptosis in these cells, we therefore, sought to investigate its effect on Akt. **Figure 4A** shows that DHA treatment strongly suppressed Akt phosphorylation in these cells (lanes 2, 4, 6) indicating that DHA appears to mediate its growth suppressive effects, at least in part, via inhibiting Akt-dependent survival signals. Extracellular signal-regulated kinases (ERKs) are another group of bona fide molecules involved in mediating proliferative signals. We also investigated the effect of DHA on ERK signaling pathway and as shown in **Figure 4B**, DHA inhibited ERK1/ERK2 phosphorylation and thus, their activation in these cells. Taken together, these results indicate that DHA-induced cell death is associated with suppression of Akt and ERK—mediated cell proliferation and survival.

Our preceding results indicate that DHA activates caspases 8 and 9 suggesting that it engages both the extrinsic and intrinsic pathways of apoptosis. To further investigate the mechanism by which DHA induces apoptosis in human prostate cancer cells, we investigated its effect on death receptor 5 (DR5). DR5 is one of the major death receptor responsible for mediating the apoptotic signals via the extrinsic pathway. **Figure 5** shows that DHA upregulated DR5 protein levels in all three prostate cancer cell lines examined. To study the potential mechanism by which DHA-mediated DR5 upregulation, we next investigated its effect on DR5 mRNA levels and noted that DHA upregulated DR5 mRNA expression in these cells. To further investigate whether DHA-mediated upregulation of DR5 mRNA levels was transcriptional or involved post-transcriptional controls, we next, investigated its effect on DR5 gene promoter activity. For this purpose, we utilized DR5 promoter construct carrying the proximal 1224 nucleotides corresponding to DR5 promoter region fused to promoterless luciferase gene. Prostate cancer cells were transiently transfected with the DR5 promoter luciferase construct and were left untreated or treated with DHA for 24 h

