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Original article

Ascorbic acid, but not dehydroascorbic acid increases intracellular vitamin C content to decrease Hypoxia Inducible Factor -1 alpha activity and reduce malignant potential in human melanoma

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ABSTRACT

Introduction: Accumulation of hypoxia inducible factor-1 alpha (HIF-1α) in malignant tissue is known to contribute to oncogenic progression and is inversely associated with patient survival. Ascorbic acid (AA) depletion in malignant tissue may contribute to aberrant normoxic activity of HIF-1α. While AA supplementation has been shown to attenuate HIF-1α function in malignant melanoma, the use of dehydroascorbic acid (DHA) as a therapeutic means to increase intracellular AA and modulate HIF-1α function is yet to be evaluated. Here we compared the ability of AA and DHA to increase intracellular vitamin C content and decrease the malignant potential of human melanoma by reducing the activity of HIF-1α.

Methods: HIF-1α protein accumulation was evaluated by western blot and transcriptional activity was evaluated by reporter gene assay using a HIF-1 HRE-luciferase plasmid. Protein expressions and subcellular localizations of vitamin C transporters were evaluated by western blot and confocal imaging. Intracellular vitamin C content following AA, ascorbate 2-phosphate (A2P), or DHA supplementation was determined using a vitamin C assay. Malignant potential was accessed using a 3D spheroid Matrigel invasion assay. Data was analyzed by One or Two-way ANOVA with Tukey's multiple comparisons test as appropriate with $p < 0.05$ considered significant.

Results: Melanoma cells expressed both sodium dependent vitamin C (SVCT) and glucose (GLUT) transporters for AA and DHA transport respectively, however advanced melanomas responded favorably to AA, but not DHA. Physiological glucose conditions significantly impaired intracellular vitamin C accumulation following DHA treatment. Consequently, A2P and AA, but not DHA treated cells demonstrated lower HIF-1α protein expression and activity, and reduced malignant potential. The ability of AA to regulate HIF-1α was dependent on SVCT2 function and SVCT2 was not significantly inhibited at pH representative of the tumor microenvironment.

Conclusions: The use of ascorbic acid as an adjuvant cancer therapy remains under investigated. While AA and A2P were capable of modulating HIF-1α protein accumulation/activity, DHA supplementation resulted in minimal intracellular vitamin C activity with decreased ability to inhibit HIF-1α activity and malignant potential in advanced melanoma. Restoring AA dependent regulation of HIF-1α in malignant cells may prove beneficial in reducing chemotherapy resistance and improving treatment outcomes.

1. Introduction

The incidence of melanoma, a malignancy derived from pigment producing melanocytes, has continually risen over the past 30 years and accounts for 75% of skin cancer deaths. In 2016 an estimated 76,000 new cases of skin melanoma will be diagnosed in the United States, contributing to over 10,000 fatalities by year's end [1]. Most cutaneous melanomas can be readily cured by

Abbreviations: BRAF, Murine sarcoma viral (v-raf) oncogene homolog B1; HIF-1α, Hypoxia inducible factor-1 alpha; PHD, prolyl hydroxylase; FIH, Factor Inhibiting HIF; AA, Ascorbic acid; A2P, Ascorbate 2-phosphate; DHA, Dehydroascorbic acid; EDHB, Ethyl 3, 4-dihydroxbenozoate; SVCT, Sodium dependent vitamin C transporter; GLUT, Glucose transporter; NAC, N-acetyl cysteine; ChCl, Choline chloride.

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surgical excision [2,3], however once disseminated, metastatic melanoma is highly aggressive and difficult to treat. Five year survival of melanoma patients declines from 98 to 17% following metastasis to a distant site [1]. Even though clinical responses have seen some improvement through the use of immunotherapy, poor patient prognosis, attributed to chemotherapy resistance, highlights the need for alternative or adjuvant treatment options to improve survival in melanoma patients.

Melanoma oncogenesis is predominantly driven by the acquisition of BRAF mutations; 50% of all melanomas contain either a V600E or V600D BRAF mutant [4]. This mutation results in the constitutive activation of signaling pathways leading to unchecked cell proliferation, invasion, and metastasis. Constitutive BRAF activation is also known to contribute to elevated gene expression of hypoxia inducible factor-1 alpha (HIF-1α) [5], the oxygen responsive subunit of the HIF-1 transcription factor. HIF-1α activity in malignant tissue contributes to increased expression of proteins that drive melanoma cell motility and invasion [6]. Elevated expression of HIF-1α protein is widespread in malignant tissue, including melanoma [7], and has been linked to poor patient outcomes in a variety of cancers (reviewed in [8]). Ascorbic acid (AA; reduced vitamin C) is an essential cofactor for Fe II/2-oxoglutarate dioxygenase enzymes including the prolyl hydroxylase (PHD1-3) and factor-inhibiting HIF (FIH) hydroxylase enzymes that regulate HIF-1α protein stability and transcriptional activity respectively [9,10]. Plasma AA concentration in healthy individuals is typically between 40 and 80 μM [11]. Interestingly, cancer patients, including those with melanoma [12], have been observed to have below normal levels of plasma AA [13–16]. Likewise, tumor tissue has been found to contain decreased intracellular AA compared to paired non-transformed tissue from the same patient [17]. The degree of AA deficiency in malignant tissue also correlates with increased tissue accumulation of HIF-1α protein and with tumor stage [17,18], suggesting that inadequate intracellular AA may contribute to the development or progression of a malignant phenotype. The majority of current studies investigating AA as an anti-cancer therapy utilize I.V. administered mega or high dose (>1 mM) AA supplementation to induce cytotoxic cell death, however the potential benefit and use of AA supplementation at physiological concentrations to restore or support its cofactor functions in malignant cells has remained largely unexplored and uncharacterized. Recently, we reported that supplementation of human metastatic melanoma cells with physiological concentrations (10–100 μM) of ascorbic acid inhibits both normoxic and hypoxia-mimetic induced protein accumulation and transcriptional activity of HIF-1α, and reduces the malignant potential of these cells [19,20], emphasizing the functional importance of physiological levels of AA in malignant cells. I*n vivo* studies using Gulo −/− mice (a model of human AA dependency) inoculated with murine melanoma cells demonstrate that AA supplementation can decrease tumor accumulation of HIF-1α [21] and decrease tumor volume [21–23]. Furthermore, tumor ascorbate levels inversely correlated to the expression of HIF-1 target genes [21], providing further support for initiating the use of AA as an adjuvant cancer therapy.

Dietary vitamin C[1] is comprised of both reduced AA and the fully oxidized form, dehydroascorbic acid (DHA). The ability of AA and DHA to provide equivalent intracellular vitamin C activity has been controversial for decades with conflicting reports on the ability of DHA intervention to prevent or treat scurvy in animal and human subjects [24–27]. Recently, McCarty [28] made the speculation that DHA would be a more effective cancer therapy than AA. The rationale for this idea was that advanced malignancies, particularly those with elevated HIF activity, often overexpress glucose transporters (GLUTs), a common observation in Warburg metabolism [29]. Interestingly, DHA uptake is facilitated by GLUTs, therefore elevated GLUT1 expression, which is a known HIF-1 target gene, would support increased DHA entry into malignant cells [28]. DHA itself does not have any biological activity or act as an enzyme cofactor, however, once transported into the cell it is reduced to AA in the cytosol, providing the functional form of vitamin C (Fig. 1) [11]. To our knowledge, the capacity of using DHA rather than AA, as an effective clinical source for increasing intracellular AA levels has not been evaluated in malignant cells.

There are several physiological factors that may impair the delivery of adequate vitamin C to melanoma cells via AA or DHA supplementation. Some of these include the expression and subcellular localization of sodium dependent vitamin C transporters (SVCT1 & 2) and glucose transporters (GLUTs), which transport either AA or DHA respectively. DHA also competes with glucose for entry into the cell through the GLUT transporters and is known to be unstable at physiological pH [30]. Therefore, the effect of glucose competition on DHA uptake, poses a relevant concern for the use of DHA in the clinical setting as a means to promote intracellular vitamin C accumulation and warrants evaluation. The objective of this study was to compare the suitability of AA vs. DHA as a potential adjuvant cancer therapy to reduce the malignant potential of melanoma cells by increasing intracellular vitamin C content and subsequently restoring regulation of aberrant normoxic HIF-1α protein accumulation and activity in human melanoma cells.

