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Human Fortilin Is A Molecular Target of Dihydroartemisinin

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Abstract

Dehydroartemisinin (DHA) is an effective anti-malaria agent. Fortilin is an anti-apoptotic molecule overexpressed in many human cancers. Here, we show that DHA binds human fortilin, increases the ubiquitination of fortilin, shortens fortilin's half-life in a proteasome-dependent fashion, and reduces cellular levels of fortilin in varieties of cells. DHA induced DNA fragmentation in U2OS cells in a fortilin-dependent manner. The fortilin-knocked-down cells were less susceptible—and fortilin-overexpressing cells more susceptible—to DHA than were wild type cells, suggesting that apoptotic effects of DHA are—at least partly—conferred through fortilin. Together, these data suggest that fortilin is a molecular target of DHA. DHA and its derivative may prove to be viable anti-cancer agents in fortilin-overexpressing cancers. (115 words)

Keywords

Fortilin; dihydroartemisinin; DHA

Introduction

Fortilin, also known as translationally controlled tumor protein (TCTP) and histamine releasing factor (HRP), possess potent anti-apoptotic function [1]. In human cells, fortilin overexpression protects cells against apoptosis induced by chemotherapeutic agents such as etoposide [1] and 5-fluorouracil (5-FU) [2] while fortilin depletion either precipitates spontaneous apoptosis or increases sensitivity to cytotoxic stimuli [1,3]. Importantly, the expression of human fortilin is far greater in cancerous cells and tissue than their normal counterparts [3]. Although these observations suggest that human fortilin inhibitors are viable anti-cancer agents, there has been no such compound reported.

Dehydroartemisinin (DHA, Fig. 1B) is a metabolite of artemisinin—an active ingredient of sweet wormwood *Artemisia annua* (*Qing Hao*) (Fig. 1A) [4,5]. Both artemisinin and DHA are highly effective anti-malaria agents and are well-tolerated [6–8]. Bhisutthibhan and others reported that DHA binds malaria fortilin in vivo [9–11]. Based on the fact that fortilin is highly conserved across species—human (NP_003286) and malaria (AAZ73158) fortilins shares 33% and 58% of identity and homology, respectively, we hypothesized that DHA also binds and inhibits human fortilin.

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Here, we report that the DHA binds human fortilin at the dissociation constant (K_d) of 38 μM as assessed by the surface plasmon resonance-based methods. We further report that the binding of DHA to human fortilin results in the shortening of the half-life of fortilin, via the ubiquitination of fortilin and subsequent proteasome-mediated degradation.

We demonstrate that DHA reduces cellular fortilin levels in many human cell lines despite the transcriptional activation of fortilin gene. We establish that fortilin is required for the full apoptotic effects of DHA in U2OS cells. We propose that DHA is a novel human fortilin inhibitor.

Materials and Methods

Generation of recombinant proteins

Recombinant GST-human fortilin and GST-alone were produced as previously described [12]. Detailed description on Materials and Methods can be found in Supplemental Materials.

Generation of U2OS stable transfectants

U2OS cells stably over-expressing fortilin (U2OS-fortilin) or harboring empty plasmid (U2OS-empty) were generated as described previously by us [2]. U2OS-sh-fortilin, U2OS cells that stably harbor a short-hairpin RNA against fortilin (shRNA-fortilin) and express much lower levels of fortilin, were generated as described in detail within Supplemental Materials.

Measurement of surface plasmon resonance (SPR)

All analyses were carried out using a Biacore T100 (Biacore Inc., Part of GE Healthcare, Piscataway, NJ) as described previously [13,14]. GST-human-fortilin (will be called GST-fortilin hereafter) or GST alone were directly coupled to a Series S CM5 chip. For binding assays, the flow rate of running buffer—HBS-EP⁺ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05 % v/v surfactant P20)—was maintained at 30 $\mu\text{L}/\text{min}$ at 25°C. For each sample cycle, DHA at the concentrations between 0–100 μM were injected for a period of 30 s followed by an un-disturbed dissociation period of 10 s. Sensorgrams for various concentrations of DHA were obtained and subjected to steady state affinity analysis [13,14]. Data were double reference subtracted and solvent (DMSO) corrected.

Western blot analysis

Western blot analyses—using anti-fortilin and anti-GAPDH antibodies—and quantitative signal analyses were performed as previously described by us [1,15].

