Dihydroartemisinin inhibits the mammalian target of rapamycin-mediated signaling pathways in tumor cells

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Dihydroartemisinin (DHA), an antimalarial drug, has previously unrecognized anticancer activity, and is in clinical trials as a new anticancer agent for skin, lung, colon and breast cancer treatment. However, the anticancer mechanism is not well understood. Here, we show that DHA inhibited proliferation and induced apoptosis in rhabdomyosarcoma (Rh30 and RD) cells, and concurrently inhibited the signaling pathways mediated by the mammalian target of rapamycin (mTOR), a central controller for cell proliferation and survival, at concentrations (~3 μM) that are pharmacologically achievable. Of interest, in contrast to the effects of conventional mTOR inhibitors (rapalogs), DHA potently inhibited mTORC1-mediated phosphorylation of p70 S6 kinase 1 and eukaryotic initiation factor 4E binding protein 1 but did not obviously affect mTORC2-mediated phosphorylation of Akt. The results suggest that DHA may represent a novel class of mTORC1 inhibitor and may execute its anticancer activity primarily by blocking mTORC1-mediated signaling pathways in the tumor cells.

Introduction

Rhabdomyosarcoma (RMS) is a soft tissue (usually muscle) sarcoma, which occurs often in the head, neck, bladder, vagina, arms, legs and trunk of children (1,2). About 80% of patients are <15 years old (3). Around 70% of lesions happen in the head and neck, extremities and genitourinary tract (1). Histologically, RMS manifests in two major types, embryonal RMS and alveolar (aRMS) (1). Morphologically, embryonic type resembles to the embryonic muscle cell precursor, whereas alveolar type has clusters of round cells similar to lung alveoli (1). Treatments of RMS are routinely made of multimodality approach including surgery, radiation and chemotherapy (1–3). Fortunately, due to the improvement in treatment strategies during the last 30 years, overall survival rate of RMS has increased to ~80% (1). Current standard chemotherapy for RMS is the combination of vincristine, actinomycin D and cyclophosphamide (1). Treatment-resistant tumors are classified according to the Cancer Center, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130-3932, USA.

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cell proliferation and survival (21). Dysregulation of mTOR pathway has been frequently observed in a variety of human tumors, and these tumor cells have shown higher susceptibility to inhibitors of mTOR than normal cells (21,22). Thus, mTOR has emerged as an important target for the development of anticancer agents. mTOR functions at least as two complexes, mTORC1 and mTORC2 (21). These two complexes consist of unique mTOR-interacting proteins that determine their substrate specificity. mTORC1 is composed of mTOR, mLST8 (also termed G-protein β-subunit-like protein, GβL, a yeast homolog of LST8), raptor (regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate 40kDa) and DEPTOR (23–29). Where mTORC2 consists of mTOR, mLST8, rictor (rapamycin insensitive companion of mTOR), mSin1 (mammalian stress-activated protein kinase-interacting protein 1), proto (protein observed with rictor, also named PR5, proline-rich protein 5) and DEPTOR (26,29–38). mTORC1 regulates phosphorylation of p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (eIF4EBP1) (23–29), whereas mTORC2 phosphorylates Akt (S473), serum and glucocorticoid-induced kinase 1 (SGK1, S422), protein kinase C α (PKCα, S657), focal adhesion proteins (FAK and Paxillin), and signals to small guanosine triphosphatases (RhoA, Rac1 and Cdc42) (30–40). Many functions of mTORC1 are sensitive to rapamycin, a conventional allosteric mTOR inhibitor (21). However, the action of rapamycin on mTORC2-mediated Akt depends on the concentration and duration of rapamycin treatment (41). Acute rapamycin treatment decreases phosphorylation of insulin receptor substrate-1, which results in insulin receptor substrate-1 accumulation, thereby activating PI3K/Akt (42–44). However, higher concentrations and/or longer exposure of rapamycin can inhibit Akt by disrupting mTORC2 complex formation (41). Although the cellular functions of the mTOR complexes remain to be determined, current data indicate that mTOR is at least involved in the regulation of synthesis and/or activities of cyclins D1/A (45–47), cyclin-dependent kinases (CDKs) (48), CDK inhibitors (p21Cip1 and p27Kip1) (49–51), c-myc (52), HIF-1α (53), VEGF (54), Erk1/2 (55), FAK (39), MMP-2 (56) and NF-κB (57). Therefore, mTOR has been implicated as a central regulator of cell growth, proliferation, survival, motility and angiogenesis. Of particular interest is that among the proteins regulated by mTOR, a number of them, such as HIF-1α, VEGF, cyclin D1, c-myc, NF-κB, Erk1/2, FAK and MMP-2, are also targeted by DHA (13–20). This prompted us to study whether DHA inhibits mTOR signaling.