2. Methods

2.1. Cell culture and reagents

WM3211, SbCl2, WM3248, WM1366, WM239A, and WM9 melanoma cell lines were a generous gift from Meenhard Herlyn's lab at the Wistar Institute (University of Pennsylvania). Human Epidermal Melanocytes, neonatal, lightly pigmented (HEMnLP) were purchased from Life Technologies. All cells were cultured in a humidified incubator with 5% CO_2/95% air at 37 °C. SbCl2 cells were cultured in MCDB 153 media (Sigma) supplemented with 2% fetal bovine serum (FBS), 5 μg/mL insulin (Sigma), 1.68 mM $CaCl_2$, and 1% penicillin/streptomycin. WM3211 cells were cultured similarly expect for 5% FBS without $CaCl_2$. WM3248, WM1366, WM239A, and WM9 cells were cultured in standard RPMI 1640 media (Gibco; 1 g/L glucose as indicated in text) supplemented with 10% FBS and 1% penicillin/streptomycin. HEMnLP cells were cultured in Medium 254 (ThermoFisher) supplemented with Human Melanocyte Growth Supplement (HMGS; ThermoFisher) and 1% gentamicin/amphotericin B. L-Ascorbic Acid (AA), Dehydroascorbic acid (DHA), L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P), Ethyl 3, 4-dihydroxbenozoate (EDHB), and Cobalt Chloride ($CoCl_2$) were purchased from Sigma. Glucose Transporter Inhibitor III; STF-31 was purchased from EMD Millipore.

[1] Typically, it is convention to use the terms vitamin C and ascorbic acid (AA) interchangeably when discussing human physiology. However, in this manuscript we are evaluating two distinct forms of vitamin C, reduced vitamin C (i.e. AA) and oxidized vitamin C, dehydroascorbic acid (DHA). In the present study the term vitamin C is used to describe both forms of ascorbate (AA or DHA), total ascorbate (the summation of AA and DHA), or when the form of vitamin C cannot be distinguished because of assay limitations, thus the terms vitamin C and AA are not necessarily interchangeable.

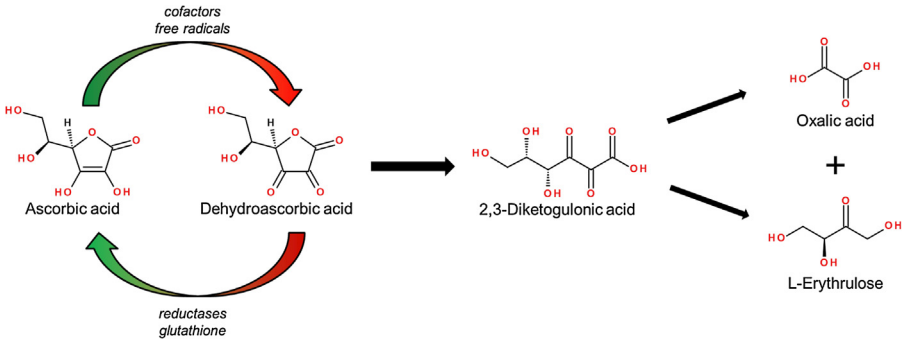

Fig. 1. Major degradation products of ascorbic acid. Ascorbic acid (AA) becomes fully oxidized to dehydroascorbic acid (DHA) following the donation of electrons to either enzyme cofactors or free radicals. Intracellular DHA can subsequently be reduced and recycled to AA by multiple DHA reductases or glutathione, facilitating comparatively higher levels of AA in tissues and plasma. Alternatively, impaired reductase or glutathione activity promotes the spontaneous and irreversible degradation of DHA to 2,3-diketogulonic acid (2,3-DKG) resulting in the loss of vitamin C. Cleavage between the second and third carbon atoms of 2,3-DKG causes the formation of L-erythrulose and oxalic acid, the latter being a primary metabolite of AA.

2.2. Western blots

Membrane protein enrichment was prepared by trypsinizing and resuspending cells in a non-detergent buffer (250 mM sucrose, 10 mM Tris, 1 mM EDTA; pH 7.5) and passing through a 26G needle. Homogenate was centrifuged for 1 h at 16000 x *g* (4°). The resulting pellet was lysed (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.75% Trition X-100, 0.25% SDS; pH 7.5) for 1 h on ice and centrifuged for 10 min at 16000 x *g* (4°) to collect the supernatant. Nuclear protein extracts were isolated using the NePER Nuclear and Cytoplasmic Extraction Kit (Pierce) following the manufacturers protocol. All buffers were supplemented with Complete Mini Protease Inhibitor Cocktail (Roche). Protein lysates were separated by SDS-PAGE on 4–20% MP-TGX precast polyacrylamide gels (BioRad) and transferred to nitrocellulose using BioRad MINIProtean3 system. Membranes were immunoblotted with antibodies against SVCT2 (1:500; Santa Cruz), GLUT1 (1:5000; abcam), HIF-1α (1 μL/mL; R&D Systems). Equal lane loading was accessed by visualization of total protein using MemCode™ Reversible Protein Stain Kit (Thermo Scientific) or probing for βactin (1:10000; 1.5 h at RT; Sigma). Immunoblots were visualized using an enhanced chemiluminescence detection kit (ECL Prime; GE Healthcare) and imaged on a PhotoDyne Imaging system (PhotoDyne Technologies). All western blot images are representative of 3 different experiments or biological repeats as indicated in figure legend with similar results.

2.3. Luciferase reporter assay

Assay was conducted as previously described [19]. Briefly, 2.0×10^5 cells were seeded in 60 mm culture dishes 24 h prior to transfection. Cells were incubated overnight with transfection mixture containing 1.5 μg HIF-1 pTLLuc(5′-3′: GTGACTACGTGCTGCCTAGGTGACTACGTGCTGCCTAGGTGACTACGT GCTGCCTAGGTGACTACGTGCTGCCTAG; Affymetrix, LR0128) and 0.1 μg pSV-β-galactosidase plasmids (Clontech) and eXtreme Gene 9 transfection reagent (Roche) following the manufacturers protocol in OptiMEM Reduced Serum media (ThermoFisher). Transfection media was replaced with standard RPMI the following day, and cells treated as described. Luciferase activity was measured using the Luciferase Assay Kit (Promega) and normalized against β-galactosidase activity that was measured using the β-galactosidase Assay Kit (Promega). Luciferase and β-gal were measured separately on a SpectraMax L and SpectraMax M2e plate reader (Molecular Devices) respectively.

2.4. Reverse transcription (RT) and PCR

Total RNA was isolated from WM9 cells using an RNeasy Mini Kit (Qiagen) following the manufacturers protocol. mRNA quality and quantity was assessed spectrophotometrically using a NanoDrop 200 UV/Vis spectrophotometer. cDNA was synthesized from total mRNA using the Advantage RT-for-PCR kit (Clonetech Laboratories) following the manufacturers protocol. PCR analysis of target sequences were generated using the Advantage cDNA kit (Clonetech Laboratories) with the following PCR primers; GLUT1: 5′ – TCATCAACCGCAACGAGGAG – 3′(Forward), 5′ – CAAAGATGGCCACGATGCTC – 3′ (Reverse); BNIP3: 5′ – TGGACGGAGTAGCTCCAAGA – 3′ (Forward), 5′ – TCATGACGCTCGTGTTCCTC – 3′ (Reverse); βactin: 5′ – GCTGCTCGTCGACAACGGCTC – 3′ (Forward), 5′ – CAAACATGATCTGGGTCATCTTCTC – 3′ (Reverse). PCR condition: 95 °C 1 min; 25 cycles of 95 °C 30 s, 57 °C 1 min, 72 °C 2 min; 72 °C 5 min. PCR products were separated on 1% agarose gel containing GelRed™ nucleic acid stain (Biotium) and visualized by UV using a PhotoDyne Imaging system (PhotoDyne Technologies). cDNA and PCR products were generated using MJ Mini Personal Thermal Cycler (Biorad).

2.5. Confocal imaging

Melanoma cells were grown under standard conditions on chambered tissue culture slides (BD Falcon™). To visualize the plasma membrane, cells were incubated with wheat germ agglutinin (WGA) conjugated with Alexa Fluor® 594 (Invitrogen) for 10 min (2 μg/mL) before being fixed using 3.7% formaldehyde (10 min). Cells were washed 3 X with PBS-T then permeabilized using PBS-T containing 0.1% Trition X-100 (5 min) and blocked with 5% BSA for 1 h at RT. Fixed cells were incubated overnight at 4° with SVCT2 (1:50; Santa Cruz) or GLUT1 (1:250; abcam) antibodies. Following PBS-T wash, cells were incubated with donkey anti-goat IgG-FITC (1:100; Santa Cruz) or donkey anti-rabbit IgG H&L (Alexa Fluor® 488) (1:500; abcam) respectively for 2 h. Slides were mounted using VECTASHIELD Hard Set mounting medium (Vector Laboratories). Images were acquired at The Marshall University Molecular and Biological Imaging Center using a Leica SP5 TCSII (Leica Microsystems). A 63 x glycerol lens (NA 1.3) was used to collect a series of images in z-axis with focus steps of 0.5 μm. Total thickness of z-series (approximation of cell monolayer thickness) ranged from 2.5–4 μm. Z-series are shown here as single image maximum intensity projections. Image format is 2048×2048 pixels representing a square area of 156 μm x 156 μm (each pixel 76 nm x 76 nm). Emissions from Alexa Fluor® 594

(plasma membrane) were collected by photo-multiplier tube (PMT) through a slit passing light 575–650 nm with excitation from 561 nm solid state laser. Emissions from FITC and Alexa Fluor® (SVCT2 and GLUT1) were collected by PMT through a slit passing light 500–550 nm with excitation from 496 nm line of argon gas laser. Images were processed using ImageJ v 1.50 g.

