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Dihydroxyacetone induces G2/M arrest and apoptotic cell death in A375P melanoma cells

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Abstract

The active ingredient in sunless tanning products (STPs) is a simple sugar, dihydroxyacetone (DHA). Several studies have demonstrated that DHA is absorbed within the viable layers of skin and not fully contained within the stratum corneum. Additionally, spray tanning and other aerosolized application methods have increased the risk of internal exposure through mucous membranes and inhalation. Beyond its presence in STPs, DHA also occurs as an endogenous by-product of fructose metabolism, and an excess of DHA in cells can induce advanced glycation end products (AGE) and oxidative stress. Therefore, exogenous and endogenous exposures to DHA may be harmful to cells, and it has already been demonstrated that exogenous exposure to DHA is cytotoxic in immortalized keratinocytes. Still, little is known about the exogenous DHA exposure effects on other skin components. In the present study, we explore the effects of exogenous DHA exposure in a human melanoma cell line, A375P. Melanoma cells were sensitive to DHA and displayed a transient burst of reactive oxygen species within an hour of exposure. Cell cycle arrest at G2/M was observed within 24 h of exposure, and apoptosis, monitored by the cleavage of PARP-1 and Caspase-3, was detected within 72 h of exposure to DHA. Together, these demonstrate that exogenous exposure to DHA has cytotoxic effects in our selected cell model and indicates the need to further investigate the exogenous exposure effects of DHA in other relevant exposure models.

Keywords

dihydroxyacetone; sunless tanning; melanoma; apoptosis; oxidative stress; mitochondria; cell cycle arrest

1. Introduction

Skin cancer is the second most common cancer among young adults ages 15–29 years, with white females being at highest risk [1]. The increased incidence of skin cancer in this

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population is often attributed to increased exposure to ultraviolet (UV) radiation through tanning behaviors [2–6]. Concern about UV exposure in this population has prompted bans on tanning beds and sunlamps for individuals under the age of 18, and educational campaigns and interventions that promote the use of sunless tanning products (STPs) as an alternative to UV tanning [7–9]. Adoption of STP use in young female populations has grown steadily, with 25% of girls 12–18 years of age reporting use of over-the-counter STPs and spray tanning [10].

Most over-the-counter STPs contain dihydroxyacetone (DHA), the most popular and longest-used FDA-approved browning agent [11]. DHA is a keto sugar that undergoes a Maillard-like reaction with the amines in the skin's surface to produce a brown color within 2 to 4 hours of application [12]. Darker skin is achieved by increasing the percentage of DHA in the STP (typically between 2–15%) and by increasing the frequency of application [12].

There has been a prevailing attitude that DHA is “safe,” largely because it is a common by-product in carbohydrate metabolism and because its absorption has been thought to be limited to the outermost dead layer of skin, the stratum corneum [13]. Several studies have also reported that DHA has a Sun Protective Factor (SPF) of 1–3 within the first 24 hours of exposure [14–16], although a contradictory report indicated an increase in free radicals when DHA is co-exposed with UV [17]. These conflicting reports and the increasing popularity of spray tanning have raised questions about the safety of DHA, especially since internal exposure through inhalation and absorption through mucous membranes are now a risk through aerosolized application [16, 18].

Reports indicate that up to 20% of an applied 5% dose of DHA penetrates into and remains within the underlying viable tissue (epidermis and dermis) [16, 19, 20]. While there is still some disagreement about DHA penetrating into the viable skin, FDA scientists have reported that up to 11% of applied DHA penetrates into viable tissues with 0.5% penetrating into the bloodstream [19, 21]. This penetration into viable skin, coupled with the exogenous exposure to other tissues through aerosolized application and/or inhalation requires further investigation, yet there is a lack of existing literature examining exogenous exposures to DHA.

One report has examined consumer relevant exposures of DHA to the immortalized keratinocyte cell line, HaCaT, finding that doses higher than 5 mM induced apoptosis through G2/M cell cycle arrest and DNA damage [22]. Given the observed effects in immortalized keratinocytes, we sought to first evaluate the effects of DHA exposure on a melanoma cell line.