Here, for the first time, we show that DHA inhibited proliferation and induced apoptosis in cells derived from RMS (Rh30 and RD). Concurrently, DHA, at pharmacological concentrations (<3 μM), potently suppressed mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 in the tumor cells. Unlike rapamycin, DHA did not obviously affect mTORC2-mediated phosphorylation of Akt. Our results suggest that DHA may represent a new class of mTORC1 inhibitor and execute its antitumor activity by primarily targeting mTORC1 signaling.

Materials and methods

Materials

Artemisinins, including artemisinin, DHA, artemunate and arteether (all purity >98% by high-performance liquid chromatography) were purchased from TCI America (Portland, OR), dissolved in 100% ethanol to prepare a stock solution (10 mM), aliquoted and stored at −20°C. IGF-1 (PeproTech, Rocky Hill, NJ) was rehydrated in 0.1 M acetic acid to prepare a stock solution (10 μg/ml), aliquoted and stored at −80°C. RPMI 1640 and Dulbecco’s modified Eagle’s medium were obtained from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA), whereas 0.05% trypsin–ethylenediaminetetraacetic acid was from Mediatech. Enhanced chemiluminescence solution was from Perkin-Elmer Life Science (Boston, MA). CellTiter 96® AQ-one One Solution Cell Proliferation Assay kit was from Promega (Madison, WI). Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences (San Jose, CA). The following antibodies were used: c-myc, cyclin A, cyclin B1, cyclin D1, cyclin E, p21Cip1, p27Kip1, Cdc25A, Cdc25B, Cdc25C, CDK1 (Cdc2), CDK2, CDK4, retinoblastoma (Rb), p-Rb (S807/811), poly(ADP-ribose) polymerase (PARP), BAD, BAX, BAK, Bcl-2, Bcl-XL, Mcl-1, survivin, PARP, FRAP (mTOR), Akt, S6K1, S6 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-mTOR (S2448), phospho-S6K1 (T389), phospho-S6 (S235/236), 4E-BP1, phospho-4E-BP1 (T70), phospho-Akt (S473) (Cell Signaling, Beverly, MA), β-tubulin (Sigma, St Louis, MO), goat anti-mouse IgG-horse radish peroxidase and goat anti-rabbit IgG-horse radish peroxidase (Pierce, Rockford, IL).

Cell lines and culture

Human RMS (Rh30, p53 mutant, Argy2–Cys; RD, p53 mutant, Argy2–Cys) and Ewing sarcoma (Rh1, p53 mutant, Tyrog2–Cys) cells (58,59) were generously provided by Dr Peter J.Houghton ( Nationwide Children’s Hospital, Columbus, OH) and were grown in antibiotic-free RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO2. Mouse myoblasts (C2C12), human leukemia (K562), lymphoma (U937), prostate carcinoma (PC-3) and cervical carcinoma (HeLa) cells were from American Type Culture Collection (Manassas, VA). C2C12 and HeLa were grown in antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, whereas K562, U937 and PC-3 were grown in antibiotic-free RPMI 1640 supplemented with 10% FBS, at 37°C and 5% CO2. For experiments where cells were deprived of serum, cell monolayers were washed with phosphate-buffered saline (PBS) and incubated in the serum-free RPMI 1640.

Cell morphological analysis and cell proliferation assay

Rh30 and RD cells were seeded in RPMI 1640 supplemented with 10% FBS in 6-well plates at a density of 2×104 cells/well and grown overnight at 37°C in a humidified incubator with 5% CO2. Next day, artemisinin, DHA, artemunate or artemether (0–30 μM) was added. After incubation for 6 days, images were taken with an Olympus inverted phase-contrast microscope equipped with the Quick Imaging system. Cells were then trypsinized and enumerated using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA).

