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Ascorbate kills breast cancer cells by rewiring metabolism via redox imbalance and energy crisis

Ali Ghanem [a], Anna Maria Melzer [a], Esther Zaal [b], Laura Neises [a], Danny Baltissen [a], Omar Matar [a], Hannah Glennemeier-Marke [a], Fadi Almouhanna [a], Jannick Theobald [a], Mohamed A. Abu el Maaty [a], Celia Berkers [b,c], Stefan Wölfl [a,*]

[a] Institute of Pharmacy and Molecular Biotechnology IPMB, Heidelberg University, Germany
[b] Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research, Utrecht University, the Netherlands
[c] Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

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ABSTRACT

The idea to use megadoses of ascorbate (vitamin C) for cancer treatment has recently been revived. Despite clear efficacy in animal experimentation, our understanding of the cellular and molecular mechanisms of this treatment is still limited and suggests a combined oxidative and metabolic mechanism behind the selective cytotoxicity of ascorbate towards cancerous cells. To gain more insight into the cellular effects of high doses of ascorbate, we performed a detailed analysis of metabolic changes and cell survival of both luminal and basal-like breast cancer cells treated with ascorbate and revealed a distinctive metabolic shift virtually reversing the Warburg effect and triggering a severe disruption of redox homeostasis. High doses of ascorbate were cytotoxic against MCF7 and MDA-MB231 cells representing luminal and basal-like breast cancer phenotypes. Cell death was dependent on ascorbate-induced oxidative stress and accumulation of ROS, DNA damage, and depletion of essential intracellular co-factors including NAD^+/NADH, associated with a multifaceted metabolic rewiring. This included a sharp disruption of glycolysis at the triose phosphate level, a rapid drop in ATP levels, and redirection of metabolites toward lipid droplet accumulation and increased metabolites and enzymatic activity in the pentose phosphate pathway (PPP). High doses of ascorbate also inhibited the TCA cycle and increased oxygen consumption. Together the severe disruptions of the intracellular metabolic homeostasis on multiple levels "redox crisis and energetic catastrophe" consequently trigger a rapid irreversible cell death.

1. Introduction

Ascorbate is an essential nutrient with versatile anti-oxidant functions [1,2]. Unlike some lower mammals, primates lack the biosynthetic pathway of ascorbate [3]. Therefore, nutritional ascorbate (vitamin C) is essential, among other aspects, for maintaining healthy connective tissues, immunity, as well as heart and brain functions [4–7].

The notion of ascorbate megadoses for treating cancer dates back to the seventies of the last century, with both Linus Pauling and Ewan Cameron hypothesizing that ascorbate can counteract cancer progression, improve patients' overall health, and modestly increase survival time [8–10]. The usefulness of ascorbate as an anti-cancer agent has since been debated [11–13]. Pauling and Cameron made their case for ascorbate megadoses in the form of a clinical study on terminal cancer patients [8,9]. A later clinical trial conducted by the Mayo clinic rebutted the findings, triggering a heated debate on the issue [14]. Notably, the latter study used the oral route for ascorbate administration, while those of Cameron and Pauling resorted to intravenous injection [15]. Oral supplementation is subject to absorption limitations [16] and therefore yields a maximum plasma concentration of about 0.22 mM [17], while 100-time higher plasma concentrations could be safely achieved intravenously [16,18,19].

This suggests the full potential of ascorbate megadoses against cancer might not have been fully unleashed due to insufficient absorption via the oral route. Therefore, recent clinical and animal studies focused on intravenous megadoses of ascorbate [13,19–22]. Moreover, the earlier clinical trials on intravenous ascorbate were also restricted to relatively lower doses not exceeding 10 g daily [8,9]. Indeed, more recent trials pushed the boundary by demonstrating the safety of previously unprecedented pharmacological doses of ascorbate in combination with conventional therapies [18–20,23], with intravenous

* Corresponding author.
E-mail address: wolfl@uni-hd.de (S. Wölfl).

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List of utilized abbreviations

2-NBDG	2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)-D-Glucose
6PGD	6-Phospho-Gluconate Dehydrogenase
AC-CoA	Acetyl Coenzyme A
ACC	Acetly-CoA Carboxylase
AMPK	AMP-Activated Protein Kinase
Asc	Ascorbate
ATP	Adenosine Triphosphate
DHA	Dehydroascorbate
DHAP	Dihydroxyacetonephosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
G6PD	Glucose-6-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT1	Glucose Transpoprter 1
GPD1	Glycerol-3-Phsophate Dehydrogenase 1
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide
LC-MS	Liquid-Chromatography-Mass Spectrometry
MIMP	Mitochondrial-Inner-Membrane Potential
NAC	N-Acetyl-Cysteine
NAD^+	Nicotinamide Adenine Dinulceotide (Oxidised)
NADH	Nicotinamide Adenine Dinulceotide (Reduced)
$NADP^+$	Nicotinamide Adenine Dinulceotide Phosphate
NADPH	Nicotinamide Adenine Dinulceotide Phosphate (Reduced)
pACC	Acetly-CoA Carboxylase (Phosphorylated)
pAMPK	AMP-Activated Protein Kinase (Phosphorylated)
PARP-1	Poly(ADP-Ribose)-Polymerase-1
PI	Propedium Iodide
PPP	Pentose Phosphate Pathway
ROS	Reactive Oxygen Species
RPL-30	Riposomal Protein L30
SRB	Sulforhodamine B
SVCT1	Sodium-dependent Vitamin C Transporter 1
SVCT2	Sodium-dependent Vitamin C Transporter 1
TCA	Tricarboxylic Acid Cycle
TPI	Triose-Phosphate Isomerase
αKG	Alpha-Ketolutarate

megadoses of ascorbate up to 100 g daily correlating to plasma concentrations higher than 20 mM [18–20,23,24]. Furthermore, initial evidence from small clinical trials suggests slightly longer patient survival time and anti-metastatic effects in pancreatic cancer [18,20], glioblastoma [24], and ovarian cancer [20].

Several recent studies probed the molecular and cellular mechanisms, through which high levels of ascorbate damage cancer cells [22,25–27]. It has been shown that ascorbate induces oxidative stress selectively in cancer cells while normal cells are spared due to higher reductive capacity and more robust redox homeostasis [22,25,28]. Multiple reports also showed that the ascorbate-associated oxidative stress resulted in inhibited glucose uptake and glycolysis [22,29,30]. Concomitant ATP depletion and energy crisis in cancer cells [18,30], will impair tolerance to oxidative damage and eventually lead to cell death [20,31]. Various explanations have been provided for the glycolysis disruption observed in cancer cells treated with ascorbate. Recent reports have attributed ascorbate specific cytotoxicity to KRAS and BRAF mutations associated with an amplified expression of Glut1 [22,29]. Glut1 is known for its ability to transport DHA into the cell [32–35] and it was therefore implicated in selective DHA uptake by cancer cells [22,36].

In conflicting reports, ascorbate treatment also has been shown to inhibit Glut1 protein expression [29], which could explain the reduced glucose uptake [22,29]. In this scenario competition between DHA and glucose for the Glut1 transporter could further limit uptake. Subsequent intracellular reduction of DHA on the expense of GSH would exhaust the intracellular reductive capacity and tip the redox homeostasis towards cytotoxic oxidation [13,22,33]. The increased oxidative potential will then trigger a metabolic molecular response, including oxidative inhibition of GAPDH [13,22]. Inhibition of GAPDH will also lead to an increase in glucose-6-phosphate and upregulation of the pentose phosphate pathway as part of a protective anti-oxidative response [37,38]. However, the described oxidative modification of GAPDH is reversible [22] and may not be sufficient to explain the disruption of glycolysis upon ascorbate treatment.

Alternative to the uptake of DHA via Glut1, ascorbate -in its reduced form-is transported by the specialised sodium-dependant vitamin C transporters SVCT1, and SVCT2 [33]. These two receptors are present in various cancers, including breast and colon cancers [39,40]. The level of SVCT2 expression varies between different breast cancer cell lines [39]. Recently, depletion of SVCT2 has been correlated to breast malignancy, with lower doses of ascorbate inducing apoptosis in cultured basal-like breast cancer cells by an indirect epigenetic mechanism [26]. This suggests the possibility that megadoses of ascorbate could affect breast cancer cells by direct uptake of ascorbate via SVCT2, in contrast with the indirect route via DHA and Glut1.

Other mechanisms of ascorbate's cytotoxicity depend on the extracellular and intracellular release of H_2O_2 due to complex metal-catalysed chemical oxidation of ascorbate to DHA, potentially via Fenton chemistry and requiring no biochemical catalysis, as the mediator of ascorbate's cytotoxic effects [24,41–43]. The one-electron chain reactions of ascorbate yield the ascorbate free radical that also generates H_2O_2 [44], catalysed for example by ferrous or copper cations in the medium [11,24,44,45]. Pharmacological ascorbate concentrations also have been shown to trigger a variety of cell death modes, including apoptosis and necrosis [25,26,44,46].

The recent advancements regarding the safety of unprecedentedly high intravenous doses of ascorbate [18–20,23,24,47] and their potential efficacy against critical and virtually terminal types of cancer [16,18–20,24] has prompted us to take a fresh look on the cellular and molecular aspects of ascorbate's toxicity to cancer cells, and further elucidate molecular mechanisms, by which high ascorbate concentrations can perturb cancer cells. We focused on cultured breast cancer cells, but also included complementary experiments on colon cancer cell lines, to confirm the observed metabolic links of ascorbate treatment in another tumour type that has been studied for ascorbate treatment *in-vivo* [22,48].