The human A375P melanoma cell line was selected, in part, because of its consistency of morphology and hardiness in culture, but also due to its proximity to keratinocytes and relevance as a tissue component which can invade throughout the epidermis and dermis to be vulnerable to topical treatments. This cell line is a commonly used melanoma model derived from sequential selection of metastatic tumor formation, and is characterized by low or poor metastatic potential and a BRAF V600E mutation [23–25]. Using this model, we found that

consumer-relevant doses of DHA induced a transient increase in oxidative stress, and induced a G2/M cell cycle checkpoint that resulted in the cells becoming partially senescent, before finally entering apoptosis. A significant induction of apoptotic cell death was observed 72 h after exposure, highlighting DHA's cytotoxic effects in a skin component and demonstrating that further investigation of tissue specific cytotoxic effects of DHA is needed.

2. Materials and Methods

2.1 Chemicals

Dihydroxyacetone (CAS 26776-70-5) was purchased from Sigma-Aldrich.

2.2 Cell culture

A375P human melanoma cells were purchased from ATCC and grown at 37°C in a 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with glutamine, 10% fetal bovine serum (FBS; Atlanta Biologicals), and 1% sodium pyruvate (Gibco). Cell passages were between 4 and 10 for all reported experiments. Cells were routinely tested for mycoplasma using Lonza MycoAlert kit and found to be free of mycoplasma contamination.

2.3 Cytotoxicity studies

Cytotoxicity was determined by colony formation assay. A375P cells were plated in 6-well dishes at a density of 500 cells per well and allowed to adhere overnight. Cells were then treated in triplicate with fresh medium containing a range of DHA doses for 5–6 days. Cells were then washed twice with phosphate buffered saline (PBS), fixed in methanol for 10 min at –20°C, and stained overnight with crystal violet (Sigma Aldrich). Plates were washed with H₂O, and, once dry, colonies were hand counted over a 1 cm² area, and then these values were extrapolated to entire area of dish. Three biological replicates were performed and images are representative.

2.4 Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) were measured by staining cells with CM-H₂DCFDA (Life Technologies) and measuring the mean fluorescent intensity. A375P were seeded in black 96 well clear bottom dishes at 5,000 cells/well and treated with 5 mM DHA for up to 24 h or with 250 µM tert-butyl hydrogen peroxide (TBHP) for 1 h as a positive control for ROS induction. A 1 mM stock of CM-H₂DCFDA in anhydrous DMSO (Sigma-Aldrich) was prepared and diluted to 2.5 µM in PBS. The CM-H₂DCFDA was added to the cells at the indicated time and allowed to react for 15 min. Cells were then gently washed in PBS. Fluorescent intensity was measured by exciting at 495 nm and recording the emission at 525 nm using a Tecan Infinite M1000 plate reader. Three biological replicates were performed and the mean fluorescent intensity ± standard error of the mean (SEM) is reported for each time point.

2.5 Immunofluorescence

Strand break formation was assessed by immunofluorescent staining of γ H2AX. A375P cells were plated in 4 chamber glass slides (Nunc LabTek II, ThermoFisher) at densities of and 3×10^4 cells per chamber. Cells were allowed to adhere overnight, and then the next day cells were dosed with fresh medium containing 5 mM DHA for 24, 48, and 72 h. As a positive strand break control, cells were dosed with 250 nM camptothecin (CPT) for 24 h.

Samples were then fixed in 3.7% formaldehyde (ThermoFisher) in PBS for 10 min at room temperature (RT) and washed 3 times with PBS. Cells were permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at RT, then washed 3 times PBS and blocked in PBS with 1% BSA for 30 min at RT. The primary monoclonal antibody for γ H2AX (Millipore 05–363) was incubated at 1:750 in blocking buffer for 1 h at RT. Samples were then washed 3 times with PBS and incubated with Alexa 488 goat anti-mouse secondary antibodies (A11029, ThermoFisher) at 1:2000 in blocking buffer for 1 h at RT, protected from light. Finally, cells were washed 3 $\times$ with PBS and NucBlue® fixed cell stain (DAPI, ThermoFisher) was applied following the manufacturer's instructions.