2.6. Intracellular vitamin C assay

Intracellular vitamin C (as AA and/or DHA) content was determined using a protocol adapted from Vislisel et al. [31]. In brief, cells were seeded similarly into 10 cm plates to ensure equivalent confluence at time of collection. At the time of vitamin C supplementation, cells were incubated in transport buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4) unless otherwise indicated in text, or in RPMI. After incubation, cells were trypsinized and cell pellets lysed on ice in methanol water (60 MeOH:40H_2O ratio) for 10 min before centrifugation at 12,000 x *g* for 10 min at 4°. Supernatant along with freshly prepared ascorbic acid standards (100 μL) were aliquoted into a 96-well plate with the addition of 100 μL 4-hydroxy-2,2,6,6-tetramethyl-piperidinooxy (TEMPOL; Acro Organics; 2.32 mM) and incubated for 10 min at RT. Immediately, 42 μL of o-phenylenediamine dihydrochloride (OPDA; Thermo Scientific; 5.5 mM) were added to samples and incubated 10 min at RT in the dark before endpoint fluorescence read on a SpectraMax M2e plate reader (Molecular Devices) at Ex 345/Em 425 nM.

2.7. Tumor spheroid formation and 3D invasion assays

Multicellular tumor spheroid formation and 3D invasion assays were adapted from Vinci et al. [32]. Briefly, WM9 cells were seeded as a monolayer and pretreated with or without A2P or DHA (100 μM) for 4 days. Thereafter, cells were detached and 1×10^4 cells were seeded into round bottom 96-well plates coated with agarose and incubated for 48 h under standard culture conditions to encourage spheroid formation. Spheroids were then suspended in basement membrane matrigel matrix (final conc. 3.5 mg/mL; BD Biosciences) and overlaid with RPMI (1 g/L glucose) with or without A2P or DHA (100 μM) for up to 96 h with images collected every 24 h using a Zeiss primovert microscope (Zeiss) to measure the diameter of the spheroid including the migrating cells. Cells and spheroids were cultured in RPMI media (1 g/L glucose) for the duration of the experiment (pre and post matrigel). Images were analyzed using ImageJ v 1.50 g.

2.8. Statistical analysis

Data was analyzed using GraphPad Prism 6 software (version 6.0f; Graphpad Software, Inc.). Statistical significance was determined by One-way or Two-way ANOVA followed by Tukey multiple comparison tests as appropriate. Three individual trials were performed for each experiment and data represented as ± SEM. $p < 0.05$ was considered statistically significant. Specific p values are indicated in figure legends.

3. Results

3.1. A2P and AA inhibit hypoxia-mimetic induced HIF-1α protein stability and transcriptional activity more effectively than DHA in human melanoma cells

In our initial experiment we wanted to compare the ability of ascorbate 2-phosphate (A2P) vs. DHA to inhibit the hypoxia-mimetic ethyl 3, 4-dihydroxbenozoate (EDHB) induced stability of HIF-1α protein in WM9 and WM239A metastatic melanoma cells

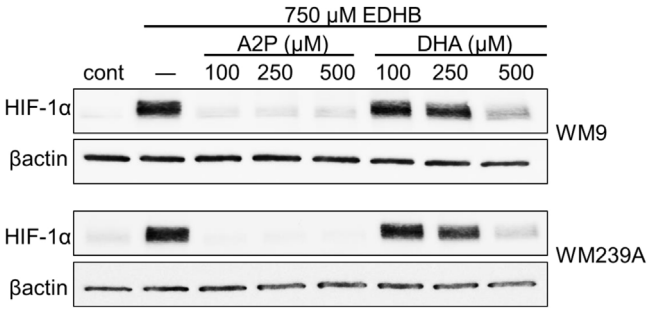

Fig. 2. Comparison of A2P and DHA to reduce HIF-1α protein accumulation in metastatic melanoma. WM9 and WM239A metastatic melanoma cells were incubated in RPMI (1 g/L glucose) containing EDHB with or without increasing concentrations (100–500 μM) of A2P or DHA as indicated for 24 h. Our data demonstrates A2P supplementation is more effective than DHA in decreasing HIF-1α stability and accumulation. Blots are representative of 3 experiments with similar results.

following a 24 h treatment. EDHB is a specific inhibitor of PHDs, binding the ascorbate and 2-oxoglutarate sites of the enzyme [33]. AA is known to have a short half-life in culture media due to rapid oxidation [34,35] therefore A2P, which is an oxidation resistant analog of AA, was utilized as our source for vitamin C in longer time point studies. Similar to our previous report [19], we demonstrated that all concentrations of A2P (100–500 μM) cause nearly complete abolishment of EDHB induced HIF-1α protein at 24 h, while only by the highest concentration of DHA (500 μM) was capable of reducing EDHB induced HIF-1α (Fig. 2).

Since AA also functions as a cofactor for FIH to regulate the transcriptional activity of HIF-1α, we wanted to compare the ability of various vitamin C compounds to decrease the transcriptional activity of HIF-1α in melanoma cell. Therefore, we transiently transfected normal human melanocytes (HEMnLP) and a panel of 5 established human melanoma cell lines with a HIF-1 HRE-luciferase reporter plasmid prior to being stimulated with the hypoxia mimetic cobalt chloride ($CoCl_2$) with or without 100 μM A2P, AA, or DHA for 24 h. As expected, HIF-1α activity was not inducible in HEMnLP cells (Fig. 3a). Interestingly, all compounds demonstrated a similar ability to decrease transcriptional activity in WM3211 and SbCl2 cells (Fig. 3b-c) which demonstrated only modest induction of HIF-1α activity. Conversely, DHA was unable to decrease HIF-1α activity in WM3248, WM1366, and WM9 cells while AA, in particularly A2P, significantly suppressed $CoCl_2$ induced HIF-1α transcriptional activity (Fig. 3d–f). Concurrent with Fig. 3f, we also found that incubation of WM9 cells with 100 μM A2P for 24 h was more effective than DHA in inhibiting HIF-1α induced mRNA expression of GLUT1 and BNIP3, classic HIF-1 target genes (Fig. 4a–c).

3.2. Human melanoma cells express SVCT2 and GLUT1 transporters necessary for AA and DHA transport

The inability of DHA supplementation to reduce HIF-1α transcriptional activity among the melanoma cell lines may be due to differences in the expression and subcellular localization of vitamin C transporters in the different cell lines. Sodium dependent vitamin C transporters (SVCT1 & 2) facilitate cellular transport of reduced vitamin C (ascorbic acid; AA). The primary function of SVCT1 is dietary absorption and reabsorption, therefore expression of this isoform is typically confined to intestinal enterocytes and renal tubule cells, as well as liver and lung tissue, providing adequate circulating AA in the plasma [36–38]. SVCT2 expression is wide spread throughout most tissues and organ systems, facilitating uptake and intracellular accumulation of AA from the plasma [36,37]. The oxidized form of AA (DHA) is