Half-life analysis of fortilin

U2OS cells (1×10^5) were seeded in wells of 6-well dishes, pre-treated with 30 mg/mL cyclohexamide (Sigma), treated with 20 μM DHA, harvested into SDS loading buffer at times 0, 1, 3, 5, and 8 hrs. When appropriate, cells were also treated by 10 μM MG-132, a proteasome inhibitor (Sigma). Samples were then subjected to Western blot analyses as described above. For the determination of steady-state level of fortilin, cells (1×10^5) were seeded in wells of 6-well dishes, treated with vehicle alone (DMSO), 20 μM and 50 μM DHA for 48 hr and harvested into SDS loading buffer. Experiments were repeated twice with the same results.

Real-time quantitative PCR (qRT-PCR) of fortilin

The qRT-PCR of fortilin was performed as previously described by us [15].

Fortilin ubiquitination assay

U2OS cells were co-transfected with pcDNA3-Ubiquitin (pcDNA-Ub) and pFLAG-fortilin, and treated with 0, 25, and 50 μ M DHA for 24 hrs. Extracted proteins were subjected to Western blot analyses using anti-FLAG (M2, Sigma) and anti-GAPDH (Fitzgerald Industries Inc.) antibodies.

DNA fragmentation assay

U2OS-fortilin, U2OS-empty, U2OS-sh-fortilin, and U2OS-sh-empty (0.2×10^4 per well) were seeded into 24-well plates, challenged by 20 μ M DHA or vehicle (DMSO), incubated for 48hrs, and subjected to a DNA fragmentation assay using the Cell Death Detection ELISA^{PLUS} kit (Roche). After the assay, cells were subjected to SDS-PAGE and Western blot analysis using anti-fortilin and anti-GAPDH antibodies.

Statistical analyses

All data were expressed as the mean $\pm$ standard deviation (SD). A *P* value of less than 0.05 was considered to be statistically significant.

Results

DHA binds human fortilin

In order to determine whether DHA (Fig. 1B) binds human fortilin, we performed surface plasmon resonance (SPR) experiments. Representative sensorgrams obtained for GST-fortilin and GST alone are shown in Figs. 2A and 2C, respectively. The binding of DHA to GST-fortilin occurred rapidly before achieving a steady state binding level during the sample injection. Subsequently, a fast dissociation phase—almost instantaneous at the end of DHA injection—was observed. The equilibrium dissociation constant of DHA binding to fortilin was calculated at 38 μ M (Fig. 2B), using the steady state affinity analysis model [13,14]. There was no significant binding of DHA to GST alone observed (Fig. 2C).

DHA shortens the half-life of fortilin

We then treated U2OS cells with cyclohexamide and then with DHA and subjected cells to Western blot analyses at 0, 1, 3, 5, and 8 hours. In the absence of DHA, the intracellular levels of fortilin gradually decreased over an 8 hr period (Vehicle, Fig. 3A and DHA [−], Fig. 3B), with the extrapolated half-life of 11.6 hr. In this system, the presence of DHA was associated with much faster decrease in fortilin levels (fortilin expression indices at 8hrs = 56.9 vs. 30.8 % for DHA [−] and DHA [+], respectively, Fig. 3B), with the estimated half-life of 6.3 hr. Taken together with the fact that DHA specifically binds fortilin (Figs. 2A & B), these data suggest that the binding of DHA to fortilin facilitates the degradation of fortilin *in vivo*.

DHA-induced shortening of fortilin's half-life is mediated by ubiquitin-proteasome pathway

Next, we repeated the same experiment described above, in the presence of MG132, a potent inhibitor of the proteasome pathway [16]. In the absence of DHA, fortilin signal slowly decreased over time, with fortilin expression index reaching at 74.7% of time zero by 8hrs (Top panel, Fig. 4A and DHA [−][open circles], Fig. 4B). In the presence of 20 μ M DHA, fortilin signals decreased more rapidly, with fortilin expression index reaching at 51.6% of time zero at 8hrs (Middle panel, Fig. 4A and DHA+[closed circles], Fig. 4B). In this system, the addition of MG132 increased fortilin levels in all time points, with essentially no reduction of fortilin levels over 8hrs (98.3% at 8hrs, Bottom panel, Fig. 4A and DHA+MG132 [closed triangles], Fig. 4B).