Immunostained cells were imaged using a 20 $\times$ C-Apochromat (NA 0.75) air immersion objective on the Nikon A1rsi confocal microscope. Multichannel images were collected using 405 and 488 nm laser lines to acquire the two fluorescent channels at 1024 $\times$ 1024 resolution. The NucBlue® signal was used to delineate a nuclear region of interest, and the intensity of the Alexa 488 γ H2AX signal was measured within this area. The mean fluorescent nuclear intensity of γ H2AX was determined for a minimum of 100 cells per condition, over two independent replicates. Values are reported as mean fluorescence intensity $\pm$ SEM.

2.6 Flow cytometry

For cell cycle analysis, A375P cells were seeded in 100-mm dishes at 0.1×10^6 per dish. Cells were allowed to attach overnight and were either left untreated or treated with 5 mM DHA for 24 h. At the end of dosing, cells were washed with PBS, detached with 0.05% trypsin (Gibco), and resuspended in PBS. Cells were pelleted, resuspended in 100 μ l cold PBS, and fixed by slowly dropping into 70% ethanol, then stored overnight at 4°C. Cells were pelleted, washed in PBS, and pelleted again. The cell pellets were then resuspended in 1 ml PBS containing 40 μ g/ml of propidium iodide (PI) and 50 μ l of RNase A stock solution (10 mg/ml). After a 30 min incubation at 37°C, samples were analyzed on a BD FACSCanto II (BD Biosciences) using FACSDiva software.

For evaluation of cell death, the annexin V-FITC Apoptosis Detection kit (Leinco) was used following the manufacturer's instruction. Briefly, cells were seeded in 100 mm dishes at 0.1×10^6 per dish. Cells were allowed to attach overnight and were left untreated, treated with 5 mM DHA, for 24, 48, and 72 h, or as a positive control, treated with 1 μ M camptothecin for 24 h. At the end of dosing, cell growth medium was collected into 15 ml tubes. Adherent cells were detached with 0.05% trypsin, and collected in previously aspirated supernatant. Cells were pelleted, washed with ice-cold PBS, pelleted again, and then re-suspended in 0.5 ml of binding buffer. 100 μ l of each sample suspension was placed into 5 ml flow tubes for

staining. Samples were then stained with annexin and propidium iodide (PI), incubated in dark at RT for 15 minutes, just prior to analysis 400 µl of PBS was added to samples then ran on a BD FACSCanto II (BD Biosciences) using FACSDiva software.

2.7 Western blotting

A375P cells were seeded in 100 mm dishes at 0.5×10^6 cells/dish and treated when cells were 70% confluent with 5 mM DHA for the time period indicated. At the designated time points, cells were washed twice with ice-cold PBS, scraped, and flash frozen. Pellets were stored overnight at -80°C , then lysed in two volumes of lysis buffer (25 mM β -glycerolphosphate, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, and 0.3% NP-40) plus Halt protease and phosphatase inhibitor (Pierce) and incubated on ice for 30 min. The lysates were centrifuged at $20,800\times g$ for 30 min at 4°C , and the supernatant fraction was removed. Protein concentrations were determined by Bradford Quick Start protein assay (Bio-Rad). 30 µg of protein was separated via 4–20% SDS-PAGE, and transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% skim milk in TBS containing 0.1% Tween 20 (TBST) and then incubated with the following primary antibodies: anti-PARP-1 (1:1,000, #9532), p21(1:1,000, #2947), and Cyclin B1(1:1,000, #12231); Caspase 3 (1:1,000, #9664) from Cell Signaling; and anti-Tubulin (1:10,000, T9026) from Sigma-Aldrich as a loading control.