Cell viability assay

Cell viability was evaluated using CellTiter 96® AQ-one One Solution Cell Proliferation Assay kit (Promega) containing MTS [3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and phenazine methosulfate. Briefly, cells suspended in the growth medium were seeded in a 96-well plate at a density of 1×104 cells/well (in triplicates) and grown overnight at 37°C in a humidified incubator with 5% CO2. Next day, artemisinin, DHA, artemunate or arteether (0–30 μM) was added. After incubation for 48 h, each well was added 20 μl of one solution reagent and incubated for 1 h. Cell viability was determined by measuring the OD at 490 nm using a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Wellesley, MA).

Cell cycle analysis

Cell cycle analysis was performed, as described previously (60). Briefly, Rh30 or RD cells were seeded in 60 mm dishes at a density of 2×104 cells/dish in RPMI 1640 supplemented with 10% FBS and grown overnight at 37°C in a humidified incubator with 5% CO2. Cells were then treated with DHA at 0–30 μM for 3 h or at 10 μM for 72 h. Subsequently, the cells were harvested with PBS and trypsinized. Cell suspensions were centrifuged at 1000 r.p.m. for 5 min, and pellets were fixed and stained with the Cellular DNA Flow Cytometric Analysis Kit (Roche Diagnostics Corp., Indianapolis, IN). Percentages of cells within each of the cell cycle compartments (G0/G1, S, or G2/M) were determined using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT analyzing software (Verity Software House, Topsham, ME). Cells treated with vehicle alone (100% ethanol) were used as a control.

Apoptosis assay

Rh30 or RD cells were seeded in 60 mm dishes at a density of 2×105 cells/dish in the growth medium and grown overnight at 37°C in a humidified incubator with 5% CO2. Cells were then treated with DHA (0–30 μM) for 72 h, followed by apoptosis assay using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA), as described previously (60). Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson). Cells treated with vehicle alone (100% ethanol) were used as a control.

Western blot analysis

Western blotting was performed as described previously (61). Briefly, following treatment, cells were washed with cold PBS. On ice, cells were lysed in radioluminometric precipitation assay buffer, containing 50 mM Tris pH 7.2, 150 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1% Triton-X 100; 10 mM NaF; 1 mM Na3VO4; protease inhibitor cocktail (1:1000, Sigma). Lysates were sonicated for 10 s and centrifuged at 14 000 r.p.m. for
10 min at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as standard (Pierce). Equivalent amounts of protein were separated on 6–12% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% non-fat dry milk to block non-specific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science).

Analysis of 4E-BP1-eIF4E binding
A functional assay of 4E-BP1 was performed, as described previously (62). Briefly, Rh30 cells (3 × 10⁶ cells/100 mm dish) were seeded and cultured overnight. Cells were then treated with DHA (0–10 μM) for 24 h or with rapamycin (100 ng/ml) for 2 h. The cells were scraped into 1 ml of ice-cold lysis buffer [50 mM Tris, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis (aminoethyl ether)-tetraacetic acid, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na₃VO₄, 50 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride and protease cocktail inhibitor (1:1000, Sigma)]. Lysis was accomplished by three freeze-thaw cycles and sonication. To pull down eIF4E, 30 μl of 7-methyl-GTP Sepharose (Amersham Biosciences, Piscataway, NJ) was added to the lysates and incubated overnight on a rotator at 4°C. The beads were pelleted by centrifugation and washed once with the lysis buffer and three times with PBS, followed by western blotting for 4E-BP1 and eIF4E, as described above.

Statistical analysis
Results were expressed as mean values ± standard error. The data were analyzed by one-way analysis of variance followed by post hoc Dunnett’s t-test for multiple comparisons. A level of P < 0.05 was considered to be statistically significant.