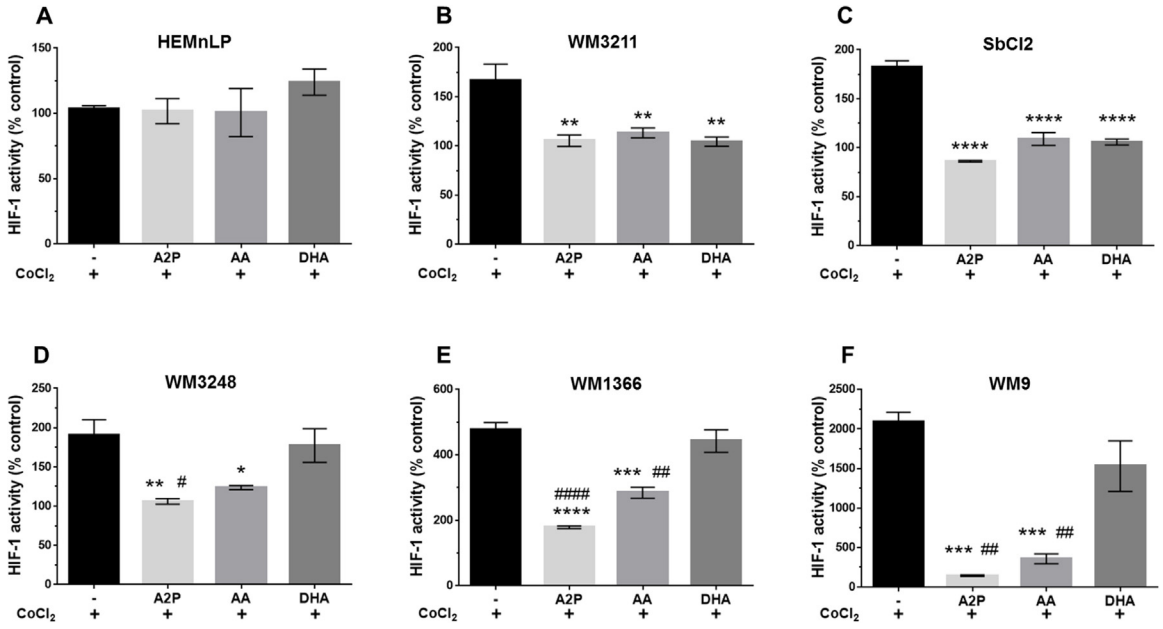

Fig. 3. Survey of HIF-1 transcriptional activity following supplementation of vitamin C compounds in melanoma cells. Normal melanocytes (HEMnLP) and a panel of melanoma cells were transiently transfected with an HIF-1 HRE-luciferase reporter vector. Following overnight transfection, cells were treated with the hypoxia-mimetic $CoCl_2$ (100 μM) with or without 100 μM A2P, AA, or DHA for 24 h. Our results demonstrate A) HEMnLP cells do not have inducible HIF-1 activity, while B) WM3211 and C) $SbCl_2$ cells are responsive to all vitamin C compounds. D) WM3248, E) WM1366, and F) WM9 cells were all responsive to A2P and AA, but not DHA. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. * denotes statistical significance from $CoCl_2$ induced cells $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. # denotes statistical significance from DHA treated cells $p < 0.05$; ## $p < 0.01$; #### $p < 0.0001$. Data was normalized as percent of control (control = 100%; not shown).

transported by glucose transporters (GLUTs), primarily GLUT1 & 3 [11,37,39]. Following uptake, DHA can be reduced back to AA enzymatically by DHA reductases or, to a lesser extent, non-enzymatically by glutathione (Fig. 1) [30]. To evaluate the potential contribution of differential transporter expression and localization on aberrant HIF-1α activity and subsequent ability of AA and DHA to modulate HIF-1α stability and activity, we first evaluated the protein expression of SVCT2 and GLUT1 transporters in HEMnLP and melanoma cells by western blot. All cell lines were found to express both SVCT2 and GLUT1 proteins (Fig. 5a). Subsequent PCR analyses confirmed that SVCT2 and GLUT1 are the primary isoforms expressed in these cell lines (data not shown).

To effectively transport extracellular AA or DHA into the cell, SVCT2 and GLUT1 transporters must be localized to the plasma membrane. Therefore, we examined the subcellular localization of SVCT2 and GLUT1 in WM3211 and WM9 melanoma cells. These cells were chosen for this experiment because they possess similar protein expression of GLUT1 (Fig. 5a), yet were either responsive (WM3211) or non-responsive (WM9) to DHA. Our results demonstrate that the majority of SVCT2 protein appears to be

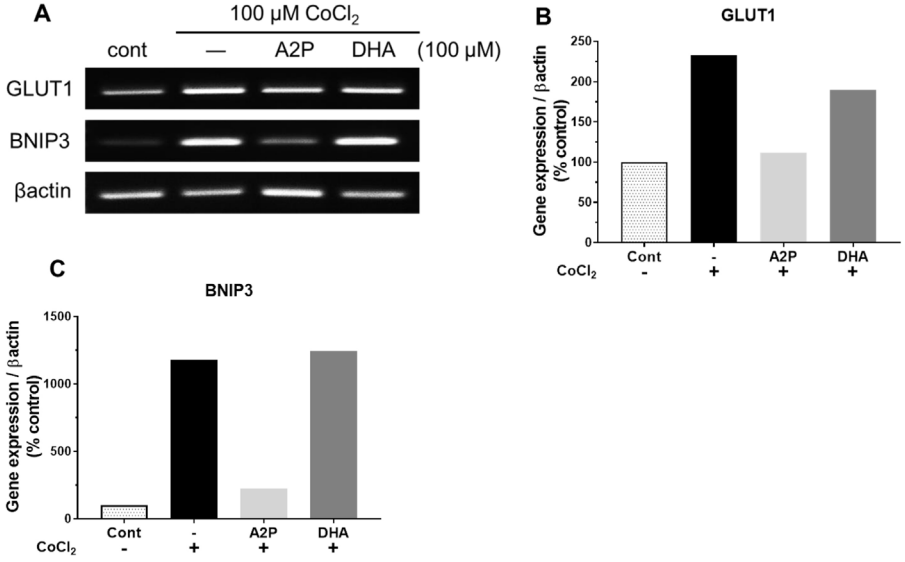

Fig. 4. Regulation of HIF-1 target gene expression by A2P or DHA. To evaluate the impact of vitamin C compounds on HIF-1 target gene mRNA expression, HIF-1 transcriptional activity was stimulated in WM9 cells using $CoCl_2$ (100 μM) and co-treated with 100 μM A2P or DHA for 24 h before cDNA was generated from isolated total RNA. A) Traditional PCR using primers for classic HIF-1 target genes (GLUT1 and BNIP3) reveals A2P supplementation more effectively decreases HIF-1 target gene mRNA expression compared to DHA. B, C) Densitometry of GLUT1 and BNIP3 mRNA expression. Data is representative of multiple individual experiments with similar results.

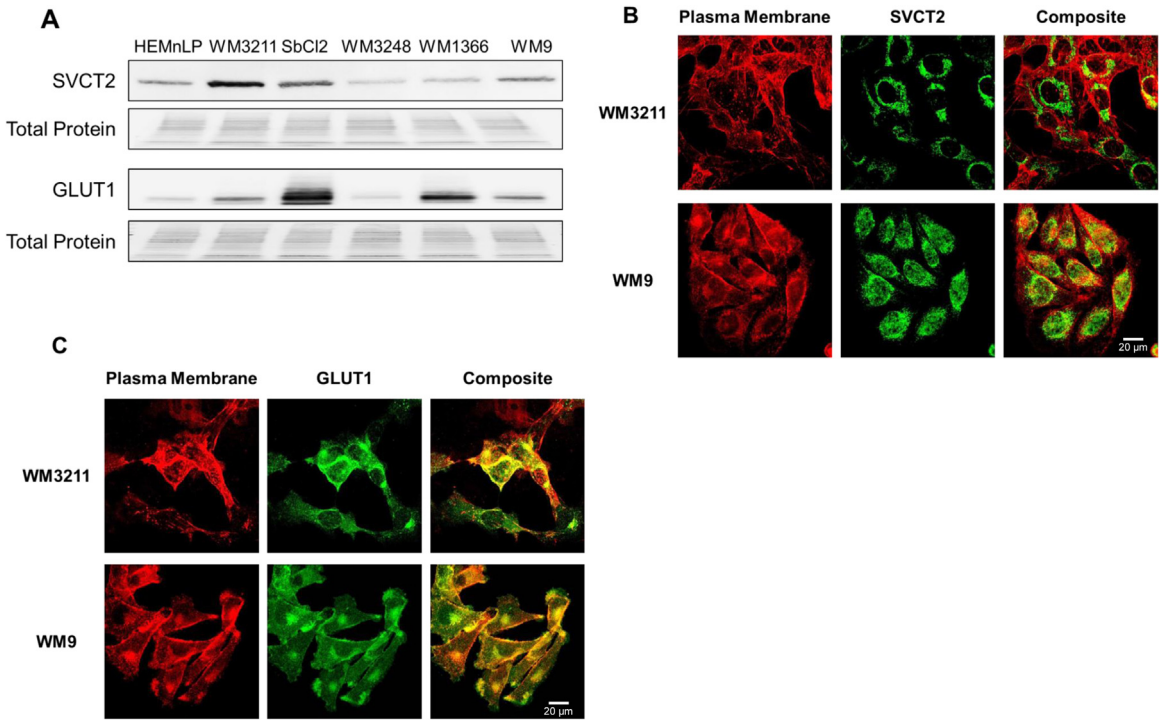

Fig. 5. Protein expression and subcellular localization of SVCT2 and GLUT1 in human melanoma. A) Western blot analysis of cell membrane enriched protein extracts reveal varying expression of SVCT2 and GLUT1 isolated from melanocytes (HEMnLP) and a variety of melanoma cell lines. B) Confocal analysis of WM3211 and WM9 cells depicts the majority of SVCT2 protein is internalized and not presence within the plasma membrane. Conversely, C) the expression of GLUT1 as primarily limited to the plasma membrane. Confocal images are representative of multiple experiments.

internalized in both cell lines (Fig. 5b) while GLUT1 appears to be localized primarily to the plasma membrane (Fig. 5c). Thus, the ability of melanoma to utilize DHA as a source of AA to reduce HIF-1α activity does not appear to be a consequence of altered or differential GLUT1 protein expression or localization.