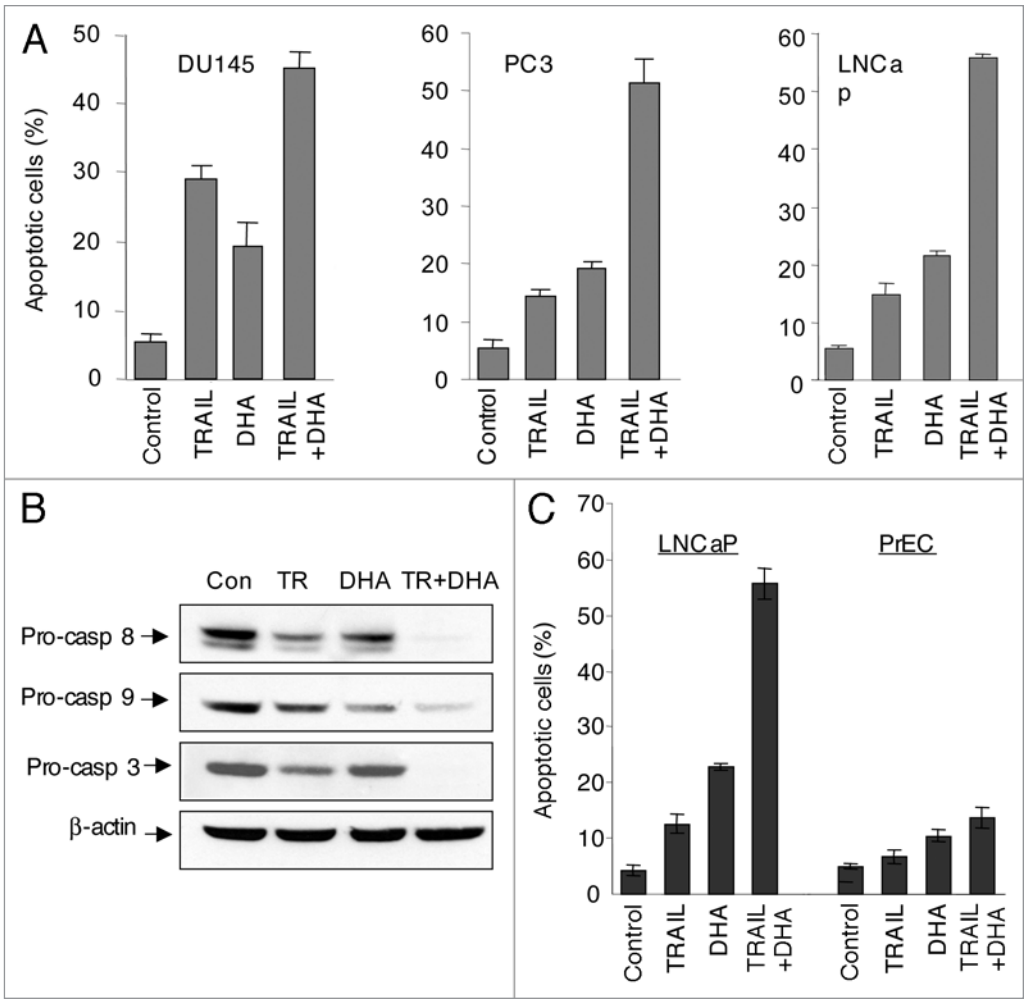

Figure 6. (A) DHA and TRAIL cooperate to induce apoptosis in prostate cancer cells. Cells were either left untreated or treated with DHA for 24 h and apoptotic cells were evaluated as we previously described.[27] For DU145 and PC3 cells, 20 ng/ml TRAIL (TR) and 30 μM DHA were used. For LNCaP cells, 10 ng/ml TRAIL and 10 μM DHA were used. (B) DHA and TRAIL combination further enhances activation of caspases 8, 9 and 3. DU145 cells were either left untreated (Con) or treated with DHA (30 μM) and TRAIL (TR, 20 ng/ml) for 24h. Cell lysates were analyzed by immunoblotting using pro-caspases 8, 9 and 3 antibodies. (C) DHA and TRAIL combination has minimal cytotoxic effect on normal prostate epithelial cells. LNCaP prostate cancer cells and HPrEC normal prostate epithelial cells were left untreated or treated with DHA (10 μM) and TRAIL (TR, 10 ng/ml) for 24h. Apoptotic cells were analyzed as we previously described.[27]

and cell lysates were harvested to analyze promoter activity. Our results shown in **Figure 5C** indicate that DHA also increased DR5 promoter activity in these cells. Taken together these results indicate that DHA upregulates DR5 levels, at least in part via transcriptional controls.

TRAIL is a natural ligand for DR5 and is currently in clinical trials to explore its potential for the treatment of a number of human malignancies (http://clinicaltrials.gov). Given that DHA increased the expression of DR5, we next investigated whether TRAIL cooperates with DHA and sensitizes cells to DHA-mediated apoptosis. To this end, DU145, PC3 and LNCaP cells were left untreated or treated with DHA, TRAIL or the combination of the two reagents and cell death was evaluated after 24 h treatment. **Figure 6A** shows that TRAIL and DHA induced apoptosis when used as a single agent and cell death was clearly enhanced when both agents were used in combination.

We also examined the activation of caspases under these experimental conditions and as shown in **Figure 6B**, combination of DHA and TRAIL strongly enhanced the activation of caspases 8, 9 and 3. Taken together, these results clearly indicate that DHA and TRIAL cooperate with each other to strongly induce apoptosis in these cells.

In order for the DHA and TRAIL combination to be eventually developed as a novel cancer therapeutic approach, it is important to asses their effects, in combination, on normal cells. Therefore, next we sought to investigate their effect, in combination, on normal prostate epithelial cells. Normal prostatic epithelial cells HPrEC and LNCap cancer cells were similarly treated with DHA, TRAIL or the combination of these two agents or left untreated. Results shown in **Figure 6C** indicate that whereas the combination of DHA-TRAIL strongly induced apoptosis in LNCaP cells, this combination had only minimal effect on

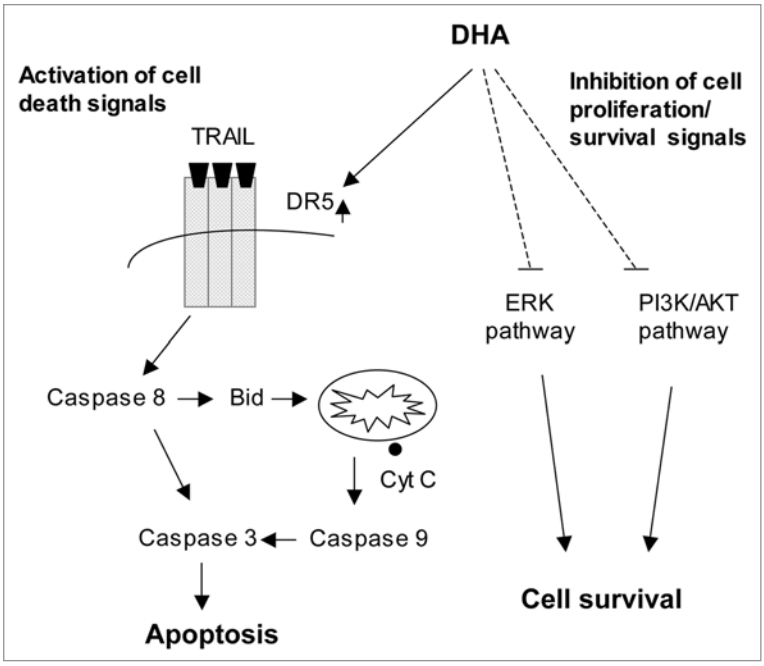

Figure 7. Proposed mechanisms for DHA-mediated cell death in prostate cancer cells. DHA exerts its tumor suppressing effect by inducing activation of DR5-dependent death signaling pathway as well as by inhibiting the cell proliferation/survival pathways.

HPrEC, the normal epithelial cells. These results therefore, indicate that DHA and TRAIL combination-induced apoptosis is more specific to malignant cells.