Results
DHA inhibits proliferation in RMS cells
 Artemisinins, including artemisinin, DHA, artesunate and artemether, as antimalarial agents, have been extensively studied (6, 7). Pharmacokinetic studies have demonstrated that the maximum plasma concentrations of artemisinin, DHA, artesunate and artemether range from 2 to 30 μM, when given to Sprague–Dawley rats at 10 mg/kg body weight, by intravenous or intramuscular injection, or by oral gavage (8). To determine which artemisinin compound is most potent as an anticancer agent, at the very beginning, RMS (Rh30) cells were treated with artemisinin, DHA, artesunate and artemether for 6 days at concentrations of 0–10 μM that are pharmacologically relevant (8). We found that all compounds inhibited cell proliferation in a concentration-dependent manner (Figure 1A). Both DHA and artesunate had similar antiproliferative activity (IC⁵₀ = 3–4 μM) and were more potent than artemisinin and artemether (IC⁵₀ >10 μM) (Figure 1A). Furthermore, DHA and artesunate not only exhibited a stronger cytostatic effect at low micromolar concentrations, but also exerted a stronger cytotoxic effect at the higher micromolar concentrations than artemisinin and artemether, as the original 20 000 seeded cells almost died out at 10 μM concentrations of DHA or artesunate after exposure for 6 days, as detected by morphological analysis (Supplementary Figure 1, available at Carcinogenesis Online). This is further supported by cell viability (MTS) assay. When the cells were exposed to the compounds for 48 h, DHA and artesunate also displayed higher cytotoxicity than artemisinin and artemether (Figure 1B). To determine whether this is cell type dependent, more tumor cell lines were employed, including Rh1 (Ewing sarcoma), K362 (leukemia), U937 (lymphoma), PC-3 (prostate cancer) and HeLa (cervical cancer) cells. We found that again, both DHA and artesunate had similar inhibitory effects on the cell growth and were more potent than artemisinin and artemether in all cell lines tested (data not shown), suggesting that the anticancer effects of artemisinins are not cancer cell type dependent. As artesunate is metabolized to DHA in the body very rapidly (within 3–4 h), treatment with DHA for 36 h was chosen for our further studies.

To test whether DHA is a selective anticancer agent for RMS, we further investigated its anticancer activity in two representative RMS cell lines, Rh30 (alveolar type) and RD (embryonic type) and a normal skeletal muscle cell line, mouse myoblasts (C2C12). Interestingly, treatment with DHA (0–10 μM) for 6 days dramatically inhibited proliferation of Rh30 and RD cells, but not C2C12 cells, in a concentration-dependent manner (Figure 1C). This was consistent with our morphological analysis, as Rh30 cells were very sensitive to the treatment with DHA (10 μM, 6 days), but C2C12 cells were considerably resistant to the same treatment (Supplementary Figure 1, available at Carcinogenesis Online). The results reveal that DHA is a promising anticancer agent for RMS selective treatment.

DHA arrests the cell cycle at G₀/G₁ and G₂/M phases in RMS cells
To understand how DHA inhibits cell proliferation in Rh30 cells, cell cycle analysis was performed. As the doubling time of Rh30 cells is 36 h (our unpublished observation), the cells were treated with DHA (0–30 μM) for 36 h, followed by propidium iodide (PI) staining and flow cytometry. As shown in Figure 2A, treatment with DHA for 36 h...
induced cell cycle arrest at G$_I$/G$_S$ and G$_M$ phases in Rh30 cells in a concentration-dependent manner. DHA at 3 μM significantly increased the proportion of cells in the G$_I$/G$_S$ phase from 34% (control) to 58%, and at 10 μM significantly increased the fraction of cells in the G$_I$/M phase from 15% (control) to 24%. Noticeably, DHA remarkably increased sub-G$_I$ population at 10–30 μM (Supplementary Fig. 2A, available at Carcinogenesis Online), implying cell death induced at these concentrations. In addition, treatment with DHA (10 μM) for up to 72 h also induced a time-dependent cell cycle arrest at G$_I$/G$_S$ and G$_M$ phases in Rh30 cells (Fig. 2B), as well as increased sub-G$_I$ (Supplementary Fig. 2B, available at Carcinogenesis Online), consistent with the reduced cell viability (Fig. 1C). Similar results were also observed in RD cells. Since both Rh30 and RD cells express mutant p53 alleles (Rh30 Arg$^{273}$→Cys; RD Arg$^{248}$→Trp), losing the function of p53, our findings imply that DHA is able to arrest cells in the G$_I$/G$_S$ and G$_M$ phases and inhibits cell proliferation in a p53-independent manner.