Next, we performed fortilin ubiquitination assays where U2OS cells were co-transfected with pcDNA-Ub and pFLAG-fortilin plasmid vectors, divided into 3 dishes, and treated by 0, 25, and 50 μ M DHA for 24 hrs. The cells were harvested and extracted proteins were subjected to Western blot analyses, using anti-FLAG and anti-GAPDH antibodies. As is shown in Fig. 5, DHA dose-dependently increased the ubiquitination of fortilin. Taken together with the data presented in Fig. 4, these findings suggest that ubiquitin-proteasome pathways contribute to the degradation of DHA-bound fortilin.

DHA decreases the steady state levels of fortilin in U2OS cells

Next, we treated U2OS cells for 48hrs with DHA in the absence of cyclohexamide. Western blot analyses showed that fortilin levels dose-dependently decreased with chronic DHA treatment, even in the absence of cyclohexamide (fortilin expression index = 100 ± 4.2 , 55.3 ± 0.7 , 25.1 ± 10.5 %, for 0, 20, and 50 μ M DHA, respectively, $P = 0.003$ by one-way ANOVA, Figs. 6A and B). These data suggest that a long-term treatment of cells with DHA is associated with reduced levels of intracellular fortilin levels and that DHA can be utilized to reduce the levels of intracellular fortilin in vivo, without protein synthesis inhibitors.

The reduction of intracellular fortilin concentration is associated with increase in fortilin messages

Next, we quantified fortilin message levels of U2OS cells that had been treated with vehicle, 20, 35, and 50 μ M DHA for 48hrs in the absence of cyclohexamide. As is shown in Fig. 6C, fortilin message increased significantly as cells were treated with increasingly high concentrations of DHA (fortilin message index [fg of fortilin mRNA per 1ng of total RNA loaded] = 3.93 ± 0.47 , 3.97 ± 0.21 , 4.86 ± 0.40 , and 5.12 ± 0.60 , for 0, 20, 35, and 50 μ M DHA, respectively, $P = 0.02$ by one-way ANOVA). These data suggest that the long-term administration of DHA, despite the transcriptional activation of fortilin gene (Fig. 6C), reduces the intracellular levels (Figs. 6A & B) of fortilin protein through the shortening of fortilin's half-life (Fig. 3) via the activation of the ubiquitin-proteasome pathway (Figs. 4&5).

DHA reduces intracellular fortilin levels in many cell types

We then treated various cell lines with 20 μ M of DHA for 48hrs, in the absence of cyclohexamide, and subjected them to Western blot analyses. As is shown in Fig. 7, DHA substantively reduced cellular fortilin levels in VSMC, A549, H1299, U2OS, MDA468, and MCF7 cells. These data suggest that DHA's effects on fortilin are not limited to U2OS cells.

The lack of fortilin reduces the sensitivity of U2OS cells to DHA-induced DNA fragmentation

Finally, we evaluated if and how DHA induces DNA fragmentation in U2OS cells. We first generated U2OS-sh-fortilin, U2OS cells with reduced fortilin expression via the sh-RNA against fortilin (see Materials and Methods for details) and U2OS-sh-empty, their control cells. In addition, we had generated U2OS-fortilin, U2OS cells stably overexpressing fortilin, and U2OS-empty, their control cells. We treated these cells with 20 μ M DHA and subjected them to DNA fragmentation assays. DHA at 20 μ M caused more apoptosis (DNA fragmentation) in U2OS-sh-empty cells than in U2OS-sh-fortilin cells (Fig. 8A), suggesting that the lack of fortilin decreased the sensitivity of U2OS cells to DHA. Consistently, DHA caused more apoptosis in U2OS-fortilin cells than in U2OS-empty cells (Fig. 8B). These data suggest that the presence of fortilin was required for DHA to exert its *full* apoptotic effects and that DHA kills cells—at least partly—through the targeting, inactivation and elimination of fortilin (Fig. 9).

Discussion

The significance of the current work is that we established the presence of binding of DHA (Fig. 1) to human fortilin ($K_d = 38 \mu\text{M}$) and the biological significance of the binding. To our knowledge, the fact that DHA binds human fortilin and decreases cellular fortilin levels has not been reported. The K_d fits well with the results of in vivo experiments where 20–50 μM DHA decreased cellular fortilin levels (Figs. 3, 4, 6, & 7).