3.3. AA and DHA supplementation leads to the accumulation of vitamin C in WM9 metastatic melanoma cells

To further elucidate why DHA appeared to be ineffective at enhancing the intracellular cofactor function of AA, WM9 cells were used as the study model in the remainder of this investigation because they represent an advanced metastatic phenotype, providing a more clinically relevant model for evaluating the use of AA or DHA as an adjuvant therapy in melanoma. To evaluate intracellular vitamin C accumulation, we compared the ability of AA and DHA to increase intracellular vitamin C content in WM9 metastatic melanoma cells. Cells were incubated for 30 min with AA or DHA (100 μM). Cells were treated with AA rather than A2P because A2P must first be dephosphorylated by cell surface phosphatases or esterases before it can be transported by SVCTs [40,41]. This dependence on phosphatases is known to delay the initial transport of AA [31] an observation our laboratory also confirmed (data not shown). Mechanistically, the vitamin C assay operates by oxidizing the AA present in the sample to DHA before the addition of o-phenylenediamine (OPDA). DHA and OPDA then react to form a fluorescent condensation product [31], thus this assay quantifies total intracellular vitamin C (AA and DHA) content and does not distinguish between AA and DHA. At short time points, intracellular vitamin C content was significantly higher following DHA incubation compared to AA (Fig. 6a), supporting previous findings [42]. However, the effect of glucose competition on DHA transport was not accounted for under these transport conditions. Therefore, studies were repeated in the presence of physiological glucose (1 g/L; 5.5 mM) and demonstrated that glucose significantly attenuated the accumulation of intracellular vitamin C by DHA while having no impact on the ability of AA to raise intracellular vitamin C levels. The addition of glucose to the incubation buffer resulted in similar initial accumulation of intracellular vitamin C using either the SVCT2/AA or GLUT1/DHA transport system (Fig. 6a). To validate that vitamin C accumulation following DHA incubation was due to DHA uptake by GLUT1 transport, vitamin C accumulation studies were also done in the presence of the highly selective GLUT 1 inhibitor STF-31 (10 μM). Inhibition of GLUT1 significantly reduced DHA uptake and confirmed that DHA transport was mediated by GLUT 1 (Fig. 6a).

To confirm that AA or DHA supplementation results in functionally equivalent intracellular AA, the protein accumulation of HIF-1α in WM9 cells was evaluated by western blot. During a short incubation (30 min), all concentrations (10–50 μm) of AA nearly eliminated hypoxia-mimetic (EDHB) induced HIF-1α accumulation, while only the highest concentration of DHA (50 μM) was able to reduce HIF-1α protein levels (Fig. 6b).

3.4. Prolonged incubation with DHA results in the loss of intracellular vitamin C

Our results demonstrated that the presence of glucose attenuated the initial DHA transport into cells, therefore we wanted to determine if this would impact the ability of cells to accumulate and maintain intracellular vitamin C over time, thus representing a more physiologically and clinically relevant scenario to assess the therapeutic potential of AA and DHA. To evaluate the accumulation of vitamin C at longer time points, WM9 cells were supplemented with 100 μM A2P, AA, or DHA for 24, 48, and 72 h under standard culture conditions (Table 1). To maintain

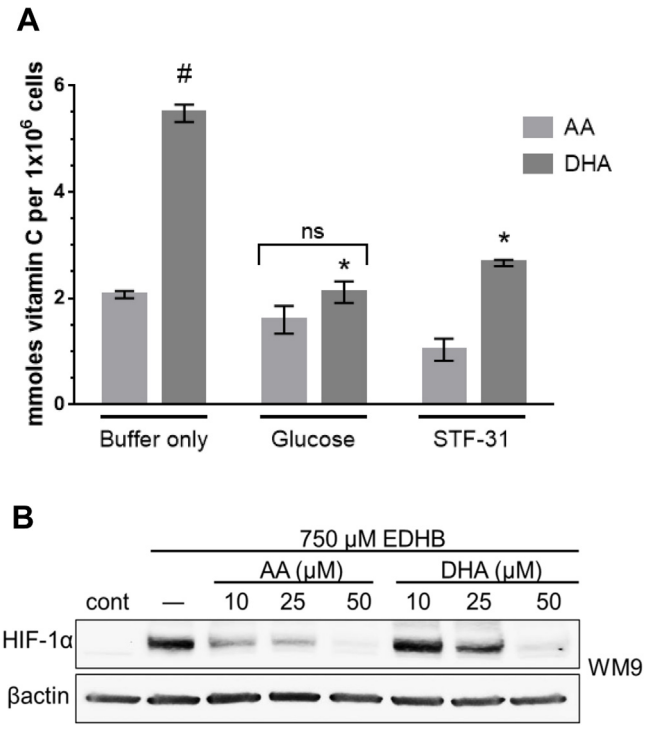

Fig. 6. Initial transport and activity of AA or DHA in WM9 melanoma cells. To evaluate the contribution of GLUT1 to vitamin C accumulation in metastatic melanoma, A) WM9 cells were incubated with AA or DHA (100 μM) for 30 min in a standard transport buffer with or without glucose (1 g/L) or the GLUT1 inhibitor STF-31 (10 μM), before being collected and intracellular vitamin C measured. B) To compare the initial ability of AA or DHA to decrease HIF-1α protein stability and accumulation WM9 cells were incubated with EDHB (750 μM) for 2 h before the media was exchanged with fresh RPMI (1 g/L glucose) containing low concentrations (10–50 μM) of AA or DHA for 30 min before nuclear protein extracts were analyzed by western blot. Our results demonstrate that in the absence of glucose DHA appears to be preferentially transported across the plasma membrane over AA, resulting in a higher initial intracellular vitamin C content. However the addition of glucose significantly attenuates DHA transport as seen by loss of intracellular accumulation of vitamin C. The use of STF-31 similarly inhibits DHA transport confirming GLUT1 is the primary mechanism for DHA uptake. Western blot analysis indicates that at all concentrations AA was more effective at regulating HIF-1α protein stability than DHA. Data was analyzed using Two-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. # denotes statistical difference from buffer only AA, $p < 0.0001$. * denotes statistical difference from buffer only DHA, $p < 0.0001$. ns = not significant. AA content was not detectable in control cells so significance is not indicated.

Table 1
Intracellular accumulation of vitamin C following incubation with various vitamin C compounds.

	Vitamin C (100 μM)	mmoles per 10^6 cells
24 h	A2P	4.700 ± 0.333
	AA	2.907 ± 0.184 **
	DHA	0.163 ± 0.029 *** #
48 h	A2P	2.500 ± 0.411
	AA	0.847 ± 0.428 *
	DHA	0.020 ± 0.020 **
72 h	A2P	0.390 ± 0.189
	AA	ND
	DHA	0.033 ± 0.015

WM9 cells were incubated with 100 μM A2P, AA, or DHA for the indicated time period in RPMI media containing 1 g/L glucose. Incubation with A2P resulted in the greatest vitamin C content compared to AA and DHA. Interestingly, minimal vitamin C accumulation resulted from DHA incubation. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test within each time period. Data shown as ± SEM, n = 3. * denotes significance from A2P at the indicated time period, $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. # denotes significance from AA at the indicated time period, $p < 0.001$. ND = not detectable.

the health of the cells during the extended time point incubations, transport buffer was substituted with standard RPMI media containing (1 g/L) glucose. Following incubation, cells were collected and the total vitamin C content measured using the vitamin C assay. Our results show supplementation with A2P resulted in significantly higher intracellular vitamin C compared to either AA or DHA at both 24 and 48 h. Intriguingly, in contrast to shorter time points, incubation with DHA at all longer time points (24–72 h) resulted in minimal vitamin C content in our cells (Table 1), suggesting a limited therapeutic capacity for DHA compared to AA and A2P.