CDKs play an essential role in the regulation of cell cycle progression (63). A CDK (catalytic subunit) has to bind to a regulatory subunit, cyclin, to become active (63). Also, the activity of a CDK is regulated by CDC25 positively and by CDK inhibitor(s) negatively (63). Cyclin D-CDK4/6 and cyclin E-CDK2 complexes control G$_I$ cell cycle progression, whereas cyclin A-CDK2 and cyclin B-CDK1 regulate S and G$_M$ cell cycle progression, respectively (63). Therefore, perturbing expression of CDKs and/or the regulatory proteins, such as cyclins, CDC25 and CDK inhibitors, may contribute to the altered cell cycle distribution. Since DHA induced cell cycle arrest at G$_I$/G$_S$ and G$_M$ phases (Fig. 2), we next examined protein expression of CDK1/2/4/6, cyclins A/B1/D1/E, CDC25A/B/C and CDK inhibitors (p21$^{Cip1}$ and p27$^{Kip1}$). As shown in Fig. 3A, treatment of Rh30 with DHA for 24 h remarkably inhibited cellular protein expression of cyclin D1, CDC25A and CDC25C in a concentration-dependent manner. Expression of cyclin B1, CDK1 and CDK4 was slightly downregulated. Of notice, starting at 3 μM, DHA reduced expression of cyclin D1 very sharply. Protein levels of other molecules including CDK2, CDK6, cyclin A, cyclin E, CDC25B and p21$^{Cip1}$ were not obviously altered (Fig. 3A). Unexpectedly, expression of a CDK inhibitor, p27$^{Kip1}$, was downregulated (Fig. 3A). In time course studies, we also observed that DHA remarkably inhibited expression of cyclin D1, CDC25A and CDC25C in a time-dependent manner (Fig. 3B). Treatment with DHA at 3 μM for 12 h was able to inhibit expression of cyclin D1 profoundly. Our results suggest that DHA may inhibit expression of cyclin D1, CDK4 and CDC25A, resulting in cell cycle arrest at G$_I$/G$_S$ phase and inhibit expression of CDK1, cyclin B1 and CDC25C, leading to cell cycle arrest at G$_M$/phase.

As Rh, one of the most important G$_I$ phase cyclin/CDK substrates, functions as a tumor suppressor and a regulator of cell cycle progression in the late G$_I$ phase (63), we further investigated the effect of DHA on Rh phosphorylation. By western blot analysis, Rh was detected as a 110 kDa band in vehicle-treated control Rh30 cells (Fig. 3A). After DHA (3–30 μM) treatment for 24 h, a lower band, which migrates rapidly and represents the dephosphorylated protein, was observed (Fig. 3A), indicating that DHA inhibited phosphorylation of Rh. This was further verified by using the antibodies against specific phospho-Rh (S807/811) (Fig. 3A). Similarly, DHA (3 μM) also inhibits phosphorylation of Rh in a time-dependent manner (Fig. 3B). The data indicate that DHA arrested cells in G$_I$/G$_S$ phase of the cell cycle due to inhibition of Rh, a consequence of inhibition of G$_I$ CDKs.

**DHA induces p53-independent apoptosis in tumor cells**

To further define whether DHA-induced cell death is due to apoptosis, we carried out Annexin V-PI staining, a conventional approach to detect apoptosis. As shown in Fig. 4, treatment with DHA for 72 h induced apoptosis of Rh30 in a concentration-dependent manner. A representative fluorescence-activated cell sorting assay result is shown in the Supplementary Fig. 3, available at Carcinogenesis Online. Exposure to DHA (10 μM, 72 h) induced significant increase of the proportion of cells positive for Annexin-V/PI (~52%) as compared with non-treated cells (~5%). Treatment with the compound (10 μM) increased the number of Rh30 cells undergoing apoptosis by ~10-fold. Similar results were also seen in another RMS cell line (RD) and Ewing sarcoma cell line (Rh1) (data not shown).