Discussion

In this study, we have investigated the potential of DHA as novel anticancer drug for prostate cancer. Our results indicate that DHA induces apoptosis in dose and time-dependent manner. Furthermore, DHA induces apoptosis equally well in both the androgen-sensitive and -insensitive cells, which highlights the value of DHA as a potentially important therapeutic for prostate cancer. Our results also indicate that DHA although strongly induces apoptosis in prostate cancer cells, it only minimally affects the normal prostate epithelial cells. In addition, our results also indicate that DHA efficiently triggers cell death signaling by engaging both the intrinsic and extrinsic cell death signaling pathways.

There are several recent reports that have examined the effects of ART and its derivatives in animals. For example, Hou et al. investigated the effect of ART and ART derivatives such as DHA, atmether and artesunate on human hepatic cancer and normal cells.[11] They found that ART and DHA inhibited tumor growth in mice xenograted with HepG2 and Hep3B hepatic cancer cells.[11] Willoughby et al. have also reported that administration of ART (100 mg/kg/d) (DHA is a derivative of ART) strongly inhibited LNCaP prostate cancer cell growth in mice and that the experimental animals did not show signs of toxic side effects.[10] Thus, these studies including ours, suggest that ART and DHA have a significant anticancer potential and further in-depth studies are clearly needed to delineate the mechanisms by which these agents mediate their anticancer effects and to firmly establish them as novel cancer therapeutic agents.

Our present results indicate that DHA inhibits Akt and ERK activation and thus, appears to mediate its effect partly via inhibition of the PI3-K/Akt and ERK pathways, the two major cell proliferation and survival pathways. Our results also indicate that DHA upregulates DR5 protein levels and that the mechanism of DR5 upregulation involves increased expression of DR5 gene as is evidenced by DHA-mediated upregulation of DR5 mRNA and promoter activity. Thus, our data indicate that DHA exerts its antitumor effect by activating the cell death signaling pathway as well as by suppressing the cell proliferation/survival signaling pathways (**Fig.** 7). We also demonstrate that DHA-mediated upregulation of DR5 is functionally relevant as DHA and TRAIL display clear cooperation to induce apoptosis in these prostate cancer cells. The cooperative effect of TRAIL and DHA in inducing apoptosis appears to be synergistic rather than additive which is consistent with our findings that DHA engages additional signaling events other than TRAIL death receptors. Our novel finding highlighting the cooperation between DHA and TRAIL is clinically relevant. TRIAL, as a promising anticancer agent either alone or in combination with other agents is currently in clinical trials. For example, TRAIL in combination with Rituximab is being evaluated for the treatment of non-Hodgkin's lymphomas (http://clinicaltrials.gov). Similarly, a combination of TRAIL and Bevacizumab for the treatment of non-small cell lung cancer (NSCLC) is also being evaluated (http://clinicaltrials.gov). Our results presented here indicate that a DHA and TRAIL combination more strongly induces prostate cancer cell death than either agent alone while only minimally affecting normal prostate epithelial cells. These results thus, provide strong rationale to undertake more in-depth studies to explore the utility of DHA and TRAIL combination as a novel therapeutic strategy for prostate cancer as well as for other malignancies.

Materials and Methods

Reagents, cell lines and cell culture. DU145, PC3 and LNCaP cells were respectively maintained in DMEM, Ham's and RPM1 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Glabasas, CA). DHA is obtained from Institute of Tropical Medicine, Guangzhou University of Traditional Medicine.

MTT assay. MTT assay was performed as we previously described.[26] Briefly, cells were first incubated in growth medium containing MTT salt with a final concentration of 1 mg/ml for 2 h. After incubation, medium was removed, the precipitate was dissolved in isopropanol with 0.04 M HCl and then read with a Bio-Rad Smart-Spec 3100 at 570 nm with background subtraction read at 650 nm.

Analysis of apoptosis. DU145, PC3 and LNCaP cells were left untreated or treated with DHA for approximately 24 h and then processed for apoptosis detection by counting floating and adherent cells that exhibited morphologic features of apoptosis using a phase contrast microscope as reported previously.[27]

Western blotting. Western blot analyses were performed by standard procedures as described previously.[28] DR5 was detected using a monoclonal anti-human DR5 antibody (Oncogene Science, San Diego, CA). Anti-human pro-caspase 3 (BD Bioscience, San Jose, CA), anti-human pro-caspase 8 antibody and anti-human pro-caspase 9 antibody (Assay designs/Stressgene, Ann Arbor, MI) were used to detect caspases 3, 8 and 9 activation, respectively. β-actin was detected using a monoclonal antibody purchased from Sigma Chemicals (St. Louis, MO).

Northern blotting. RNA extraction and northern blot analyses were performed by standard procedures as we have described previously.[27,28] DR5 mRNA expression was detected using a full-length human DR5 cDNA as probe. Ethidium bromide staining of the gel was used to indicate RNA integrity.

Acknowledgements

This work was supported in part by grants from the NIH (CA128096 to Y.H., and ES014489 to M.S.S.).

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