3.5. A2P reduces HIF activity and invasive potential more effectively than DHA

Since our data demonstrated that incubation with A2P results in significantly higher intracellular accumulation of vitamin C compared to DHA over an extended time period, we wanted to evaluate the possible therapeutic potential of A2P and DHA by assessing changes in malignant potential during that time. Examination of the ability of 100 μM A2P, AA, or DHA to inhibit HIF-1α transcriptional activity at 48 and 72 h in WM9 cells indeed demonstrated persistent inhibition of HIF activity following A2P treatment, while DHA treatment proved to be ineffective (Fig. 7a–b). In order to assess and compare the ability of A2P vs. DHA to decrease the malignant potential of metastatic WM9 cells, we conducted 3D tumor spheroid invasion assays. Cells were incubated in the presence or absence of A2P or DHA (100 μM) for 4 days prior to the generation of spheroids and throughout spheroid formation. Following seeding and formation of tumor spheroids, spheroids were directly imbedded in matrigel matrix (with the addition of 100 μM of the appropriate vitamin C) and the migration of invasive cells into the matrix was monitored over the next 96 h. Our findings show that A2P effectively prevented the migration and invasion of cells, limiting spheroid progression to approximately 3%, while control and DHA supplemented cells demonstrated a 20% and 23% increase in spheroid diameter respectively (Fig. 8a-b). This further demonstrates the reduced efficacy of using DHA as a potential therapeutic agent to re-establish intracellular AA levels. Cell counts prior to spheroid formation following the 4-day pretreatment of cells confirmed vitamin C treatment did not result in altered cell proliferation (data not shown).

3.6. AA mediated regulation of HIF-1α is dependent on SVCT2 activity

After confirming that the non-responsiveness of WM9 cells to DHA is not due to the absence of GLUT1 expression or function, we wanted to confirm that modulation of HIF activity by AA is dependent on SVCT2 activity, resulting in increased intracellular accumulation of vitamin C (as AA) rather than unrelated extracellular stimuli triggered by AA supplementation. To demonstrate the role of SVCT2 in AA mediated HIF-1α regulation, the activity of SVCT2, a sodium dependent transporter, was functionally inhibited by the replacement of sodium chloride (NaCl) with choline chloride (ChCl) in the transport buffer. Since SVCTs are the only transporters capable of transporting AA [38], the impact of sodium deprivation from the media would effectively prevent any extracellular transport of reduced vitamin C. To measure the impact of sodium removal on AA accumulation, WM9 cells were incubated for 30 min in either NaCl or ChCl containing transport buffer with 100 μM AA before intracellular vitamin C content was determined. Substitution of Na in the transport buffer resulted in complete inhibition of vitamin C accumulation in the cells (Fig. 9a). To demonstrate the functional impact of SVCT transporter inhibition on AA mediated HIF-1α regulation, cells were pretreated

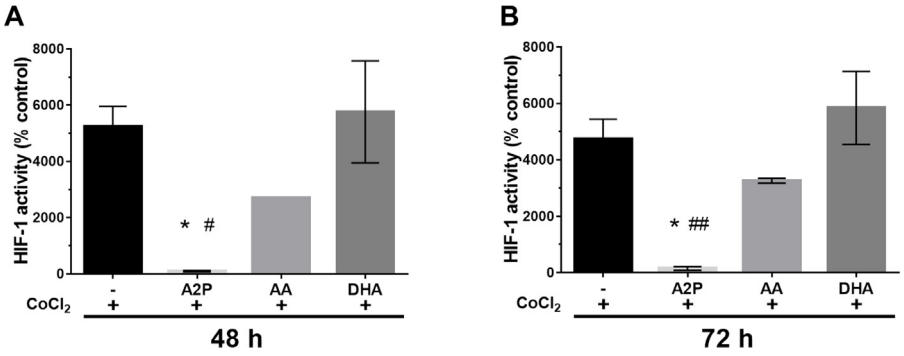

Fig. 7. Comparison of vitamin C compounds to decrease HIF-1 transcriptional activity over time in WM9 melanoma cells. Similar to Fig. 3f, WM9 were transiently transfected with an HIF-1 HRE-luciferase reporter vector. Following overnight transfection, cells were treated with the hypoxia-mimetic $CoCl_2$ (100 μM) with or without 100 μM A2P, AA, or DHA for A) 48 h and B) 72 h. Our findings show, A2P supplementation significantly attenuates HIF-1 activity up to 72 h. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. * denotes statistical significance from $CoCl_2$ induced cells $p < 0.05$. # denotes statistical significance from DHA treated cells $p < 0.05$; ## $p < 0.01$. Data was normalized as percent of control (control = 100%; not shown).

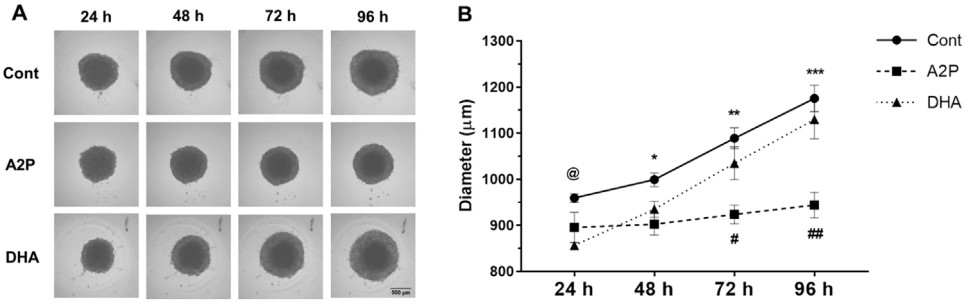

Fig. 8. A2P, but not DHA, inhibits the invasive potential of human melanoma. WM9 cells were pretreated with or without A2P or DHA (100 μM) for 4 days prior to the generation of tumor spheroids. Following their suspension in matrigel, A) images of the tumor spheroids were collected up to 96 h to visualize cell migration/invasion. B) The diameter of the expanding tumor spheroids, including the migrating cells, was measured to access the impact of A2P or DHA on cancer cell migration/invasion. Our results demonstrate supplementation with A2P is capable of limiting the invasive potential of WM9 metastatic melanoma cells. Not surprising, DHA was largely ineffective at inhibiting cell migration/invasion. Images are representative of multiple experiments. Data was analyzed using Two-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. @ denotes statistical significance between control and DHA treated cells $p < 0.05$. * denotes statistical significance between control and A2P treated cells $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. # denotes statistical significance between A2P and DHA treated cells $p < 0.05$; ## $p < 0.0001$.

with EDHB (750 μM) for 2 h in standard RPMI media to induce HIF-1α before the media was exchanged with NaCl or ChCl transport buffer with or without AA (100 μM) for 30 min. As determined by western blot, inhibition of SVCT2 function also prevented the AA mediated decrease in HIF-1α protein stability (Fig. 9b), demonstrating that the functionality of SVCT2 is directly associated with AA accumulation and anticancer activity within these cells.

The tumor microenvironment of aggressive malignancies is frequently found to be slightly acidic (pH 6.5–6.9) [43]. Because SVCT2 function can be diminished by decreasing pH [39], potentially contributing to reduced tumor AA levels, as well as presenting a challenge for the implementation of AA therapy we wanted to examine the impact of pH on the ability of AA vs. DHA to be transported across the plasma membrane. To do this WM9 cells were pretreated for 30 min with a concentration of AA or DHA (100 μM; empirically determined from Fig. 6b) known to inhibit HIF-1α protein stability in a standard transport buffer (1 g/L glucose) adjusted to pH 6.5, 7.0, or 7.4. Following pretreatment, buffer was exchanged with fresh RPMI containing EDHB (750 μM) for 2 h before cell collection. Using HIF-1α protein degradation as an indicator of intracellular AA accumulation and thus functional transport activity, western blot analysis showed that reduced pH, representative of the tumor microenvironment, did not alter the transport activity of SVCT2 or GLUT1 (Fig. 9c), suggesting that the reduced pH of the microenvironment would likely have a negligible impact on the uptake of therapeutic AA or DHA.

3.7. Inhibition of HIF-1α depends on cofactor function and not antioxidant properties

Since intracellular AA can function as an enzyme cofactor or as an antioxidant (free radical scavenger) we wanted to confirm that the AA associated inhibition of HIF-1α protein stability is a result of its HIF hydroxylase (PHD1-3, FIH) cofactor function rather than an indirect function of its antioxidant properties. Therefore we examined the protein stability of EDHB-induced HIF-1α following short (30 min) or long term (24 h) incubation with increasing concentrations (10–500 μM) of N-acetyl cysteine (NAC), another common antioxidant. Supplementation with NAC failed to reduce HIF-1α protein accumulation at all concentrations and time points (Fig. 10a-b) confirming AA mediated decrease of HIF-1α is due to its function as a PHD/FIH cofactor and is not the result of its antioxidant properties.