In addition, we also observed that DHA induced PARP cleavage, a hallmark of caspase-dependent apoptosis in Rh30 cells, in a concentration-dependent manner (Fig. 5). This is consistent with our finding that DHA increased cleavage of caspase 3 (Fig. 3), indicating activation of caspase 3. It appears that treatment with DHA for 24 h did not affect expression of proapoptotic proteins, such as BAD, BAK and BAX, but obviously reduced expression of antiapoptotic proteins, including Bcl-2, Bcl-xL, Mcl-1 and survivin, in a concentration-dependent manner (Fig. 5). Besides, in the time course study, we observed that treatment with DHA at 3 μM increased the expression of cleaved caspase 3 and cleaved PARP in a time-dependent manner (Supplementary Fig. 4, available at Carcinogenesis Online). This was correlated to increased expression of BAD, and decreased expression of Mcl-1 (Supplementary Fig. 4, available at Carcinogenesis Online). Similar results were also observed RD and Rh1 cells. As p53 is mutated in Rh30, RD and Rh1 cells, our results indicate that DHA can induce p53-independent apoptosis in the tumor cells.

**DHA inhibits mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 but does not affect mTORC2-mediated phosphorylation of Akt**

Increasing evidence has implicated that mTOR is a central controller of proliferation and survival (21). The present study and others (13–20) have demonstrated that DHA potently inhibits cell proliferation and induces apoptosis in RMS and many other cell lines. In particular, among the proteins regulated by mTOR, a number of them, such as HIF-1α, VEGF, cyclin D1, c-myc, NF-kB, Erk1/2, FAK and MMP-2, are also targeted by DHA (13–20). We therefore hypothesized...
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that DHA might be disrupting these cellular processes by primarily inhibiting the mTOR signaling pathway. To test this, we set out to examine the effect of DHA on the mTOR signaling pathway in tumor cells. By western blot analysis, we found that DHA inhibited phosphorylation of mTOR (S2448) in a concentration- and time-dependent manner (Figure 6A). Consistently, DHA also inhibited phosphorylation of S6K1 and 4E-BP1, two best-known downstream effector molecules of mTORC1. Dose–response experiments indicated that treatment with 1 μM DHA (for 24 h) obviously inhibited phosphorylation of S6K1 (T389) (Figure 6A). Similarly, phosphorylation of 4E-BP1 (T70) was also inhibited (Figure 6A). As 4E-BP1 functions as a suppressor of eIF4E, and hypophosphorylated 4E-BP1 binds to and inhibits eIF4E, instead of probing more phosphorylation sites (e.g. T37/46, S65, etc.) of 4E-BP1 (21), we directly studied whether DHA affects the interaction of 4E-BP1 with eIF4E. Our 7-methyl-GTP Sepharose pull-down assay demonstrated that DHA increased the binding of 4E-BP1 to eIF4E in a concentration-dependent manner in Rh30 cells (Supplementary Figure 5, available at Carcinogenesis Online). Of note, 3–10 μM of DHA was able to induce similar amount of 4E-BP1 bound to eIF4E, as 100 ng/ml of rapamycin did (Supplementary Figure 5, available at Carcinogenesis Online). The data strongly suggest that DHA suppresses mTORC1-mediated eIF4E pathway, by inhibiting phosphorylation of 4E-BP1. In addition, our time course studies showed that treatment with DHA (at 3 μM) for 16 h remarkably inhibited phosphorylation of S6K1 and 4E-BP1 (Figure 6B). DHA also potently inhibited phosphorylation of S6, a substrate of S6K1 (Figure 6A and B). However, treatment with DHA for 24–48 h did not exhibit an obvious inhibitory or stimulatory effect on phosphorylation of Akt (S473), a best characterized...
substance of mTORC2, in Rh30 cells (Figure 6C). Of note, treatment with DHA for 24 h did not appear to affect the total cellular protein levels of these proteins (Figure 6A–C).

To exclude the possibility that DHA inhibition of mTOR signaling is cell type dependent, we extended our studies using Ewing sarcoma (Rh1). When serum-starved Rh1 cells were treated with DHA (0–10 μM) for 24 h and then stimulated with IGF-1 (10ng/ml) for 1 h, western blot analysis revealed that IGF-1 stimulated phosphorylation of S6K1, 4E-BP1 and Akt (Supplementary Figure 6, available at Carcinogenesis Online). Consistent with the findings in Rh30 cells (Figure 6A), DHA exhibited an inhibitory effect only on phosphorylation of S6K1 and 4E-BP1, but not on phosphorylation of Akt, in the cells (Supplementary Figure 6, available at Carcinogenesis Online). Taken together, our results indicate that DHA, at pharmacological concentrations (1–10 μM), inhibited mTORC1-mediated phosphorylation of S6K1 and 4E-BP1, but did not affect mTORC2-mediated phosphorylation of Akt, which was independent of the nature of cancer cell lines.