The blots were washed with TBST, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Azure Biosystems). Bound antibody was detected using enhanced chemiluminescence (Advansta). The membrane was stripped by incubating with buffer containing 62.5 mM Tris-HCl, pH 6.8, 100 mM β -mercaptoethanol, and 1% SDS for 30 min at 50°C , or with Review Western Blot Stripping Buffer as suggested by the manufacturer, then washed twice for 30 min with room temperature TBST.

2.8 Senescence assay

A375P cells were plated in 35 mm fluorodishes (World Precision) at a density of 10,000 cells per dish. Cells were allowed to adhere overnight, and then dosed with fresh medium containing 5 mM DHA for 24, 48, and 72 h. Senescence was detected using Cellular Senescence Assay Kit (Biotium) following manufacturer's instructions. Briefly, after the designated time points, untreated and treated cells were washed twice with PBS and fixed for 5 min. Cells were then washed three times in PBS and stained overnight with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). The staining solution was then removed and cells were washed a final time in PBS, then stored in 70% glycerol until imaged with a Nikon Eclipse wide field microscope. Three biological replicates were performed and images are representative.

2.9 Mitochondrial membrane potential

A375P cells were seeded in black 96 well clear bottom plates at 1,000 cells per well. Cells were allowed to adhere overnight, and then dosed with fresh medium containing 5 mM DHA for 24, 48, and 72 h. Membrane potential was assayed using tetramethylrhodamine ethyl ester (TMRE) Mitochondrial Potential Assay Kit (Biovision). For a negative control, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 20 µM) was dosed into one

well and incubated at 37°C for 20 minutes. All wells were then treated with TMRE (200 nM) and incubated at 37°C for 30 minutes. Wells were then gently washed and filled with assay buffer and the fluorescence intensity was measured on a Tecan Infinite M1000 plate reader using 549 nm excitation and 575 nm emission. To control for fluctuations in cell number the TMRE signal was normalized to nuclear content by staining with NucBlue® live cell stain (ThermoFisher) after TMRE fluorescence was recorded. Nuclear intensity was recorded by exciting at 360 nm and recording fluorescence at 460 nm. Cell count normalized results were then expressed relative to control. A minimum of three biological replicates were performed and the values are expressed as relative fluorescence intensity $\pm$ SEM.

3. Results

3.1 DHA induces cytotoxicity in melanoma cells

To determine if DHA induces cytotoxicity in the melanoma cell line, A375P, we evaluated the inhibition of cell growth by colony formation assay over a range of DHA doses. Colony formation was significantly inhibited at doses higher than 1 mM, with the IC₅₀ and IC₉₀ values determined to be 1.9 mM and 4.9 mM DHA, respectively (Fig. 1).

3.2 DHA exposure increases oxidative stress without inducing cytotoxic DNA damage

The sensitivity of the A375P cells to DHA may be linked to the higher levels of oxidative stress experienced by cancer cells. DHA is a triose sugar, which if absorbed into the cell, could be incorporated into the pentose phosphate pathway altering the balance between dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate and altering cellular levels of NAD(H)/NADP(H) and stimulating the generation of ROS [26].

To evaluate whether DHA exposure generates cytotoxic ROS in the melanoma cells, we examined changes in the levels of intracellular ROS using the fluorescent reporter, CM-H₂DCFDA. A375P cells were exposed to 5 mM DHA for 1, 2, and 24 h and the levels of intracellular ROS examined by measuring fluorescent intensity of the reporter.

DHA exposure induced a transient increase in ROS within 1 h of exposure, but these levels were reduced by 2 h and were below control levels by 24 h (Fig. 2A). This suggests that DHA exposure alters the redox balance in the cell but does not induce cytotoxic DNA damage, which is consistent with the reduced level of ROS generated as compared to TBHP (Fig. 2A). This lack of DNA damage was confirmed by examining the formation of strand breaks using the general strand break marker, γ H2AX. A slight increase in γ H2AX signal was observed 24 h after exposure, but this increase was not significant compared to cytotoxic CPT exposure (Fig. 2B).