In this work, we confirm the cytotoxic efficacy of ascorbate against luminal breast cancer cells and also demonstrate similar toxicity towards basal-like breast cancer cells. In both cell types glucose utilisation was decreased and ATP depleted. Using metabolite quantification, we show a clear disruption of glycolysis at the triose phosphate level. This disruption is common to both luminal MCF-7 and basal-like MDA-MB231 breast cancer cells in response to ascorbate. To further elucidate this disruption, we performed various enzymatic activity measurements and did not observe inactivation of GAPDH and TPI, the two enzymes catalysing the steps at which glycolysis is disrupted. In contrast, we detect an accumulation of lipid droplets following exposure to high concentrations of ascorbate, and upregulation of glycerol-3-phosphate dehydrogenase 1 (*GPD1)*, which links glucose metabolism to

triglyceride synthesis [49] via reduction of DHAP into glycerol-3-phosphate and coupled oxidation of NADH to NAD^+ [50]. We also provide evidence for a lack of cytotoxicity of DHA towards both breast cancer cells and colon cancer cells. Additionally, we demonstrate that ascorbate promotes spontaneous H_2O_2 generation, and that extracellular H_2O_2 scavenging prevents ascorbate cytotoxicity. Using pharmacological inhibition of the sodium-dependent vitamin C transporters SVCTs we also show that SVCTs are essential for ascorbate uptake, but not for ascorbate cytotoxicity.

2. Results

2.1. Comparison of toxic levels of ascorbate concentrations in breast cancer and colon cancer cells

Several reports described cytotoxic effects of high ascorbate concentrations on tumour cell lines in culture (20,40,41). To assess ascorbate's cytotoxicity in our experimental setting, we performed cell growth and survival assays with two breast cancer cell lines representing basal-like (MD-MB231) and luminal (MCF-7) phenotypes and as an additional comparison analysed HT-29 cells as a reference for colon cancer. SRB assays of cells treated with a gradient of ascorbate concentrations showed a dose-dependent increase in cell death 48 h upon treatment (Fig. 1a). Ascorbate toxicity in both breast cancer cell types (MDA-MB231, MCF-7) is comparable and even higher than measured for the colon cancer cells (HT29).

2.2. High concentrations of ascorbate cause rapid cell death in MCF7 and MDA-MB231 breast cancer cell lines

Using FACS analysis and AnnexinV/PI staining we observed that rapid cell death occurred within 4 h of exposure to high concentrations of ascorbate (Figure S1 a). Interestingly, we also observed cell death using much lower concentration 0.6 mM and a longer exposure of 24 h. We also established a direct correlation of IC50 to cell density. Longer incubation with high concentrations of ascorbate (3 mM) completely dissipated cells leaving no material to investigate. Further analysis of various cell density/concentration/duration combinations explains that treatments need to be adjusted to the requirements of each experiments and the investigated biochemical effect (Figure S1 b).

We next sought to define the mode and mechanism of cell death occurring in the breast cancer cells analysed. Hence we probed apoptotic cell death markers and analysed the activation of caspase-3 and PARP-1 cleavage. Taking into consideration the significant difference of activity of high and low concentrations, analysis was done with 3 mM ascorbate after 7 h and with 1.5 mM after 18 h exposure. MCF-7 exhibited full caspase3 cleavage within 7 h (3 mM ascorbate) and in addition apoptotic PARP-1 cleavage (89 kD fragment) (Fig. 1 b). In contrast, in MDA-MB231 cells pro-caspase3 remained intact and no clear apoptotic PARP-1 cleavage was visible (Fig. 1 b). Alongside the apoptotic PARP-1 fragment, also a smaller non-apoptotic (possibly necrotic) fragment (55 kD) is seen for MCF-7 (Fig. 1 b). Intriguingly, co-treatment with the global caspase inhibitor zVAD-fmk correlated to an enrichment of the non-apoptotic PARP-1 fragment on the expense of the apoptotic one (Fig. 1 b). Hence indicating a mixed mode of cell death that can be skewed via caspase inhibition [51]. Noteworthy is the absence of direct inhibition of caspase3 cleavage by zVAD-fmk (Fig. 1 b), which is consistent with observations that this caspase inhibitor can induce caspase cleavage [52,53].

Using real-time fluorescence microscopy (Incucyte®) with suitable medium-stable dyes we observed the activation of executioner caspases 3 and 7 over-time. Subsequently, cells stained increasingly for AnnexinV and CytoTox, indicating both apoptosis induction and subsequent cell death (Fig. 1c). Co-treating with zVAD-fmk attenuated the observed caspase3/7 induction and slightly delayed the positive AnnexinV staining. In contrast, this co-treatment also correlated to a faster increase of complete cell death. Compared to etoposide, ascorbate treatment induced a much faster onset of the apoptotic and cytotoxic events (Fig. 1 c). Menadione was included as a positive control for oxidative stress, compared to ascorbate it led to non-apoptotic PARP-1 cleavage pattern in MCF-7 at both 12.5 and 25 μM for 7 and 18 h, respectively. Consistently 12.5 μM menadione elicited no caspase activation in MCF-7 in the real-time microscopy setting, and the annexin activation was very marginal compared to ascorbate, hence indicating a non-apoptotic cell death with menadione.

2.3. Ascorbate, not DHA, is the source of cytotoxicity

Recently, indirect evidence has been put forward in favour of DHA being the cytotoxic form of ascorbate [22]. DHA is mainly taken up by Glut1 and is assumed to exhaust cells' GSH pool leading to uncontrollable oxidative distress [22]. Therefore, we compared the effects of DHA to those of ascorbate. SRB ad PI cytotoxicity assays showed that DHA was less effective than ascorbate, with much higher doses required to elicit toxicity (Fig. S2). This is corroborated by absence of cell death in AnnexinV/PI staining and absence of other hallmarks of energy and oxidative distress upon DHA treatment in contrast to treatment with ascorbate (Fig. S3). This difference was seen in all cell lines analysed, breast and colon cancer (Fig S3 c).

2.4. Ascorbate and DHA uptake in MCF-7 and MDA-MB231 cells and inhibition by phloretin

Having established ascorbate in its reduced from as the source of cytotoxicity we next aimed to assess the uptake dynamics and the intracellular fate of ascorbate. We detected comparable levels of intracellular ascorbate in both MCF-7 and MDA-MB231 following either ascorbate or DHA treatments, while non-treated cells exhibited no detectable intracellular ascorbate levels (Fig. S4 a&b). Moreover, ascorbate was still detected intracellularly in MCF-7 cells 4 h after ending the treatment by replacing medium (Fig.S4 c). Phloretin is known to block the sodium-dependent ascorbate transporters SVCT1 and SVCT2 [54,55]. We used phloretin co-treatment with ascorbate to probe if SCVTs account for the detected ascorbate uptake. Indeed, the co-treatment with phloretin completely prevented ascorbate uptake as seen by diminished intracellular ascorbate in both MCF-7 (Fig.S4 d) and MDA-MB231 (Fig.S4 e). The co-treatment with phloretin slightly alleviated the cytotoxicity of ascorbate in MCF-7 cells, exhibited by a shift in the survival curve (Fig. 4 f), this indicates that intracellular ascorbate only partially accounts for the cytotoxicity.

2.5. High concentrations of ascorbate induce oxidative stress, ROS accumulation, mitochondrial hyperpolarisation, and increased respiration

We next aimed to confirm the oxidative origin of concentrated ascorbate in accordance with previous reports [22,25,41,46,56]. Using DHE we microscopically observed significant increase in ROS in both MDA-MB231 and MCF7, reactive oxygen species are the main perpetrator of cellular oxidative damage and therefore are the best proxy for assessing oxidative stress. FACS measurements also indicated increased ROS accumulation in MCF-7, 2 and 4 h after treatment (Fig. 2 a). Since the mitochondria are both a major source and effector of ROS and oxidative damage [57,58], we probed the mitochondrial membrane polarisation as a proxy for mitochondrial integrity and function upon exposure to concentrated ascorbate. Both MDA-MB231 and MCF-7 cells exhibited mitochondrial hyperpolarisation (MIMP) in response to ascorbate at 3 mM. Interestingly, MIMP was even further increased using 6 mM ascorbate. Live cell imaging (IncuCyte®) also indicated increased mitochondrial superoxide formation with 1 mM and 3 mM ascorbate similar to reference treatments with H_2O_2 and menadione. We next found that the observed mitochondrial hyperpolarisation did not correlate to increased ATP levels. While 0.3 mM ascorbate strongly