Since DHA, at concentrations of <3 μM, did not obviously inhibit cell proliferation in normal cells (C2C12) (Figure 1C), we further investigated whether this was related to poor inhibition of mTORC1 signaling in the cells. Interestingly, as expected, treatment with DHA (3 μM) for up to 24 h did not obviously inhibit mTORC1 signaling, as phosphorylation of mTOR (S2448), S6K1 (T389), p-S6 (S235/236) and p-4E-BP1 (T70) was not apparently suppressed in C2C12 cells (Supplementary Figure 7, available at Carcinogenesis Online). The results indicate that DHA, at pharmacological concentrations, fails to inhibit mTORC1 signaling in normal myoblasts, and this may be the reason why DHA did not obviously inhibit proliferation in C2C12 cells (Figure 1C).

Discussion

Here, we have shown that DHA, an antimalarial drug, at pharmacological concentrations (<10 μM) (8), inhibited proliferation and induced apoptosis in cells derived from various cancer types including RMS (Rh30 and RD), but not in normal cells such as mouse myoblasts (C2C12). This suggests that DHA is possibly a selective anticancer agent and has a great potential to be repurposed for RMS cancer therapy.

Approximately 50% of human cancers, including RMS, contain a mutated form of p53, one of the most important tumor suppressor proteins in cells (64). Wild-type p53 responds to DNA damage in cells and can arrest growth of the cells to allow time for DNA repair to occur or can induce apoptosis of the cells with irreparable DNA damage (64). Cancer cells, especially those with p53 mutations, are able to escape cell cycle arrest and apoptosis despite their large number of genetic mutations/malfunctions (64). Here, we have also observed that DHA effectively inhibited cell proliferation by arresting the cells in the G0/G1 and G2/M phases of the cell cycle and induced cell death in RMS (Rh30 and RD) and Ewing sarcoma (Rh1). As these cells are p53 mutant, losing p53 function (Rh30, p53 mutant, Arg172→Cys; RD, p53 mutant, Arg179→Trp and Rh1, p53 mutant, Tyr245→Cys) (58,59), our results indicate that DHA can inhibit cell proliferation and induce cell death through a p53-independent mechanism. This observation implies that DHA may have potential applications as a chemotherapeutic agent against those p53 mutant tumor cells, which are resistant to radiotherapy or other chemotherapies. In this study, we found that treatment with DHA downregulated expression of some proteins related to cell cycle progression or cell survival, such as cyclin D1, Cdc25A, Cdc25C, Bcl-2, Bcl-xL, Mcl-1 and survivin. However, it is not clear whether DHA inhibits expression of those proteins at transcriptional, translational and/or posttranslational level. Further research is needed to address this issue.

Although DHA has been in clinical trials as a new anticancer agent for treatment of types of cancer (6,11,12), the anticancer mechanism remains elusive. Studies have revealed that DHA targets a number of cellular proteins, which are critical for cell proliferation, survival, motility and invasion, as well as angiogenesis (13–20). Of particular interest is that among the proteins regulated by mTOR, most of them, such as HIF-1α, VEGF, cyclin D1, c-myc, NF-kB, Erk1/2, FAK and MMP-2, are also targeted by DHA (13–20). This led us to investigate whether DHA primarily inhibits mTOR signaling, thereby inhibiting expression or activities of those proteins. Here, for the first time, we demonstrate that DHA inhibited cell proliferation and induced apoptosis in RMS cells, and concurrently inhibited the signaling pathways (S6K1 and 4E-BP1) mediated by mTOR, a central controller of cell proliferation and survival (21,22). DHA inhibition of mTOR signaling was also observed in Ewing sarcoma cells (Rh1) (Supplementary Figure 6, available at Carcinogenesis Online). During the preparation of our manuscript, Zhao et al. (65) reported that DHA also inhibits mTOR-mediated phosphorylation of S6K1 induced by interleukin-2 in Cd4+ T cells. These findings indicate that DHA inhibition of mTOR signaling is not cell dependent. Collectively, our results support the hypothesis that DHA may execute its anticancer activity by primarily targeting mTORC1-mediated signaling pathways.