To further verify that DNA strand breaks are not induced in the melanoma cells, we also performed an alkaline comet assay, which revealed no significant induction of strand breaks 1, 2, 4, 8, 12, 16 or 24 h after exposure with 5 mM DHA (<2% breaks, data not shown).

3.3 DHA induces G2/M cell cycle arrest within 24 h but onset of apoptosis is delayed

Given the lack of DNA damage induction by DHA in the melanoma cells, we evaluated whether DHA induced cell cycle arrest, which could contribute to the inhibition of cell

growth. Cells were dosed with 5 mM DHA for 24 h, then PI staining was utilized to observe cell cycle changes. The percentage of cells observed in G2/M more than doubled 24 h after exposure to 5 mM DHA (Fig. 3A&B). This was confirmed by increased levels of Cyclin B1 at 24 h and p21 at 24 and 48 h after exposure (Fig. 3C). Cells failed to recover from the induced arrest, as demonstrated by the sustained loss of Cyclin B1 48 and 72 h after exposure (Fig. 3C).

To evaluate whether the induction of cell cycle arrest initiates cell death, we used annexin/PI staining to examine apoptotic and necrotic cell death 24, 48 and 72 h after exposure to 5 mM DHA. No significant induction of apoptosis or necrosis was observed until 72 h after the addition of DHA, when a significant fraction of cells underwent apoptotic cell death (Fig. 4A). This delayed induction of cell death was also observed through appearance of cleaved PARP-1 and cleaved Caspase 3 at 72 h, though a small fraction of cleaved PARP-1 is observed 48 h after the addition of DHA (Fig. 4B).

With this in mind, we re-examined γ H2AX signaling at 48 and 72 h after DHA exposure and observed that γ H2AX signaling increased significantly at 72 h, consistent with the fragmenting of the DNA during apoptosis (Fig. 5). This indicates that sustained cell cycle arrest contributes more significantly to the induction of apoptosis in the melanoma cell line than DHA-induced DNA damage.

3.4 DHA induces a low fraction of senescent cells with 24 and 48 h of exposure

With the observed delay in the induction of apoptosis, we examined morphological changes in the A375P cells exposed to 5 mM DHA and evaluated whether these cells entered senescence prior to cell death. A375P cells were treated with 5 mM DHA for 24, 48, and 72 h, and cellular senescence was evaluated by the presence of β -galactosidase (β -gal). As shown in Fig. 6, significant morphological changes were induced after exposure to DHA, with cytoplasmic volume increasing and nuclei swelling as a function of exposure time. While there was not a dramatic increase in β -gal staining, there was a noticeable bluish tint in cells exposed to DHA for 24 and 48 h, and this signal is lost at 72 h when the cells lose their integrity. This increased portion of senescent cells is consistent with increased p21 protein levels 24 and 48 h after exposure.

3.5 DHA exposure may induce cell stress and apoptosis by altering mitochondrial function

Together, these data suggest DHA exposure induces significant cellular stress, which cannot be overcome, and eventually causes the initiation of cell death. Given the lack of nuclear DNA damage observed by DHA exposure, the mechanism of action by which DHA induces this stress is still unclear. However, since DHA can participate in cellular metabolism and alter the intracellular balance of NAD(H)/NADP(H) and ATP, we examined the effect DHA has on the mitochondrial membrane potential using TMRE. Changes in the fluorescence intensity generated by TMRE provide a qualitative measure of the polarization of the mitochondrial membrane, which is related to the cell's ability to generate ATP by oxidative phosphorylation. Cells were treated with DHA for 24, 48, and 72 h then stained with TMRE and the relative fluorescent intensity was measured. Variations in cell number were

controlled for so the loss in fluorescent signal reflects loss in potential, not decrease in cell number.

An increase in fluorescence, though not significant, was observed 24 h after exposure to DHA, indicating more polarized mitochondria (Fig. 7). The TMRE signal is then reduced 48 and 72 h after exposure, with the 72 h time point showing similar fluorescence intensity to mitochondria that have been decoupled by FCCP.