The fact that DHA induce cytotoxicity and apoptosis in cancerous cells has been reported by several groups. Lai and others treated molt-4 lymphoblastoid cells with 200 μM DHA and showed their survival was less than 50% after 8hr incubation [17]. Kim and others showed that a glioma cell line—U373MG—is susceptible to DHA with IC_{50} of 70 μM [18]. Our current observation (Fig. 8) is consistent with above findings and add that the apoptosis by DHA of U2OS cells is at least partly mediated by its targeting of fortilin, since the knockdown (Fig. 8A) and overexpression (Fig. 8B) of fortilin made U2OS cells less and more sensitive to DHA, respectively. In other words, fortilin was required for DHA to exert its *full* apoptotic effects.

The fact that DHA induces apoptosis by targeting fortilin does not exclude a possibility that DHA induces apoptosis by activating other apoptosis pathways. In fact, DHA's binding to fortilin is moderate at best—DHA is only an imperfect fortilin antagonist. To make the matter complex, the other apoptosis pathways activated by DHA can directly or indirectly be antagonized by fortilin, an anti-apoptotic molecule. It is also possible that DHA-bound fortilin may become pro-apoptotic. Further investigation is called for to identify and characterize the pathways by which DHA induces apoptosis without directly attacking fortilin.

It is shown here that DHA is a viable lead compound to develop more specific fortilin inhibitors and that human fortilin is a critical target of DHA and its derivatives. Such fortilin inhibitors would negate the biological function of fortilin simply by facilitating the degradation of fortilin in cells (Fig. 9). Since fortilin is required to sustain the life of many cancerous cells [1,2,15, 19], such fortilin inhibitors would cause cells to go into severe fortilin deficiency and cell death and prove to be potent anti-cancer drugs in fortilin-over-expressing cancers. Encouragingly, DHA and its derivatives are extremely well tolerated medication, with reported median lethal doses in mice being 4228 mg/kg [5], suggesting that derivatives of DHA would also be well tolerated by patients. That said, further biological analyses using whole organisms will be required to definitively determine the efficacy of the fortilin-targeting strategies in cancer chemotherapies. In the initial screening of such DHA derivatives, SPR (Fig. 2) and fortilin-knock-down cell (Fig. 8) systems described in the current work will prove to be invaluable tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DHA
dihydroartemisinin

ROS	reactive oxygen species
TCTP	translationally controlled tumor protein
HRP	histamine releasing factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
ANOVA	analysis of variance
SPR	surface plasmon resonance

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Fig. 1. Chemical structure of artemisinin and dihydroartemisinin (DHA)

A. Artemisinin ($C_{15}H_{22}O_5$, Molecular weight [M.W.] = 282.33) is a sesquiterpene with endoperoxides (a peroxy group). **B. Dihydroartemisinin** (DHA, $C_{15}H_{24}O_5$, M.W. = 284.35) is a metabolite of artemisinin.

Fig. 2. DHA binds human fortilin as detected by the surface plasmon resonance (SPR) measurement GST-fortilin, GST-fused to the N-terminus of human fortilin; GST, GST-only protein; RU, resonance units; K_d , equilibrium dissociation constant. **A.** Sensorgrams of DHA binding to human fortilin. **B.** Calculation of K_d by the steady state affinity analysis model. **C.** Sensorgrams showing the lack of interaction between DHA and GST. All analyses were carried out using a Biacore T100 (Biacore Inc., Part of GE Healthcare, Piscataway, NJ) as described in the Materials and Methods section. GST-fortilin (**A**, **B**) or GST alone (**C**) was immobilized to the carboxymethylated (CM) sensor surface of a Series S CM5 chip. DHA at the concentrations between 0–100 μM were injected for a period of 30 s followed by an un-disturbed dissociation

period of 10 s. Sensorgrams for various concentrations of DHA were obtained. Data were double reference subtracted and solvent (DMSO) corrected.

Fig. 3. DHA shortens the half-life of fortilin protein

A: Western-blot analysis of fortilin and glyseraldehyde-3-phosphate dehydrogenase (GAPDH) in cells treated with either 20 μ M DHA or Vehicle (DMSO). **B:** Quantitative analyses of fortilin signals. Open circles: fortilin expression indices of cells not treated by DHA. Closed circles: fortilin expression indices of cells treated by DHA. Cells were pre-treated with cycloheximide and then with DMSO (vehicle) or 20 μ M DHA (**A**), followed by the calculation of fortilin expression indices, fortilin signals normalized to corresponding GAPDH signals and expressed as a percentage of the values at time zero (**B**).