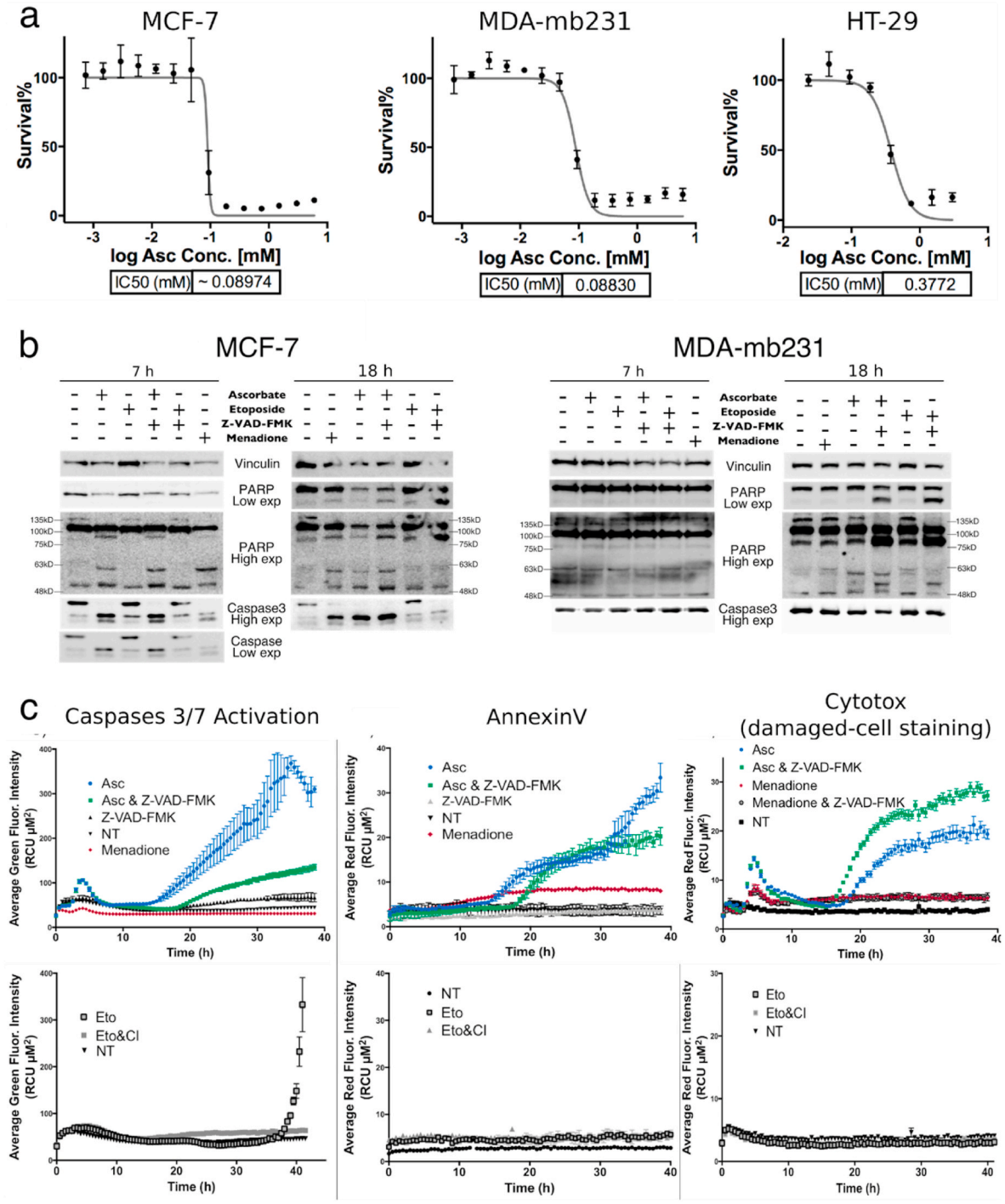

Fig. 1. Charactarisation of ascorbate cytotoxicity and the mechanism of associated cell death

a) SRB assays of ascorbate cytotoxicity for MCF7 (ductal breast cancer), MDA-MB231 (basal like breast cancer) and HT29 (colon cancer) cells (MV±SD; N = 5). **b)** Immunoblotting of PARP-1 and its cleaved variants and pro- and activated Caspase 3 in fully denatured MCF-7 and MDA-MB231 cell lysates, respectively. 6×10^5 cells/well were treated for 7 h (right) and 18 h (left) and then lysed in 6 M urea buffer. Ascorbate was used at a concentration of 3 mM for 7 h and 1.5 mM for 18 h, etoposide was used as reference genotoxic and apoptosis inducing agent at a concentration of 60 µM for 7 h and 25 µM for 18 h. The Caspase inhibitor z-VAD-FMK was used at a concentration of 10 µM. Menadione, a cytotoxic redox cycling ROS generator, was used at 12.5 µM and 25 µM for 7 and 18 h respectively. **c)** Quantitative analysis of apoptosis and cell damage markers using real-time fluorescent microscopy of live-stained MCF-7 cells (IncuCyte®). Activation of caspases 3 and 7 was detected using the IncuCyte® Caspase 3/7 Green reagent, early apoptosis was visualized using IncuCyte® AnnexinV Red Reagent, and severely damaged cells were distinguished using IncuCyte® Cytotox Red dye. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

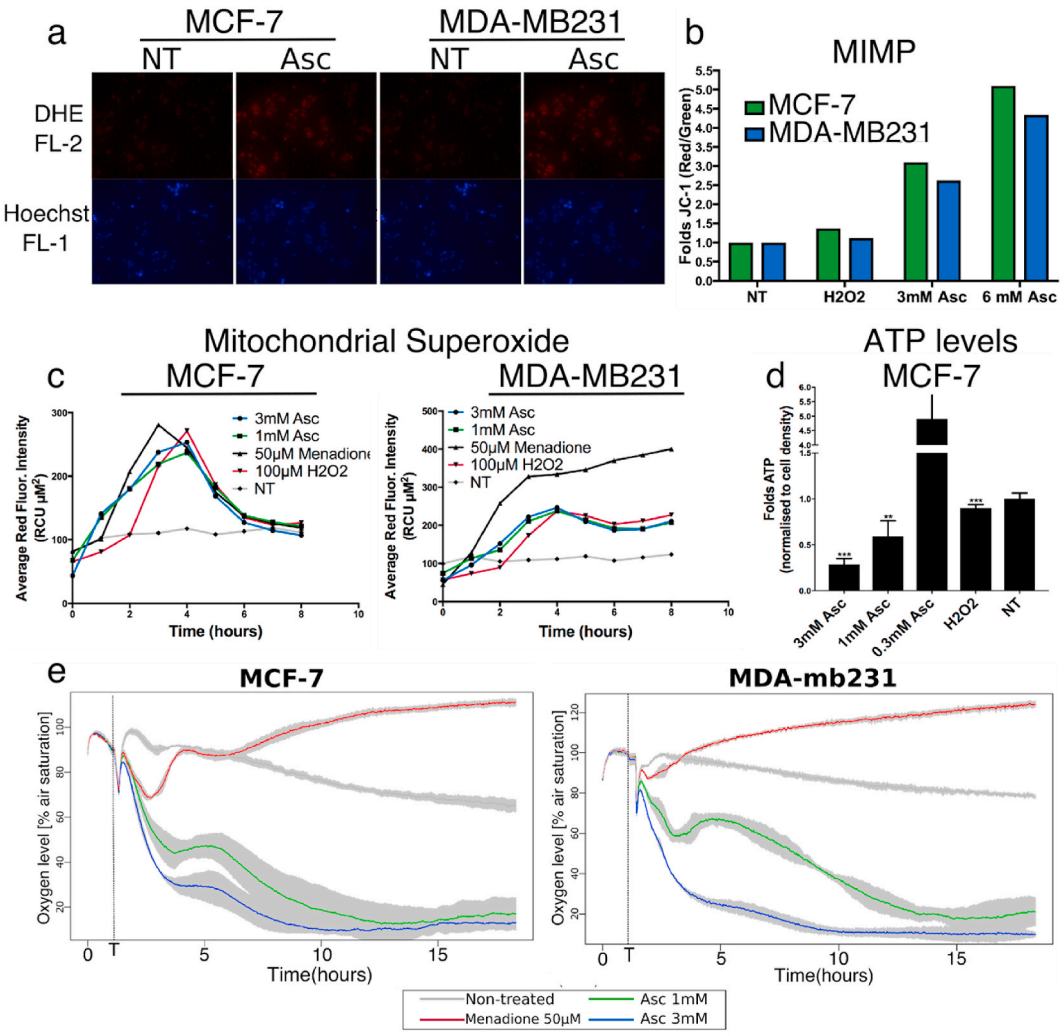

Fig. 2. Ascorbate treatment triggers oxidative stress, ROS accumulation, and compensatory mitochondrial hyperpolarization
a) Microscopic depiction of DHE fluorescence in MCF-7 and MDA-MB231 upon 2-h 0.6 mM ascorbate treatment; MCF7 (top-right), MDA-MB231 (bottom right). **b)** FACS measurements of MIMP in JC-1 stained MDA-mb231 and MCF-7 cells, 6 h following ascorbate or H_2O_2 treatment. c) Mitochondrial superoxide staining monitored overtime using the IncuCyte® fluorescence microscopy system. **d)** ATP levels in MCF-7 and HT-29 cells following ascorbate or H_2O_2 treatments, bar charts feature the mean values (N = 4, error-bars: SD). **e)** Online measurements of O_2 levels in the culture medium of MCF7 and MDA-MB231 cells. Curves feature the mean - value of the continuously measured (every 2 min) oxygen levels for each condition (N = 3, error-regions: SD).

induced ATP formation, 1 and 3 mM correlated to diminished intracellular ATP (Fig. 2d) even stronger than H_2O_2 used as control. Next we asked if the mitochondrial hyperpolarisation was an indication of increased respiration. Indeed, real-time oxygen measurements in medium revealed that both cell lines exhibited a remarkable increase in cellular respiration with 1 mM and 3 mM ascorbate as indicated by accelerated oxygen depletion in medium compared to non-treated cells (Fig. 2e). Menadione, used as a positive control for oxidative stress, also caused a quick spike in oxygen consumption followed by cell death leading to renewed oxygen saturation.

2.6. ROS scavenging abrogates ascorbate induced cytotoxicity

To further validate the causal relationship between oxidative distress and cytotoxicity in response to concentrated ascorbate, we tested how essential ROS accumulation accounted for the observed cytotoxicity. For this, we assessed the impact of co-treatment with ROS-scavengers on the outcome of exposure to concentrated ascorbate. Supplementing the medium with glutathione not only rescued MCF-7 cells from ascorbate cytotoxicity but also correlated to an almost 3-fold increase in proliferation (Fig. 3 a). Consistently, 10 mM glutathione were also sufficient to prevent the induction of ROS and ablation of glucose uptake upon ascorbate treatment (Fig. 3 b and c). Catalase and NAC also prevented ascorbate cytotoxicity up to a concentration of 3 mM (Fig. 3 d and f). Moreover, GSH protected cultured cells, reducing the loss of cell adhesion of MCF-7 cells in flow-through tissue culture used for real time measurements (Fig. S11). Superoxide dismutase, on the other hand, was less effective, leading only to a limited rescue in cell survival upon ascorbate treatment (Fig. 3 e). This is consistent with a lack in effectively neutralising ROS since superoxide dismutase also yields H_2O_2. Together, these observations using ROS scavengers as co-treatment agents clearly indicate a key role for oxidative stress and ROS accumulation in the ascorbate-elicited metabolic disruption and cytotoxicity.