4. Discussion

DHA is a popular browning agent used in a variety of cosmetics, including sunless tanning lotions and creams and spray tanning. Additionally, DHA is found in cells as DHAP, a common by-product of fructose metabolism [26, 27]. Endogenous increases in DHA and DHAP have been well-studied in diabetes and in cases of triosephosphate isomerase (TIM or TPI) deficiency, where increases in three carbon metabolism have been linked to increased protein damage through advanced glycation end products (AGE) [27–29]. DHA itself can act as a glycation agent or can be metabolized into methylglyoxal. AGE protein modifications are responsible for many physiologically detrimental events, most notably in the complications of diabetes mellitus, while also being implicated in the progression of Alzheimer's, as well as in the formation of cataracts [27, 28]. However, the effects of exogenous exposure to DHA are less well understood.

Previous study by Petersen et al. examined the exogenous exposure effects of DHA on the immortalized keratinocyte cell line, HaCaT [22]. In the present study, the A375P melanoma cells demonstrate more sensitivity to DHA, with an IC_{50} of 1.9 mM (Fig. 1), compared to the HaCaT cells [22]. Exposure of the A375P cells to 5 mM for 24 h did induce a similar G2/M cell cycle arrest; however, the induction of DNA damage observed 1 h after exposure in the HaCaT cells by comet assay [22] was not observed in the A375P cells (Fig. 2).

However, a transient increase in ROS was observed within an hour of exposure to DHA; this increase was quickly resolved by the redox environment of the cells (Fig. 2). This indicates that DHA is being absorbed into the cell and likely altering metabolic pathways. We also observed changes in mitochondrial membrane potential as a function of exposure, suggesting that apoptosis in these melanoma cells is likely induced by metabolic pathway changes that increase the production of AGEs. Further work is required to elucidate the mechanism by which DHA is transported into the cell and identify the metabolic products that alter mitochondrial function.

Together, our results demonstrate that DHA exposure induces cell death in the A375P melanoma cells by inducing cell cycle arrest and promoting metabolic changes that induce apoptosis. Unlike the previous report using HaCaT cells, induction of nuclear DNA damage does not appear to contribute to the induction of apoptosis [22]. These findings are in line with the cytotoxicity observed in the HaCaT cells, but it is clear the different cell types respond to exogenous exposure to DHA differently. Absorption of the DHA into cells and its conversion into metabolically active DHAP, likely dictate the degree to which DHA can perturb metabolic pathways and induce oxidative stress. This is consistent with recent work

injecting hyperpolarized [2-¹³ C] DHA into mice, which showed rapid distribution of DHA to the liver and kidneys and metabolism of DHA to DHAP by liver and kidney cells, but showed different conversion rates of DHAP to glyceraldehyde 3-phosphate depending on tissue type [30].

Melanocytes and melanoma cells have the potential to be exposed to DHA throughout the epidermis and dermis, and the bioenergetics of the melanoma make it likely that exogenous DHA will be metabolized [25, 31]. While the transport mechanism by which DHA enters into cells still needs to be determined, it is clear that exogenous DHA can enter cells and alter metabolism in a tissue and cell specific manner [30]. Thus, it is important to evaluate potential exposure effects, noting that there have been no studies examining the effect of DHA exposure on mucous membranes, which may occur during spray tanning [16, 19, 21].

Overall, both our results and those of Petersen et al. support the conclusion that exposing viable cells to exogenous sources of DHA significantly alters their microenvironment and promotes the induction of cell death [22]. While these results do not directly reflect potential biological effects of DHA in the complex skin microenvironment, they raise concerns about exogenous exposures to DHA particularly, via internal exposures from inhalation, absorption into mucous membranes, or through broken skin. More work is required to understand the complex metabolic events induced by exposure to DHA, whether this exposure arises from exogenous sources or endogenous sources at pathologically-elevated levels.