Pharmacokinetic studies have demonstrated that when Sprague-Dawley rats were given DHA at 10 mg/kg body weight, by intravenous or intramuscular injection, or by oral gavage, maximal plasma concentrations of DHA at 23.5±13.0, 5.5±1.6 and 2.7±0.8 μM are reachable, respectively (8). In this study, we found that treatment with DHA for 24 h at 1 μM potently inhibited mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 (Figure 6A). The results imply that DHA should be able to inhibit mTORC1 signaling well within the range of pharmacologically achievable concentrations in vivo. Of interest, we observed that DHA inhibition of mTORC1 did
not obviously activate or inhibit the phosphorylation of Akt, a best characterized substrate of mTORC2 in RMS (Rh30) (Figure 6C) and Ewing sarcoma (Rh1) cells (Supplementary Figure 6, available at Carcinogenesis Online). This is in contrast to the effects of the conventional allosteric mTORC1 inhibitors (rapamycin and its analogs, such as CCI-779, RAD001, AP23573, etc.) or the new generation of adenosine triphosphate-competitive mTOR inhibitors (e.g. Torin1, PP242, WAY-600, Ku-0063794, OSI-027, NVP-BEZ235, PI-103, etc.). It has been observed that treatment with rapamycin for short time (e.g. 2h) or at low concentrations (e.g. 10–100nM) is able to inhibit mTORC1-mediated phosphorylation of S6K1 and 4E-BP1, but activate mTORC2-mediated phosphorylation of Akt (S473) (21,42–44). However, higher concentrations (e.g. >1μM) and/or longer exposure (e.g. >24h) of rapamycin can inhibit Akt by disrupting mTORC2 complex formation (41). Adenosine triphosphate-competitive mTOR inhibitors can inhibit both mTORC1 and mTORC2 (21,22,66). Therefore, our findings highlight that DHA may represent a new class of mTORC1 inhibitor and is promising for targeted cancer therapy.

A new question that arises from the current work is how DHA inhibits mTORC1 signaling. mTORC1 is positively regulated by IGF-1R or PI3K and negatively regulated by phosphatase and tensin homolog and adenosine monophosphate-activated protein kinase (21,22). Further research is required to determine whether DHA inhibits mTORC1 by inhibiting IGF-1R or PI3K and/or by activating phosphatase and tensin homolog or adenosine monophosphate-activated protein kinase.

mTORC1 mainly consists of mTOR, mLST8 and raptor (23–29). The function of mTORC1 is greatly affected by the complex integrity, especially its association with raptor (23,24). The classic mTOR inhibitor, rapamycin, disrupts the interaction of raptor with mTOR (23,24). In particular, at high concentrations (~20μM), rapamycin induces apoptosis in breast cancer cells (MDA-MB-231 and MCF-7) by dissociating raptor from mTOR, leading to 4E-BP1 dephosphorylation (67). 4E-BPs are the major regulators of cell cycle progression and proliferation (66,68). In this study, we found that DHA potently inhibited proliferation and induced apoptosis in RMS cells. Also, DHA inhibited phosphorylation of not only S6K1 but also 4E-BP1 (Figure 6). Particularly, DHA increased 4E-BP1 binding to eIF4E (Supplementary Figure 5, available at Carcinogenesis Online), suggesting suppression of eIF4E pathway. Possibly, DHA may inhibit mTORC1 by disrupting mTORC1 formation/stability.

In addition, it has been described that PRAS40 binds the mTOR kinase domain and negatively regulates Rheb-guanosine triphosphate-induced mTORC1 activity (27,28). DEPTOR binds to and negatively regulates mTORC1 as well (29). If DHA enhances association of PRAS40 or DEPTOR to mTOR, this may also result in inhibition
of mTORC1 phosphorylation of S6K1 and 4E-BP1. More studies are on the way to address whether DHA inhibits phosphorylation of S6K1/4E-BP1 by disrupting mTORC1 formation and/or by enhancing the association of PRAS40 or DEPTOR with mTOR.

Supplementary material

Supplementary Figures 1–7 can be found at http://carcin.oxfordjournals.org/

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References

20. Loewith, R. et al. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell, 10, 457–468.


68. Dowling, R.J. et al. (2010) mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science, 328, 1172–1176.

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