2.7. Ascorbate spontaneously releases H_2O_2 in culture medium

Previous studies attributed ascorbate's cytotoxicity to spontaneous H_2O_2 release via chemical mechanisms including the Fenton reaction. Cancer cells are more critically affected by H_2O_2 due to the higher oxidative burden resulting from rapid proliferation [25]. We used an amplex red/horseradish peroxidase based reaction to colourimetrically/fluorometrically detect the release of hydrogen peroxide

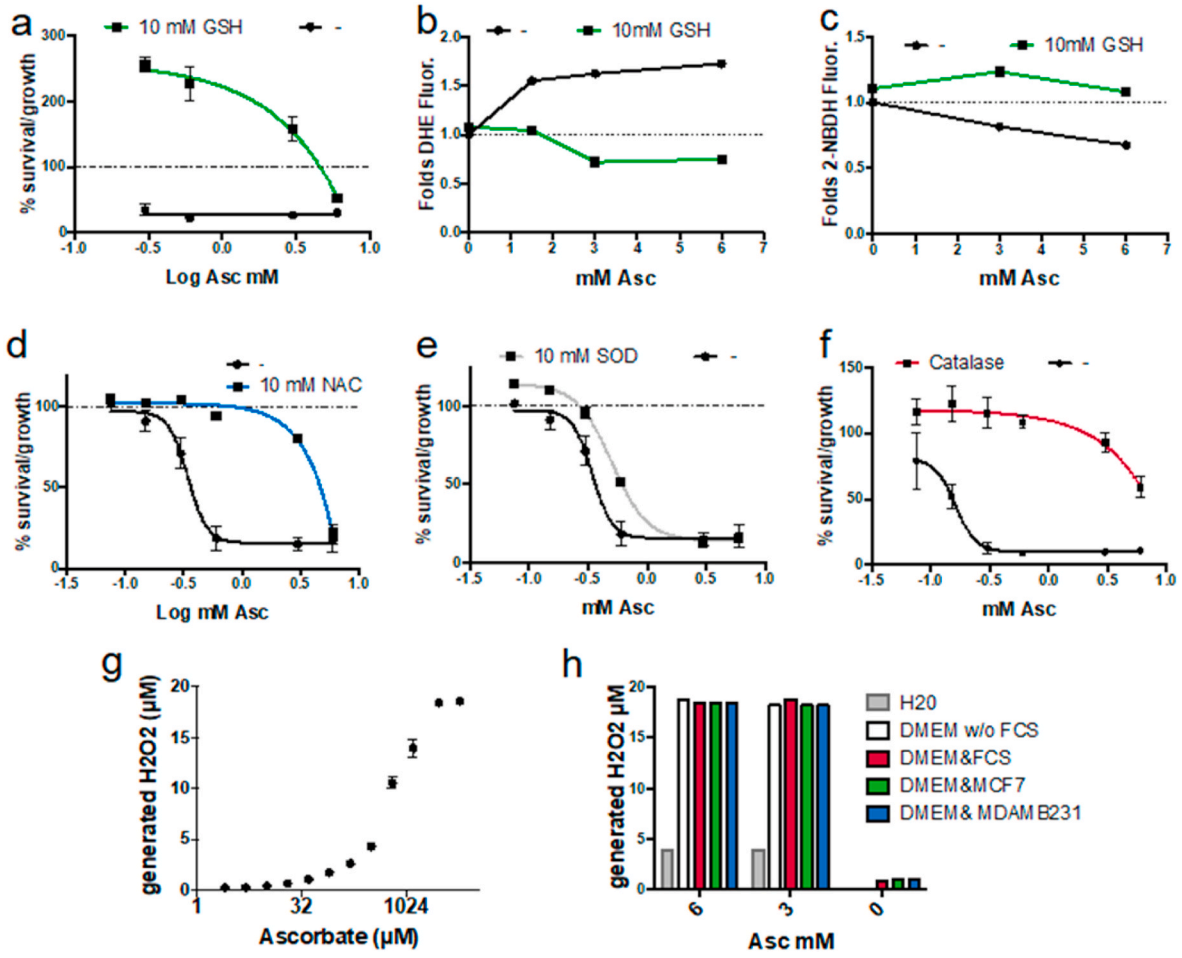

Fig. 3. The alleviation of oxidative stress mitigates ascorbate's activity. Ascorbate spontaneously generates extracellular H_2O_2 into DMEM medium.
a) b) and c) the effect of glutathione co-treatment (pre-treatment for 2 h the co-treatment with ascorbate) on the impact of ascorbate on MCF-7 cell-survival (SRB, 48 h, N = 3, MV±SD), ROS accumulation (DHE, 2 h), and glucose uptake (2-NBDG, 4 h), respectively. **d) e) f)** The effects of NAC, catalase and SOD co-treatments on cell-survival (SRB, 48 h) upon ascorbate treatment. (N = 3, MV±SD) **g)** the correlation between ascorbate concentration in full DMEM and the generated concentrations of H_2O_2, 30 min post ascorbate addition. **h)** Ascorbate-mediated H_2O_2 extracellular generation in different media and cellular contexts, 30 min post ascorbate addition.

upon ascorbate addition to DMEM medium. We observed a dose-dependent sigmoidal increase in released H_2O_2 with increasing ascorbate concentrations up to 3 mM (Fig. 3 g). We next asked what components in medium contribute to ascorbate induced H_2O_2 release and whether the presence of cells will affect the detected H_2O_2 levels. There was no significant influence on the levels of ascorbate generated peroxide in medium mediated by the presence of FCS or cells (Fig. 3 h).

2.8. Ascorbate treatment disrupts redox balance of glutathione and NADP (H) redox pairs, and depletes NAD(H) and NADP(H)

The disruption of intercellular redox homeostasis is a potential outcome of ROS accumulation, and can be reflected in an imbalance in the intracellular redox cofactor pairs GSH/GSSG and $NADP^+$/NADPH. Moreover the depletion of NAD^+ has been observed in response to ascorbate treatment as a result of PARP-1 hyperactivation in response to ROS-mediated DNA damage [56,59].

Hence, to confirm the suspected oxidative source of cytotoxicity and to shed more light on these pivotal cellular co-factors we quantified glutathione, NADP(H), and NAD(H) using an array of commercially available luminescence based assays (supplementary methods XIV). The methods used allow the overall quantification of the total amount of these co-factors as well as the absolute quantification of the intracellular amount of the oxidised and reduced pools of each co-factor. In MDA-MB231 cells 2-h treatment with 3 mM ascorbate led to a decrease in GSH and an increase in GSSG (Fig. 4 a), resulting in an increased oxidised-to-total glutathione ratio (Fig. 4 b) while the overall amount of glutathione remained unchanged (Fig. 4 a). A similar but slight increase in the GSSG/GSH ratio was observed in MCF-7 cells (Fi4. c and d), albeit and contrary to earlier reports (30), this change in glutathione ratio was not-significant in our experiments. Nevertheless, total and reduced glutathione (GSH) were both diminished to a statistically meaningful level (Fig. 4 c) in MCF7 as well as in MDA-MB231 cells.

NAD^+ was severely depleted in both MCF-7 and MDA-MB231 cells upon 2 h of exposure to 3 mM ascorbate (Fig. 4 e and f). Both cell lines also showed depleted NADH and total NAD(H) (Fig. 4 e and f). This strongly reaffirms the abovementioned observation of NAD^+ depletion as a mechanistic link between the ascorbic-acid-induced oxidative damage and the metabolic disruption and energy crisis.

Consistent with increased ROS and oxidative stress we also established NADPH and total NADP(H) depletion in both MDA-MB231 and MCF-7 upon 2 h of 3 mM ascorbate exposure (Fig. 4 g and h), while oxidised $NADP^+$ showed no increase. This could be explained by the NAD^+ kinase-catalysed conversion of the resulting excess of $NADP^+$ upon ascorbate treatment to NAD^+ to compensate for the severe depletion of the latter, also resulting from the treatment.