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Abbreviations

β-gal	β-galactosidase
AGE	advanced glycation end products
CAS	chemical abstract service
CPT	camptothecin
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
NA	numerical aperture
PI	propidium iodide

ROS	reactive oxygen species
SEM	standard error of the mean
SPF	sun protection factor
STP	sunless tanning product
TBHP	tert-butyl hydrogen peroxide
TMRE	tetramethylrhodamine ethyl ester
UV	ultraviolet.

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Fig. 1. DHA is cytotoxic to melanoma cells

A375P cells were exposed to a range of DHA doses for 5 days, then cells were fixed and colonies were counted, in triplicate (see Materials and Methods). Graph represents the mean number of colonies per dose $\pm$ SEM. Images are representative of growth inhibition.

Fig. 2. Exposure to DHA increases intracellular ROS and not DNA damage

A. A375P cells were exposed to 5 mM DHA (IC_{90} dose) for 1, 2, and 24 h. Prior to the time endpoint indicated, intracellular ROS indicator, CM- H_2 DCFDA, was added and incubated for 15 min. Increases in fluorescent signal indicate an increase in intracellular ROS. Cells were dosed with oxidizer TBHP as a positive control. Fluorescent intensity was read using a fluorescence plate reader, and the mean fluorescence intensity $\pm$ SEM normalized to control is presented for three independent measurements. **B.** Induction of strand breaks was examined according to the mean fluorescence intensity of strand break marker γ H2AX in the nucleus of cells using fluorescent microscopy. Cells were treated with 5 mM DHA and

stained for γ H2AX. Treatment with 250 nM CPT was used as a positive control. A minimum of 100 cells were analyzed and mean fluorescence intensity of the nucleus $\pm$ SEM is shown.

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A. **B.** **C.**

Fig. 3. DHA exposure induces cell cycle arrest at G2/M

A. The cell cycle of A375P treated with 5 mM DHA for 24 h was determined using PI. **B.** A significant increase in the population of cells residing in G2/M was observed after 24 h. Mean of three independent experiments $\pm$ SEM are shown. **C.** Cell cycle arrest was confirmed by increases in Cyclin B1 24 h after dosing and increases in p21 24 and 48 h after dosing.

Fig. 4. Induction of apoptotic cell death by DHA 72 h after exposure

A. Annexin/PI staining was used to assess the percentage of cells undergoing apoptosis and necrosis 24, 48, and 72 h after exposure to 5 mM DHA. Apoptotic cell death did not occur until 72 h after exposure. Mean of three independent experiments $\pm$ SEM are shown. **B.** Immunoblot for apoptotic markers, cleaved PARP-1 and cleaved Caspase 3, also showed a delay in the induction of apoptosis. Tubulin was used as a loading control.

Fig. 5. Low levels of nuclear DNA damage occur after extended exposure to DHA

A. Strand breaks analysis was extended to 48 and 72 h after exposure to 5 mM DHA. The mean fluorescence intensity of the strand break marker, γ H2AX in the nucleus of cells, was examined using fluorescent microscopy. Images for untreated and DHA treated cells are shown. Images are representative with a scale bar of 10 μ m. **B.** A minimum of 100 cells were analyzed for each treatment condition, and mean fluorescence intensity of the nucleus $\pm$ SEM is shown.

Fig. 6. Changes in cellular morphology accompanied by increase in cellular senescence is observed after DHA exposure

Bright field color images of untreated and DHA treated cells were taken 24, 48, and 72 after exposure to DHA. Levels of cellular senescence were examined by staining with β -gal. Images are representative of three independent experiments. Scale bar is 20 μ m.

Fig. 7. DHA induces changes in mitochondrial membrane potential

Mitochondrial membrane potential was assessed by measuring the mean fluorescence intensity of TMRE in untreated and treated cells. FCCP uncouples the membrane potential causing a loss in fluorescence signal and serves as control. Graph shows the mean fluorescence intensity of three independent experiments normalized for cell number variations (see Material and Methods) $\pm$ the SEM.