Fig. 4. DHA-induced shortening of fortilin's half-life is mediated by proteasome pathway
A: Western-blot analysis of fortilin and GAPDH in cells treated with DMSO (DHA 0 μ M), 20 μ M DHA, or 20 μ M DHA plus MG132. **B:** Quantitative analyses of fortilin signals. Closed triangles: fortilin expression indices of cells treated with both DHA and MG132, Open circle: DMSO (vehicle) only, Closed circle: DHA only. Cells were pre-treated with cycloheximide and when appropriate also with MG132 before they were treated with vehicle (DMSO) or 20 μ M DHA (A). Fortilin expression indices were calculated by normalizing fortilin signals to corresponding GAPDH signals and expressing them as a percentage of the values at time zero (B).

Fig. 5. DHA increases fortilin ubiquitination

α -FLAG, anti-FLAG antibody; Ub, ubiquitin. U2OS cells were co-transfected with pcDNA-Ub and pFLAG-fortilin, treated with DHA, harvested into SDS-loading buffer, and evaluated by Western blot analysis. DHA dose-dependently increased the ubiquitination of fortilin.

Fig. 6. DHA decreases the steady state levels of fortilin protein and increases the transcription of fortilin messages in U2OS cells

A: Western-blot analysis of fortilin and GAPDH in cells treated with vehicle (DMSO, 0μM DHA), 20μM and 50μM DHA. **B:** Quantitative analyses of fortilin signals. ANOVA showed that the concentration of DHA had a significant negative correlation with fortilin expression index (*P* = 0.003, *N* = 2). Cells were treated with vehicle (DMSO), 20 or 50μM DHA for 48 hrs (**A**). Fortilin expression indices were calculated by normalizing fortilin signals to corresponding GAPDH signals and expressing them as a percentage of the values at 0μM DHA (**B**). **C:** Real-time qRT-PCR of fortilin message in U2OS cells treated with DHA. Fortilin message index: fg of fortilin mRNA per ng of total RNA. ANOVA reveals that the

concentration of DHA had a significant positive correlation with fortilin message index ($P = 0.02$, $N = 3$).

Fig. 7. DHA decreases cellular fortilin levels in various cell types

VSMC, vascular smooth muscle cells; A549 and H1299, lung cancer cell lines; U2OS, osteosarcoma cell line; MDA468 and MCF7, breast cancer cell lines. Cells were treated with vehicle (DMSO, DHA[-]), 20 μ M DHA (DHA[+]) for 48 hrs. Fortilin expression indices were calculated by normalizing fortilin signals to corresponding GAPDH signals.

Fig. 8. Cells lacking fortilin are less susceptible to DHA-induced DNA fragmentation

A: U2OS-sh-fortilin, U2OS cells that are stably transfected with sh-RNA against fortilin and express a smaller amount of fortilin; U2OS-sh-empty, U2OS cells stably transfected with empty sh-RNA vector; *NS*, not statistically significant; ***, $P < 0.005$. The lack of fortilin significantly reduces the sensitivity of U2OS cells to DHA. **B:** U2OS-fortilin, U2OS cells stably overexpressing fortilin; U2OS-empty, U2OS cells stably transfected with empty pcDNA vector; *NS*, not statistically significant; ***, $P < 0.005$; +, $P = 0.085$. The fortilin overexpression increases the sensitivity of U2OS cells to DHA.

Fig. 9. The biological consequences of DHA-binding to fortilin – DHA is a fortilin inhibitor (–), negative feedback; Ub, Ubiquitin. DHA binds human fortilin. Fortilin bound by DHA is recognized as altered, ubiquitinated, and subjected to proteasome-mediated degradation, which can be blocked by proteasome inhibitors such as MG132. While the sudden reduction by DHA of cellular fortilin levels causes cells to either undergo cell death or be more susceptible to other cytotoxic stimuli, cells chronically express lower levels of fortilin may be less sensitive to DHA. DHA induces cytotoxicity in both fortilin-dependent (red solid arrow) and – independent (red dotted arrow) fashions.