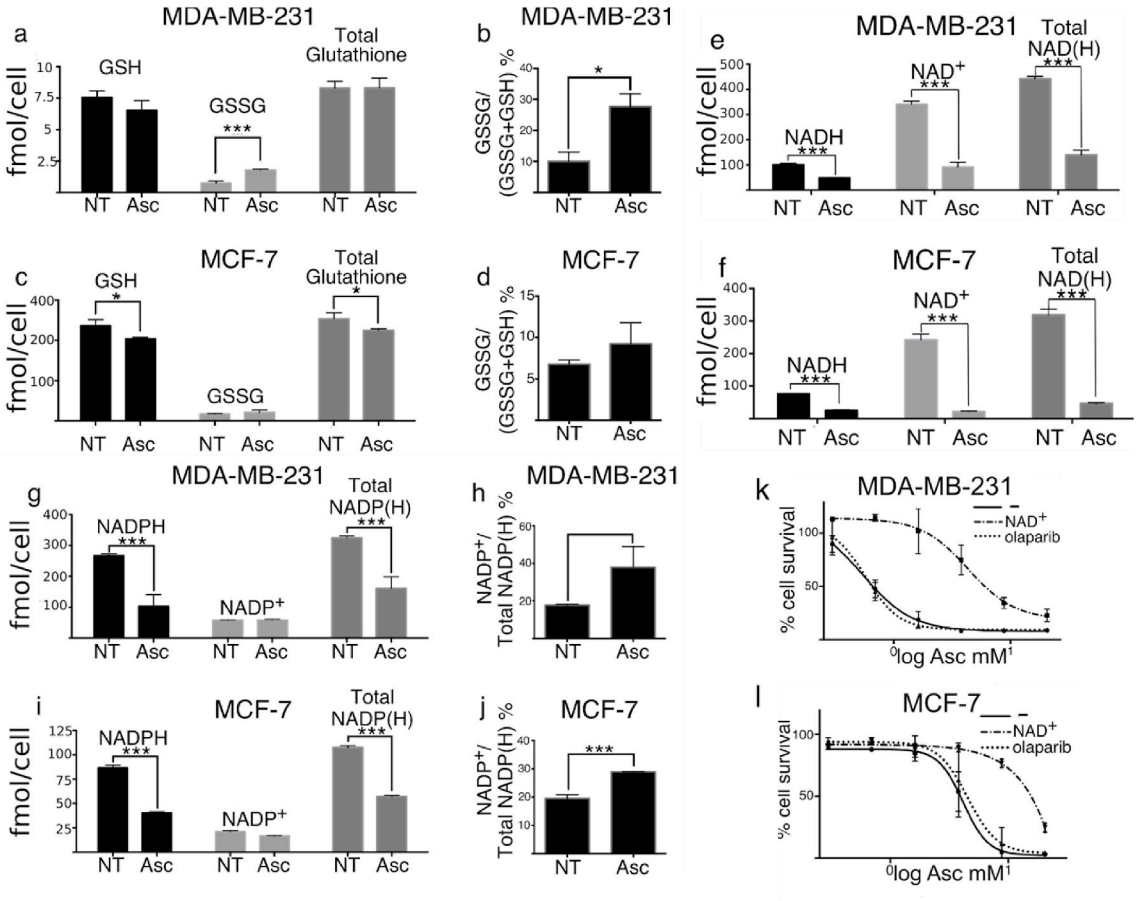

Fig. 4. Full quantification of glutathione, NAD(H) and NADP(H); ascorbate treatment shifts the redox balance towards oxidation and depletes total intracellular NAD(H) and NADP(H).
a) Reduced GSH, oxidised GSSG, and total GSG&GSSG glutathione in ascorbate treated MDA-MB231 cells (3 mM, 2 h) compared to non-treated (n = 3, MV±SD), GSSG and GSH were quantified in separate samples. **b)** Calculated percentage ratio of oxidised glutathione GSSG to total glutathione (GSH + GSSG) in MDA-MB231 cells (n = 3, MV±SD), rations calculated from quantification in separate samples. **c)** Reduced GSH, oxidised GSSG, and total GSG&GSSG glutathione in ascorbate treated MCF-7 cells (3 mM, 2 h) compared to non-treated (n = 3, MV±SD), GSSG and GSH were quantified in separate samples. **d)** Calculated percentage ratios of glutathione disulphide GSSG to total glutathione (GSH + GSSG) in MCF-7 cells (n = 3, MV±SD), ratios calculated from quantifications of GSH and GSSG in separate samples. **e)** NADH, NAD^+, and total NAD(H) in ascorbate treated MDA-MB231 cells (3 mM, 2 h) compared to non-treated (n = 3, MV±SD), NAD^+ and NADH the same samples were split for quantifying both NAD^+ and NADH. **f)** NADH, NAD^+, and total NAD(H) in ascorbate treated MCF-7 cells (3 mM, 2 h) compared to non-treated (n = 3, MV±SD), NAD^+ and NADH the same samples were split for quantifying both NAD^+ and NADH. **g)** NADPH, $NADP^+$, and total NADP(H) in ascorbate treated MDA-MB231 cells (3 mM, 2 h) compared to non-treated (n = 3, MV±SD), NAD^+ and NADPH the same samples were split for quantifying both $NADP^+$ and NADPH. **h)** Calculated percentage ratios of oxidised NADP + to total NADP(H) in MDA-MB231 cells (n = 3, MV±SD), ratios calculated from quantifications of $NADP^+$ and NADPH in the same samples. **k)** SRB survival assays (24-h treatments) showing the impact of the co-treatment with NAD^+ 10 mM or olaparib 10 mM on MCF-7 survival compared to the stand alone ascorbate treatment. Depicted are survival curves (MV±SD; N = 4). **l)** SRB assays (24-h treatments) showing the impact of the co-treatment with NAD^+ 10 mM or olaparib 10 mM on MCF-7 survival compared to the stand alone ascorbate treatment. Depicted are survival curves (MV±SD; N = 4).

2.9. Supplementation with NAD+, but not PARP-1 inhibition by olaparib, ameliorates ascorbate-induced cytotoxicity

To verify the importance of NAD^+ depletion for the cytotoxicity of ascorbate, we tested the effect of supplementing the ascorbate-containing medium with 10 mM NAD+. Indeed, and in line with earlier findings [59] we found that NAD^+ prevented ascorbate cytotoxicity in both MCF-7 and MDA-MB231 cell lines (Fig. 4 k&l). Nevertheless, PARP-1 inhibition by olaparib, a clinically established PARP-1 inhibitor in breast and ovarian cancer [60,61], did not replicate the effect of NAD^+ supplementation indicating that sparing NAD^+ by inhibiting PARP-1 is not enough to reverse the cytotoxicity. This is potentially due to the irreparable DNA damage, especially in light of *BRCA1* allelic loss in both cell lines, and MCF-7 cells exhibiting down-regulated *BRCA1* transcription [62]. Similar results have been reported in pancreatic cancer cells, with the knock-out of PARP-1 also failing to mitigate ascorbate's cytotoxicity [63].

2.10. High concentrated ascorbate disrupts glycolysis, inhibits glucose uptake, and induces the pentose phosphate pathway

Previous reports indicated an inhibition of glycolysis as a result of megadoses of ascorbate against colon, leukemic, and breast cancer cells [22,30,59], hence we used LC-MS to quantify key metabolites in breast cancer cell lines MCF-7 and MDA-MB231 in addition to HT29 colorectal cancer cells, which we included as an example of a cancer cell type well studied for its metabolic response to concentrated ascorbate [22,64]. Indeed, upon 4-h treatment with 3 mM ascorbate both MCF-7 and MDA-MB231 cells exhibited severe glycolytic disruption. This was evident by the accumulation of the upper (preparatory) glycolytic intermediates, and the depletion of lower glycolytic metabolites (Fig. 5 a). A similar glycolytic disruption was also observed for the colon cancer cells HT-29, consistent with earlier reports on ascorbate efficacy in disrupting glycolysis in colon cancer cells [22,64]. The disruption of glycolysis was albeit milder in HT29 cells, this suggests higher resistance