4. Discussion

The colonization of malignant melanoma to distant tissues coincides with a dramatic decrease in patient survivability [1]. One of the defining characteristics of metastatic melanoma is aberrant increased expression and activity of the HIF-1 transcription factor [7,44]. Widespread evidence suggests elevated HIF activity promotes the acquisition of several cancer hallmarks including,

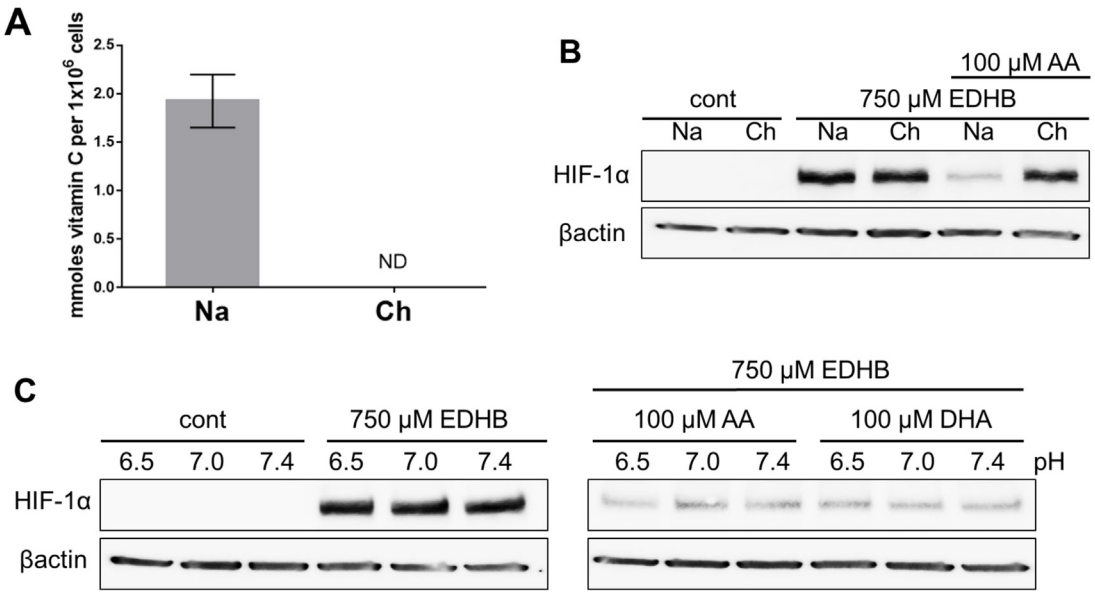

Fig. 9. SVCT2 function mediates the regulation of HIF-1α by ascorbic acid. A) WM9 cells were incubated with AA (100 μM) for 30 min in a transport buffer containing NaCl (Na) or choline chloride (Ch) before the cells were collected and intracellular AA accumulation was measured. Error bars represent SEM, n = 3. ND = not detectable. B) WM9 cells were incubated with AA for 30 min in transport buffer containing either NaCl (Na) or choline chloride (Ch). Following incubation, buffer was exchanged for fresh RPMI containing EDHB (750 μM) for 2 h. Western blot analysis demonstrates that SVCT2 activity is required to mediate AA regulation of HIF-1α stability. C) WM9 cells were incubated with AA or DHA for 30 min in standard transport buffer containing 1 g/L glucose and adjusted to pH 6.5, 7.0, or 7.4 to examine the impact of pH on transporter function. After AA or DHA pretreatment, buffer was exchanged with RPMI containing EDHB for 2 h as indicated. Using the status of HIF-1α protein as a measurement of sufficient vitamin C transporter activity, western blots indicate lower pH does not impair the ability of SVCT2 or GLUT1 to increase functional intracellular vitamin C content in our cells. Blots are representative of 3 different experiments each with similar results.

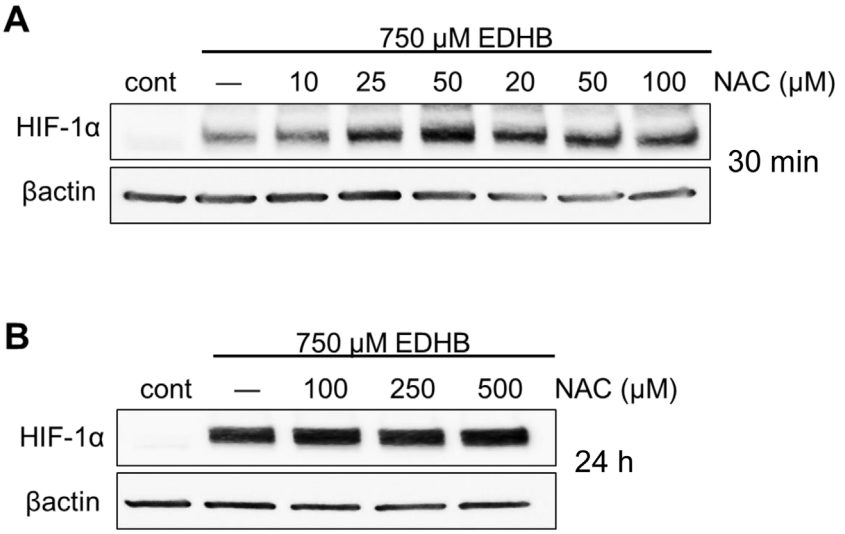

Fig. 10. Ability of NAC to decrease EDHB induced HIF-1α protein stability. A) WM9 cells were incubated with EDHB (750 μM) for 2 h before the media was exchanged with fresh RPMI containing low concentrations (10–100 μM) of NAC for 30 min before nuclear protein extracts were analyzed by western blot. B) WM9 cells were incubated in RPMI containing EDHB with or without increasing concentrations (100–500 μM) of N-acetyl cysteine (NAC) for 24 h. Western blot analysis for both experiments indicates NAC is unable to regulate the protein stability of HIF-1α. Blots are representative of biological repeats with similar results.

but not limited to, rapid tumor invasion, induction of angiogenesis, resistance to cell death, and the procurement of classic Warburg metabolism [29,45–48]. Several factors may contribute to aberrant HIF activity in melanoma including inadequate intratumoral AA, which has previously been associated with tumor accumulation of HIF-1α in a variety of different malignancies [17,18]. Our previous findings demonstrating that supplementing metastatic melanoma cells with physiological concentrations of AA decreases their malignant potential, suggests that AA deficiency plays a biologically relevant role in oncogenesis, and highlights the need to evaluate its efficacy and use in the clinical setting to improve patient response and survival. The goal of this study was to evaluate and compare the efficacy of AA and DHA supplementation ($\leq$ 0.5 mM) as therapeutic means to promote increased intracellular AA; promoting AA mediated regulation of aberrant HIF-1α protein stabilization and transcriptional activity and reducing the malignant potential of human melanoma cells.

Circulating plasma and tissue vitamin C is maintained by the dietary ingestion and intestinal absorption of AA and/or DHA. Following absorption, enterocytes reduce DHA to AA and the pool

of intracellular AA is released into the plasma by diffusion or hypothesized volume-sensitive anion channels [37,49]. The rapid reduction of DHA in the enterocytes, as well as its relative instability at physiological pH (Fig. 1), results in very low levels of plasma DHA (2–5 μM) [39], while under the same conditions, AA exists as an ascorbate monoanion [11] (commonly referred to as AA; 40–80 μM). The ability of oral DHA supplementation to treat or prevent scurvy has been vigorously contested for decades with some reports showing complete reversal of scurvy symptoms yet others demonstrate low vitamin C activity following oral DHA administration [24–27]. Regardless of the contradictory reports, it is likely that any increased vitamin C activity after DHA ingestion is derived from elevated plasma AA levels stemming from the reduction of DHA and subsequent secretion of AA from the intestinal lumen. This implies that oral DHA supplementation would be unlikely to result in elevated plasma DHA levels thus, limiting the use of DHA as a therapeutic agent to intravenous administration. This immediately demonstrates a disadvantage for any oncology patient prescribed DHA treatment because they will require additional medical assistance. Conversely, oral vitamin C supplementation in the form of AA would likely be sufficient to restore physiological AA in cancer patients. Therefore, intravenous injection of DHA would only be preferred if it provided a significant therapeutic advantage over AA.

To evaluate and compare the efficacy and functional impact of AA vs. DHA supplementation as a potential therapeutic option in melanoma, we compared the ability of A2P and DHA to decrease the protein stability and accumulation of HIF-1α in WM9 and WM239A metastatic melanoma cells. After discovering that DHA was less effective in decreasing HIF-1α protein than A2P (Fig. 2), we wanted to evaluate the impact of the various vitamin C compounds on the overall transcriptional activity of HIF-1α in a collection of human melanoma cell lines representing various stages of melanoma progression and HIF-1α expression/stability (Fig. 3). While AA acts as a cofactor for several enzymes, its impact on PHD and FIH activity is particularly important because of its regulation of HIF-1α activity, which is known to contribute to oncogenic progression not only in melanoma but other malignancies as well. Our data demonstrated all melanoma cells were sensitive to AA supplementation, particularly via A2P, while only cell lines with low HIF-1α induction (WM3211 and SbCl2; Fig. 3b-c) were significantly responsive to DHA. Noteworthy, WM3211 and SbCl2 cells are representative of early stage melanoma that is readily cured by surgical excision, minimizing the clinical relevance of this observation. Alternatively, WM1366 and WM9 cells, representative of advanced stage melanoma, were non-responsive to DHA (Fig. 3e-f).