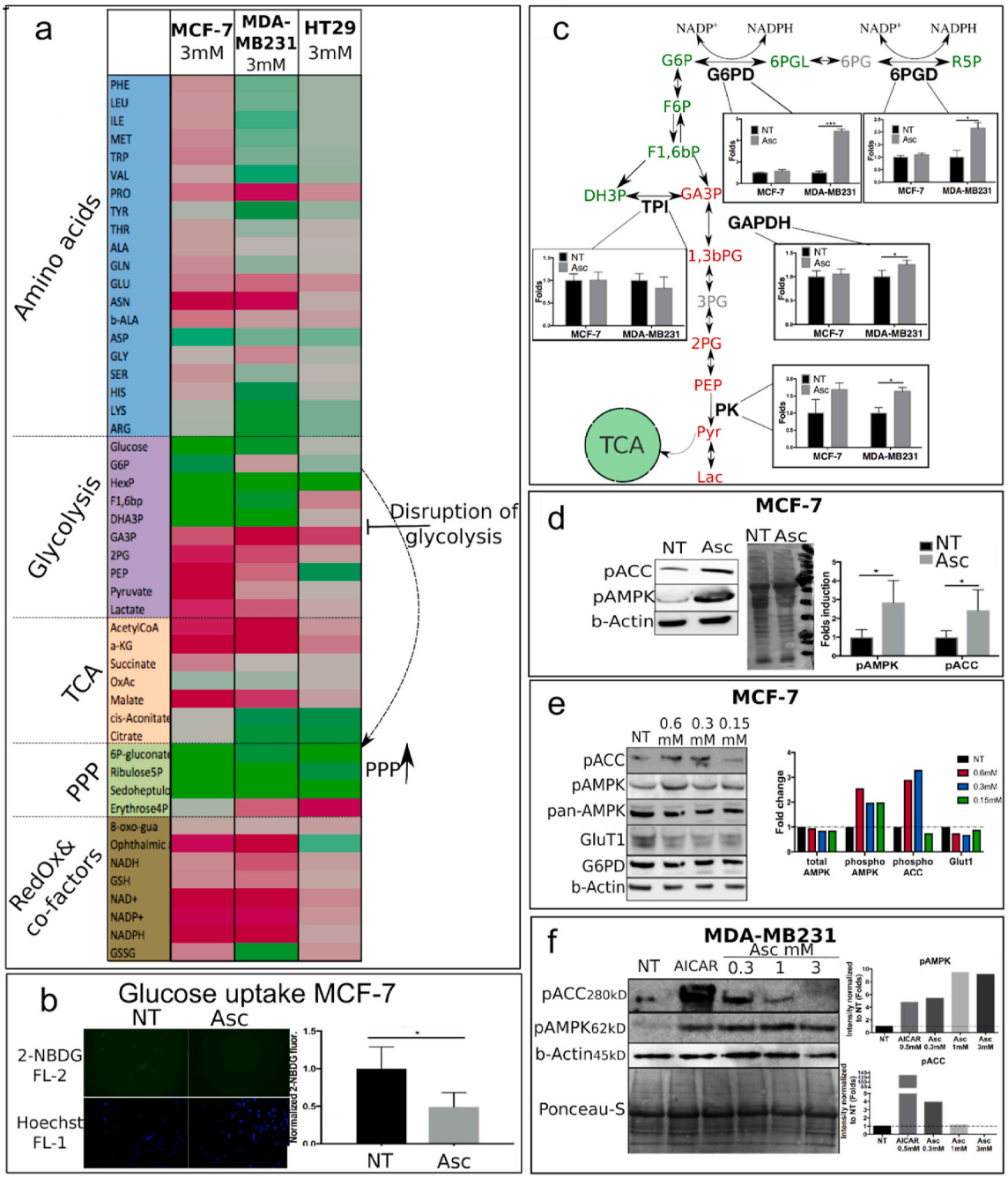

Fig. 5. High dose ascorbate disrupts glycolysis, induces compensatory pentose phosphate shunt, and triggers energy crisis a) HPLC-MS metabolite quantification. Heat-maps show the shifts in metabolite levels upon 4 h of 3 mM ascorbate treatment in HT29, MCF7 and MDA-MB231 cells. **b)** Glucose uptake in MCF7 cells (0.6 mM, 4 h). Right; microscopic fluorescence image of 2-NBDG fluorescence, Left; mean - values of 4 biologically independent replicates. (N = 4, error: SD). **c)** Catalytic activity of selected glycolysis and pentose phosphate pathway enzymes. The metabolic network features the metabolites of glycolysis and PPP, colour coded in green for enriched and red for depleted. The enzymes, whose activity was measured, are depicted in bold. The bar-plots show fold changes of enzymatic activities relative to non-treated controls for MCF-7 and MDA-MB231 cells (N = 3 or 4, SD). **d)** Western-blot showing AMPK and consequent ACC phosphorylation in total protein extracts of MCF7 cells upon a 4-h 6 mM ascorbate treatment. The bar-chart features the mean - values of quantified phospho-AMPK and pospho-ACC signals (adjusted to loading control), from 4 biologically independent replicates blotted on the same membrane (N = 4, error-bars: SD). **e)** western-blot featuring AMPK and ACC phosphorylation in total protein extracts of MCF7 upon 4 h treatment with a gradient of ascorbate concentrations. The bar-chart shows the quantified signal intensities for each target protein adjusted to the loading control. Signals for phospho-AMPK were double normalized to account for both total-AMPK and beta-actin as controls. **f)** Western-blot featuring AMPK and ACC phosphorylation in total protein extracts of MDA-mb231 upon treatment with a gradient of ascorbate concentrations, compared to AICAR as a positive control for AMPK induction, The bar-charts feature the quantified signal intensities of both phosphorylated AMPK and ACC, signals were normalized to respective beta-Actin band intensities and to the normalized value of the non-treated (non-treated = 1). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to ascorbate in this very glycolytic cell line, and also emphasizes breast cancer as a good candidate for metabolic rewiring by ascorbate. Moreover, using the fluorescently labelled glucose analogue 2-NBDG, we observed clear and statistically meaningful inhibition of glucose uptake in MCF7 cells measured over 4 h of treatment with 3 mM ascorbate (Fig. 5 b).

In contrast to other upper glycolytic metabolites, Glucose-6-phosphate was less enriched in MCF-7 and slightly depleted in MDA-MB231 following ascorbate treatment (Fig. 5 a). This adds up well with the observed strong accumulation of the pentose phosphate pathway metabolites. Thus, upon ascorbate treatment glucose-6-phosphate is funnelled away from the disrupted glycolytic pathway to fuel the oxidative branch of the pentose phosphate pathway (Fig. 5 a). To further validate this finding we measured the enzymatic activity of selected PPP and glycolysis enzymes in the native protein lysates of ascorbate treated cells. Indeed, we detected a meaningful induction in the enzymatic activity of G6PD and 6PGD, catalysing the first and third steps of the oxidative PPP, respectively (Fig. 5 c), which further corroborates the suspected PPP induction as an oxidative eustress response. On the other hand, the catalytic activity of the glycolytic enzymes GAPDH and TPI was unchanged by treatment, in contrast with earlier reports. Additionally, the LC-MS relative quantification also confirmed the increase in oxidised glutathione, depletion of NAD^+ and NADH (Fig. 3 a) (corresponding to a depletion in the NAD(H) pool) all in accordance with the absolute quantification of these co-factors (Fig. 4), reflecting a depletion of the total NAD pool. Equally remarkable was the severe depletion of ophthalmic acid, a glutathione analogue involved in redox homeostasis, in both MDA-MB231 and MCF7 cells, even below the detection level in the former cell line (Fig. 5 a). Taken together, we provide both metabolic and enzymatic evidence for an upregulated pentose phosphate pathway (Fig. 5 a&c), and clear evidence for impeded glycolysis on the metabolite level (Fig. 5 a). Additionally, we have clear evidence from two independent methods for NAD(H) depletion upon ascorbate treatment in all tested cell lines. We also showed that NAD^+ ameliorated ascorbate effects on both MDA-MB231 and MCF-7 cells. In light of the above mentioned evidence and an absence of a clear inhibition of the enzymatic activity of either GAPDH or TPI, NAD^+ depletion remains the most likely mechanism for the observed disruption in glycolysis.

2.11. Ascorbate mediated energy stress is reflected in activated AMPK signalling

To further elucidate the cellular response to the severe disruption of glycolysis by ascorbate, which at higher concentration leads to a clear reduction of ATP, as described above, we next analysed energy stress signalling following ascorbate-induced glycolysis inhibition. AMPK is the intracellular AMP/ATP sensor, rewiring metabolism towards maximal ATP production [65]. High concentration of ascorbate induced strong AMPK phosphorylation (activation), and downstream phosphorylation of ACC (Fig. 5 d). The quantification of 4 biologically independent replicates of MCF-7 protein lysates yielded statistically valid phosphorylation of both AMPK and ACC (Fig.5 d, Fig.S8 a). This reflects the ascorbate-induced energy-stress as part of its cytotoxic mechanism. Furthermore, low concentrations of ascorbate (0.6 mM and 0.3 mM) also induced AMPK and ACC phosphorylation without affecting total AMPK abundance (Fig. 5 e).

It is important to note that the observed effects with lower concentrations of ascorbate are largely dependent on cell density with higher concentrations required for comparable effects in more confluent cells. Intriguingly, a slight down-regulation of the glucose transporter Glut1 was observed (Fig. 5 e), consistent with recent findings [29]. We also asked if PPP induction is resulting from an increased G6PD expression. However, no increase in G6PD protein levels was observed indicating that the increased enzymatic activity is purely post-translational (Fig. 5 e). The induction in energy stress signalling is in harmony with the depleted ATP levels at higher concentrations of ascorbate (Fig. 2 d) and in agreement with earlier reports demonstrating ATP depletion due to increased utilisation by PARP-1 [66].

2.12. Ascorbate treatment shifts DHAP from glycolysis towards glycerol synthesis and increases lipid droplet formation

In the absence of measurable GAPDH or TPI inhibition, we postulated that the interruption in glycolysis could result from DHAP utilisation for increased glycerol synthesis. Increased de-novo fatty acid biosynthesis, incorporation in triglycerate, and lipid droplet formation [67]. Moreover, increased fatty acid biosynthesis and beta-oxidation were reported in regressed breast tumours and minimal residual disease MDR [68]. Indeed, following over-night recovery from a 2-h exposure to 3 mM ascorbate, *GPD1* (glycerol-3-phsophate dehydrogenase 1) transcription was induced in both MDA-MB231 and MCF-7 (Fig. 6 a). Treatment with a combination of ascorbate and oleate, a precursor for lipid storage, additively increased Gpd1 levels (Fig. 6 b). GPD1 catalyses the conversion of glycolytic DHAP into GA3P to make glycerol, an essential precursor for triglycerides synthesis. To investigate the possibility of an increased lipid droplet accumulation also accompanied the ascorbate induced cytotoxicity, we used the Bodipy™(493/503) dye with reported specificity to neutral lipid droplets [69]. Indeed, Bodipy™ staining showed increased lipid droplet formation in both MCF-7 and MDA-MB231 cells following over-night recovery from a 2-h treatment with 3 mM ascorbate (Fig. 6 c and d). The observed lipid droplet accumulation exceeded that observed upon oleate treatment as positive control, and adding oleate during recovery from ascorbate treatment further induced both lipid droplet accumulation and *GPD1* expression.

2.13. MCF-7 cells exhibit substantially decreased recovery rates from ascorbate treatment compared to MDA-MB231 and human fibroblasts

Since tumour cell persistence and consequently tumour recurrence is a major limitation of most cancer treatments, we further assessed the clonogenic survival of the two breast cancer cell lines, MCF-7 and MDA-MB231 and in addition human fibroblasts HFF-2 as a model for non-cancerous cells. As a proxy for clonogenic survival following exposure to concentrated ascorbate, we measured the growth and colony formation of cells passaged at low cultivation density upon ascorbate treatment. To our surprise, MCF-7 demonstrated distinct sensitivity to the utilized 3 mM ascorbate. This was visible in diminished growth of ascorbate pre-treated MCF-7 cells at days 3 and 7 upon treatment (Fig, S12 a and b). On the other hand, both MDA-MB231 and HFF-2 cells showed no significant growth inhibition upon ascorbate exposure. While we predicted the differential sensitivity between normal and cancerous cells, the variability in response between MCF-7 and MDA-MB231 cells is somehow surprising. It highlights potential variability in response to ascorbate based on the molecular type of the tumour.

3. Discussion

Ascorbate is an essential nutrient and a widely used supplement with a broad margin of safety [70]. This suggests maximum benefit of any additional applications of this molecule. Albeit controversial, the prospect of ascorbate use in cancer treatment has seen a revival with molecular, cellular and *in-vivo* evidence for its potential efficacy [18,20,22,24,29,41,56]. This renewed interest has catalysed various on-going clinical trials [71,72]. Previous reports attributed the selective toxicity of ascorbate megadoses towards cancer cells to varying possible mechanisms [72]. Those can be summed up in two major models, extracellular or intracellular [22,25]. The former suggests an abundance of hydrogen peroxide generated by the metal-catalysed auto-oxidation of ascorbate [73] selectivity against cancers depends upon the elevated oxidative burden in tumours [74]. The second model requires the

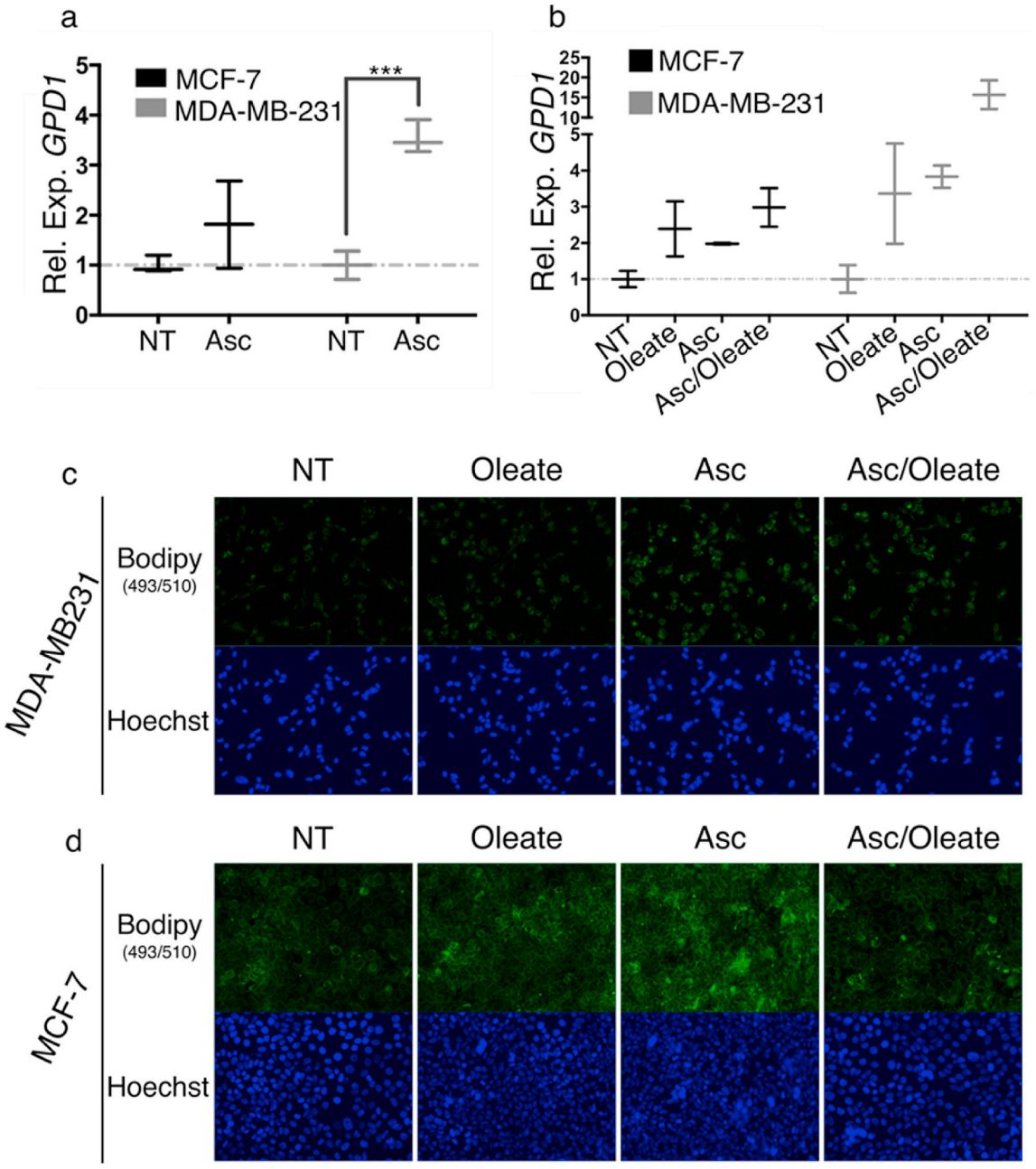

Fig. 6. High dose ascorbate treatment diverts glycolytic intermediates towards glycerol biosynthesis and induces lipid droplet formation. a) Relative mRNA levels of *GPD1* measured using real-time qPCR in MCF-7 and MDA-MB231 upon 18-h recovery following a 2-h exposure to ascorbate 3 mM. Depicted are relative expression values (based on a combined housekeeping factor of beta-Actin and RPL30) of three biologically independent replicates (N = 3). b) Relative mRNA levels of *GPD1* measured using real-time qPCR in MCF-7 and MDA-MB231 cells upon treatment with oleate, recovery from ascorbate treatment, and combined Ascorbate/Oleate treatment. Depicted are the relative expression values (based on RPL-30 as a housekeeping gene) of two biologically independent replicates and the mean - value (N = 2). **c&d)** Fluorescent microscopy of Bodipy™ stained MDA-MB231 and MCF-7 cells showing lipid droplet formation in cells following over-night recovery from a 2-h exposure to 3 mM Asc. An over-night Oleate 60 μM treatment was used as a positive control. The panel Asc/Oleate depicts cells treated with 3 mM ascorbate for 2 h, followed by 18 h of 60 μM oleate (added in the recovery phase).

cellular uptake of ascorbate, where it tips the redox homeostasis [22].

An intracellular mechanism depending on the selective uptake of DHA by cancer cells has been recently posited to explain the remarkable *in-vivo* efficacy of ascorbate against colon cancer in rats [22]. Moreover, the presence of the vitamin C transporter SVCT2 across various organs and tissues [1] suggests a mechanism of direct uptake of ascorbate.

In this work, we provided evidence indicating the efficacy of ascorbate against cell lines representing the two major molecular subtypes of breast cancer. Our results signify higher sensitivity of breast cancer cells compared to colon cancer cells (Fig. 1), a type of cancer that has already been shown sensitive to high doses of ascorbate [22]. We observed rapid cell death in all cell lines treated with ascorbate in addition to apoptotic hallmarks specifically in MCF-7 cells.

We established that elevated ROS is a common consequence of ascorbate treatment across all tested cells. Moreover, various ROS scavengers hindered the cytotoxicty, emphasising the indispensability of oxidative stress for the efficacy of ascorbate against cancer cells.

Metabolically, we provided evidence for a rewired glucose metabolism upon exposure to high concentrations of ascorbate. This coincided with an energy crisis, manifested by glycolysis disruption (Fig. 5 a,

b, c), ATP depletion (Fig. 2 d), and activated AMPK signalling (Fig. 5 e, f&g). It has been postulated that oxidative damage to GAPDH could underpin the glycolytic inhibition. Similar oxidative disruption of the triose-phosphate isomerase TPI was identified as an adaptive mechanism to oxidative damage in yeast [74]. Nevertheless, we were unable to detect any irreversible loss in the activity of either GAPDH or TPI (Fig. 5 c). This absence of the hypothesised GAPDH inhibition was also reported upon ascorbate individual treatments and in combination with menadione [75]. However, in agreement with earlier reports [18,22,30,59,63], we observed severe NAD^+ and total NAD(H) depletion upon ascorbate treatment, NAD^+ is the co-factor for the oxidation of GA3P and is therefore indispensably required for the later stages of glycolysis to occur. We also showed that the depletion of glycolytic metabolites (Fig. 5a) plausibly coincides with increased utilisation of DHAP by the enzyme GPD1 (Fig. 6a&b), potentially to generate glycerol for esterification in triglycerides as indicated by increased formation of lipid droplets upon treatment (Fig. 6 c&d). This diversion of metabolic intermediates towards glycerol synthesis also contributes to the inhibition of glycolysis and the observed metabolic rewiring, especially the depletion of all glycolytic intermediates downstream of DHAP. As predicted we also observed induction of the pentose phosphate pathway, a well characterized response to oxidative stress. Consistently, the levels of NADPH were sharply depleted upon exposure to ascorbate and this coincided with an increase in glutathione disulfide, both signifying an oxidative crisis triggered by ascorbate treatment.

Regarding the overall mechanism of action of ascorbate, our findings indicate a role of extra-cellular hydrogen peroxide generated by the partial oxidation of ascorbate in the medium [27,41]. Our measurements indicated a dose-dependent sigmoidal release of H_2O_2 in the DMEM medium with increasing ascorbate concentrations. This formation was independent of the presence of cells in the medium, and was not observed in pure water. The omission of FCS from the medium had no measurable effect of hydrogen peroxide formation from ascorbate (Fig. 3h), neither did the addition of EDTA to the medium (Fig. S13), which consists with the reported oxidizing activity of ferrous-EDTA complexes [76]. The improved tolerance to ascorbate in presence of extracellular catalase confirms the participation of hydrogen peroxide in the observed cytotoxicity of ascorbate. The implications of extracellular hydrogen peroxide on ascorbate's cytotoxicity must be taken very cautiously when considering in-vivo contexts. The use of ascorbate as a pro-drug to deliver lethal concentrations of hydrogen peroxide to tumour cells in-vivo is hindered by the anti-oxidant capacity of the blood [41].

The cytotoxic effects of DHA are much milder than those of ascorbate, and only apparent at concentrations above 10 mM. Consistently, we observed no induction of AMPK signalling with DHA compared to ascorbate, indicating an absence of energy stress in cells exposed to DHA. To rule out the effect of different stability between ascorbate and DHA as a contributor to these aforementioned differences, we assessed the cellular uptake of both molecules. Ascorbate was detected intracellularly in both ascorbate and DHA treated cells (Fig. S4), which implies that cells take up both ascorbate and DHA, with DHA being reduced inside the cells back to ascorbate. Future studies should further explore the intracellular fate of ascorbate and DHA to elucidate the dynamics of conversion between ascorbate and DHA and the interplay with other intracellular redox pairs.

Altogether, our findings indicate multiple mechanism of ascorbate cytotoxicity depending on both the extracellular H_2O_2 and the cellular uptake of ascorbate. The concern of potential haemolysis by intravenous ascorbate due to H_2O_2 is mitigated both by the evident safety in clinical trials and the finding that ascorbate generates H_2O_2 preferentially in the extracellular fluid. Nevertheless, caution should be exercised in patients with G6PD deficiencies.