Other studies in breast cancer, demonstrate a correlation between SVCT2 expression and sensitivity to AA treatment [50]. To elucidate factors that may contribute to the differential response of melanoma to AA and DHA, we investigated the prevalence and subcellular localization of SVCT2 and GLUT1 protein in our melanoma cell lines since SVCT2 specifically transports reduced vitamin C (AA), with GLUT1 transporting the oxidized form of vitamin C (DHA). SVCT2 appeared to be primarily internalized, with limited localization to the plasma membrane, which would suggest a limited capacity for AA uptake (Fig. 5b). This was somewhat unexpected given our previous and current findings following treatment of these cells with AA [19], yet this is not entirely unreasonable. Previous research has found SVCT1 & 2 can localize to various organelle membranes within the cell [51,52]. The exact purpose of intracellular localization is not fully understood, but the possibility of extracellular AA promoting the insertion of SVCTs into the plasma membrane of malignant cells is yet to be evaluated. Surprisingly, we observed both WM3211 and WM9 cells have similar expression and localization of GLUT1 (Fig. 5a,c), suggesting that other factors govern the potential anti-cancer activity of DHA.

It has been suggested that overexpression of GLUT transporters would facilitate increased uptake of both glucose and DHA by malignant tissue, however the influence of glucose competition on DHA transport was not fully considered [28]. Recent reports show the impact of glucose on DHA uptake varies greatly between cell types [36]. Therefore it was important to evaluate the impact of physiological glucose (1 g/L) on DHA transport to determine if it would limit the ability of melanoma cells to utilize DHA to reestablish functional intracellular AA. Our data clearly demonstrated physiological glucose concentrations drastically decreased the initial uptake of DHA resulting in a nearly 60% decrease in intracellular vitamin C, yielding results comparable to incubation with AA (Fig. 6a). However when we examined the ability of AA and DHA to decrease HIF-1α protein accumulation under the same conditions we found AA was more effective (Fig. 6b). Equally significant is the absence of intracellular vitamin C following DHA incubation for 24–72 h (Table 1). This suggests that although WM9 cells are capable of initially transporting equimolar concentrations of AA and DHA across the plasma membrane (Fig. 6a), they are unable to effectively recycle intracellular DHA to AA, resulting in decreased activity likely through the irreversible degradation of DHA and loss of AA necessary for downstream functions over an extended period of time (Fig. 1). The inability of DHA to be recycled is likely attributed, at least in part, to deficiencies in DHA reductase activity or glutathione. The observation that DHA is still transported into the cell after treatment with a GLUT1 inhibitor, STF-31, is likely due to the presence of GLUT3 protein, which is also known to transport DHA [53]. GLUT3 protein expression and function was not extensively examined in this study because of the minimal expression of GLUT3 mRNA compared to GLUT1 in our cell lines (data not shown).

The inability of WM9 cells to accumulate and maintain vitamin C levels following incubation with DHA raises considerable concerns as to whether clinical DHA supplementation could increase intracellular AA content sufficiently to promote its cofactor function and anticancer activity. To compare the biological significance of DHA vs. A2P supplementation we used 3D tumor spheroid invasion assays to evaluate the ability of DHA and A2P to decrease invasive potential of WM9 cells. 3D invasion assays were chosen because they more accurately model physiological tumor conditions *in vitro*. Supporting our previous findings in 2D invasion chambers [19], these assays demonstrate that incubation of WM9 cells with A2P significantly inhibits cell migration/invasion, however DHA supplementation failed to impede cell migration/invasion, resulting in spheroid formations nearly identical to non-treated control cells (Fig. 8a-b). While our WM9 tumor spheroids do not display single cell projections typically associated with an invasive phenotype in 3D culture, the morphogenesis of our spheroids are reminiscent of collective cancer cell migration/invasion [54]. We were able to exclude changes in cell proliferation as the cause of increased or decreased spheroid diameter by conducting cell counts following A2P or DHA incubation prior to the formation of spheroids (data not shown).

While the response to DHA is not solely dependent on the expression and function of GLUT1, we sought to investigate the influence of SVCT2 activity on the modulation of HIF-1α following AA intervention. In an earlier report, Hong et al. observed SVCT2 expression was an indicator of cytotoxicity for high dose (> 1 mM) AA treatment in breast cancer [50]. By evaluating the impact of inhibiting the function of SVCT2 on AA mediated HIF-1α regulation in WM9 cells, we found the ability of AA to regulate HIF-1α was entirely dependent on SVCT transporter activity (Fig. 9a-b). Also, our data demonstrated that incubation with either AA or DHA under acidic conditions (pH 6.5) did not impede the ability of the

cells to accumulate adequate intracellular AA to decrease HIF-1α protein stability, indicating that SVCT and GLUT function are not compromised at pH levels representative of the tumor microenvironment (Fig. 9c). Lastly, Fig. 10 demonstrated incubation with the common antioxidant NAC is unable to decrease the protein stability of HIF-1α, providing evidence that regulation of HIF-1 by AA was a result of its function as a PHD/FIH cofactor and not a general antioxidant property, supporting the dependence of enzyme function on the presence of AA.

Previously we have shown that melanoma progression is associated with increasing normoxic (aberrant) expression of HIF-1α [20] and that supplementing cells with physiological concentrations of AA not only decreased HIF activity but also reduced malignant properties of metastatic cells [19]. These studies demonstrate that AA may be a beneficial adjuvant therapy for melanoma patients, as well as patients with other types of malignancies, particularly those demonstrating elevated HIF activity. Previous studies have determined that individuals with plasma AA levels $< 28\ \mu M$ had a 62% increased likelihood of dying from cancer compared to those with AA levels $\geq 74\ \mu M$, and that mortality risk decreased dose-dependently with increasing plasma AA [55,56]. While the evidence for using AA as a cancer therapy continues to mount, it is speculative as to whether the use of DHA, either intravenous or dietary, would provide a therapeutic benefit to cancer patients. Most of the dispute surrounding the efficacy of DHA as a therapeutic source for AA involves its ability to alleviate scurvy, not cancer. One of the only studies examining the anti-cancer effects high-dose DHA found that DHA ($IC_{50} = 12.7–30$ mM) was much less effective than high-dose AA ($IC_{50} \approx 4–8$ mM) in decreasing the viability of several different malignant cell lines [57]. However, the cytotoxic mechanism of high dose AA (>1 mM) is dependent on the generation of H_2O_2 to induce cell death, rather than restoration of its cofactor function resulting in the down regulation of HIF activity, making the significance of this comparison difficult to interpret. In our present study we clearly demonstrated the improved efficacy of low dose AA vs. DHA (≤ 0.5 mM) to decrease invasive potential in metastatic melanoma conceivably by increasing intracellular vitamin C content and decreasing HIF activity through optimization of PHD/FIH function. The inability of DHA intervention to regulate HIF activity, and therefore invasion, is likely attributed to a combination of several factors. 1) The insufficient uptake of DHA in the presence of physiological glucose. 2) The failure of metastatic melanoma cells to reduce intracellular DHA to AA, possibly due to inefficient DHA reductase activity or a high endogenous oxidative burden depleting glutathione, and 3) the rapid and spontaneous irreversible degradation of non-recycled DHA within the cell.

5. Conclusion

These studies provide evidence to suggest that AA supplementation would in fact be more effective than DHA as an adjuvant therapy in the treatment of advanced cancer, particularly in malignancies such as melanoma, which demonstrate aberrant regulation of HIF-1α. Adequate delivery of intracellular AA is crucial for restoring or augmenting its cofactor function for critical enzymes such as the HIF hydroxylases, which regulate the protein stability and accumulation, as well as the transcriptional activity of the HIF-1α/HIF-1 transcription factor. Human melanoma is collectively sensitive to AA therapy, however in our studies, only early stage non-aggressive melanoma is significantly responsive to DHA intervention, while providing little impact in metastatic melanoma which typically requires chemotherapeutic intervention. Melanoma cells express the vitamin C transporters necessary for the uptake of both AA and DHA, however in metastatic cells the fact that the initial uptake/transport of DHA across the plasma membrane is impeded by the presence of physiological glucose, potentially impacts its clinical efficacy. This observation, accompanied by the probable degradation of unrecycled DHA, likely contributes to the inability of DHA supplementation to increase intracellular vitamin C content, leading to ineffective regulation of HIF-1α, ultimately resulting in failure to attenuate melanoma cell invasion, in comparison to AA. We also determined that the ability of AA to regulate HIF-1α is dependent on SVCT2 transporter activity, which is unaffected by decreasing pH. These finding are clinically relevant when considering interventions to restore physiological AA levels in patients diagnosed with advanced cancer and highlights the need for further investigation, particularly for clinical trials examining the benefit of restoring physiological AA not only in melanoma, but other cancer types as well.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

APF and SLM contributed to the design of the study and drafting of the manuscript. APF conducted the experiments.

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