A distinct cytotoxic role for DHA is ruled out based on the absence of cytotoxicity despite its verified entry into the cells. Oxidative stress is essential for cytotoxicity, with ROS accumulation triggering a general metabolic rewiring, energy crisis and rapid cell death. Ascorbate megadoses disrupt the oxidative and metabolic homeostasis, and induce rapid cell death in both MCF-7 and MDA-MB231 cells with activation of apoptotic markers in luminal breast cancer cells MCF-7 and an absence of these markers in basal-like breast cancer MDA-MB231. It has not escaped our attention that ascorbate exerts its cytotoxic effect by tapping into mechanisms that disrupt the fundamental oxidative, energetic, and genetic homeostasis of cancer cells. This advantage of intravenous ascorbate, along with its clinically established tolerance in combination with conventional and novel treatments of various cancers, gives it the potential of becoming a versatile treatment targeting the most common vulnerabilities of cancer cells from diverse tissue origins and molecular subtypes. This was also demonstrated in the efficacy of ascorbate against cells from two distinct subtypes of breast cancer. Similar metabolic and oxidative disruptions followed by rapid cell death were observed in both MCF-7 and MDA-MB231 cells, despite the apparent difference in cell death mode. Another advantage of ascorbate megadoses is the convergence in its cytotoxic mechanism with other well-established treatments used in cancer. The demonstrated NAD^+ depletion is a shared outcome of all DNA damaging treatments [77–79], especially in the context of BRCA mutations in breast cancers leading to increased reliance on PARP and increased NAD^+ depletion [60,80]. Consistently, we provided ample evidence, based on two independent quantification methods, for NAD^+ depletion upon ascorbate exposure. Moreover, we also provided evidence for the pivotal role of this NAD^+ depletion as a major link between the ascorbate induced oxidative cytotoxicity and the metabolic rewiring. Indeed, simultaneous NAD^+ and ATP depletion has been recently reported in pancreatic cancer cells due to consumption by PARP-1 over-activation in response to oxidative DNA damage resulting from highly concentrated ascorbate [63]. Meanwhile, combining ascorbate with other oxidative treatments yielded promising results regarding the synergy of these treatments in lethally tipping the redox homeostasis of cancer cells. We observed this with menadione (data not shown) and it has been already reported in combinations of ascorbate and auranofin [66]. Furthermore, fresh evidence demonstrated an ROS-mediated synergy of ascorbate with the combination of chemotherapy and fasting mimicking diets, here too the alleviation of oxidative stress reverted the cytotoxic effects of ascorbate [64].

The therapeutic window arising from a differential sensitivity of cancer cells towards ascorbate's oxitative cytotoxicity has been thoroughly addressed [24,25,41]. In this work we briefly compared the clonogenic survival upon ascorbate treatment in MCF-7, MDA-MB231 *vs* the non-cancerous human fibroblasts HFF-2 cells. While the full recovery of non-cancerous fibroblast was predicted, we were surprised to observe a substantial difference between MCF-7 and MDA-MB231 cells. This highlights the importance of considering the value of various molecular subtypes and clonogenic origins in predicting the sustained efficacy of ascorbate megadosing. These factors should be addressed thoroughly in future work with focus on *in-vivo* sensitivity of tumours in pre-clinical models, but they lie beyond the scope of this study.

In this work we questioned, validated, and added to the growing evidence regarding the cytotoxic effects of ascorbate on breast cancer cells. In light of the recently reported safety of previously unexplored high doses of ascorbate, and their tolerability in combination with conventional cancer therapies in addition to the convergence and synergy with other treatments, we assert that the use ascorbate megadoses in breast cancer warrants further molecular, pre-clinical, and clinical investigation.

We think that the scope of investigating the use of concentrated ascorbate in cancer treatment should expand beyond the current scope of it being experimental non-conventional last resort for a few patients with mostly incurable types of cancer and worst prognoses [18,19,23,41,47] to explore the potential of this treatment as an adjuvant therapy that could improve the lives and outcomes of numerous cancer patients at a minimal cost and risk.

4. Conclusions

We have demonstrated that megadoses of ascorbate induce rapid cell death in breast cancer cells. The mode of cell death deviated between luminal MCF-7 and basal-like MDA-MB231 cells indicating a possibly relevant difference between the two tumour subtypes. In both cell lines, ascorbate induced a metabolic shift from glycolysis towards PPP, glycerol synthesis, and increased lipid droplet formation. The TCA-cycle was also disrupted, but oxygen consumption and mitochondrial polarisation increased, most likely due to the depletion of NAD^+. Both ascorbate uptake by the cells and hydrogen peroxide release into the medium upon ascorbate addition were observed, with uptake being only partially necessary for efficacy. We are currently working on discerning the extracellular and intracellular effects of ascorbate, further identifying the intracellular fate of ascorbate upon uptake, and probing the sequence and interdependency of the rapid oxidative, genotoxic, and metabolic events triggered by ascorbate treatment.

In this work we validated and added to the growing evidence regarding the cytotoxic effects of ascorbate on breast cancer cells. In light of the recent evidence of the safety, well tolerability in combination with conventional cancer therapies, and molecular findings suggesting convergence and synergy with other treatments, we believe that the potential of ascorbate megadoses should be fully investigated with the goal of rationally using pharmacological ascorbate to bolster our arsenal in the battle against cancer with a novel, well characterised, affordable, and tolerable intervention.

5. Material and methods

Cell culture: the cell-lines MCF-7, MDA-MB231, HT-29, HFF-2, and HCT116 (ATCC) were incubated at 37° and 5% CO2. All passaging, cultivation, seeding and treatments were performed in Dublecco's modified eagle medium DMEM (Gibco, Germany) for details refer to **Supp. Methods I**.

MCF-7 and MDA-MB231, HT-29, and HCT116 cells (ATCC) were obtained in 2012, preserved in liquid nitrogen and utilized for experiments in passage numbers between 15 and 30. Cells were also tested for mycoplasma. Further details in **Supp. Methods I**.

SRB Survival and growth assays: To assess cell survival and growth, the total-protein based SRB colorimetric assay was used in a protocol derived from Vichai and Kirtika (2006) [81] as previously reported in Refs. [82,83]. Details in **Supp. Methods II**.

Flow cytometry analysis: FACS analysis was used to measure a variety of metabolic and viability markers in the cells as previously reported in Ref. [84,85]. Cells were seeded in 12 well-plates (50,000 cells/well, 1 ml medium and incubated over-night and treated on the following day. Following treatment, cells were incubated with one of the fluorescent probes: 2-NBDG, DHE, AnnexinV/PI (details and conditions for each fluorescent probe are listed in the supplementary methods) and analysed using FACS Guava (Merck-Millipore, UK). All FACS acquisitions were automated and performed in a 96 well-plate setting. Details in **Supp. Methods III**.

5.1. Protein extraction, total protein measurement, SDS-PAGE and immunoblotting

To detect the effects of ascorbate on protein expression and post-translational modifications we separated denatured proteins based on molecular weight using SDS-PAGE. Subsequently, proteins were plotted from the SDS-PAGE gels onto PVDF membranes and immunostaining via initial incubation with a primary protein-specific antibody followed by a secondary species-specific HRP-conjugated antibody. Signals were developed using chemiluminescence (ECL reagent) **Supp. Methods V**.

List of the utilized antibodies.

Target	Source organism	Provider/catalogue number
PARP	Rabbit	Cell Signaling® #9542
Caspase-3	Rabbit	Cell Signaling® #9662
AMPKα (pan)	Rabbit mAB	Cell Signaling® #5831
Phospho_AMPKα (Thr172)	Rabbit mAB	Cell Signaling® #2535
Phospho-ACC (Ser79)	Rabbit mAB	Cell Signaling® #11818
Glut1	Rabbit mAB	Cell Signaling® #12939
B-Actin	Mouse mAB	Cell Signaling® #3700
Vinculin	Rabbit	Cell Signaling® #4650

5.2. Liquid chromatography-mass spectrometry based metabolite quantification

Intracellular metabolites were extracted and quantified using an LC-MS based method as previously described in Ref. [86]. Details in **Supp. Methods VI**.

Real-time monitoring of oxygen consumption (oxygen in culture medium): we used a quenchable fluorescence sensor-based system OxoDishes® with the compatible reader system SDR (PreSens, Germany) to measure oxygen in the medium and eventually the effects of tested conditions on cellular oxygen consumption. Details in **Supp. Methods IX**. The real-time measured levels of oxygen in medium were plotted using the TReCCA analyzer [87].

Real-time monitoring of cellular respiration, acidification (glycolysis) and impedance in the Bionas flow-through system:

For the analysis of changes in hallmarks of cellular metabolism and survival upon ascorbate treatment the Bionas 2500 biosensor chip system (Bionas, Rostock, Germany) was used as formerly described [84,88]. Details in **Supp. Methods X**.

Measurements of cellular ATP production a one-step luciferase-based kit ATPLite-1step (PerkinElmer®, Weltham, USA) was used. Details in **Supp. Methods IV**.

Real-time fluorescence microscopy (IncuCyte®).

We used the IncuCyte® live cell analysis system to investigate:

Mitochondrial superoxide formation in real time using the MitoSox dye.

Caspase induction, apoptotic and cell death markers using IncuCyte® fluor, reagents: Caspase3/7 Green, AnnexinV Red and Cytotox red, respectively. Details in **Supp. Methods VII**.

Determination of hydrogen peroxide in the culture medium.

The concentrations of H_2O_2 in culture medium were determined using the Amplex™ Red Hydrogen Peroxide kit (Thermo Fischer). Details in **Supp. Methods VIII**.

Catalytic activity measurements of glycolytic and oxidative PPP enzymes.

Using spectroscopic measurements we determined the activities of GAPDH, TPI, PK, G6PD and 6PGP. Details in **Supp. Methods XI**.

Fluorescent microscopyKeyence BZ-9000 fluorescent microscope was used to image cells with the following fluorescent probes/applications; 2-NBDG for glucose uptake, DHE for ROS accumulation, and Bodipy™ 493/510 for neutral lipid droplets. Details in Supp. Methods XII.

Real-time quantitative PCR rt-qPCR.

For the quantitative PCR the qTOWER real time thermo cycler and the LightCycler® Sybr Green (Roche®) reaction mix were utilized.

Utilized primers:

GPD1 For: CATCAACACGCAGCATGAGAA; Rev: GATGGGGCACCACAAAGATCA

β -Actin For: CATTCCAAATATGAGATGCGTTCG; Rev: GCTATCACCTCCCCTGTGTG.

RPL-30 For: GGTGTCCATCACTACAGTGG; Rev: GTCAGAGTCACCTGGATCAATG.

The detailed protocol and method of the relative quantification of expression levels are in the **Supp. Methods XIII.**

Absolute quantification of Glutathione, NAD(H), and NADP(H).

The following luminescence based kits GSH/GSSG-Glo™ Assay, NAD/NADH- Glo™ Assay, and NADP/NADPH- Glo™ Assay (all provided by Promega Corporation), were used for the absolute quantification of the oxidised and reduced fractions of glutathione, NAD(H), and NADP(H), respectively. In short, cultivated cells were either treated with 3 mM ascorbate or had medium replaced, upon 2 h incubation, the oxidised and reduced fractions of each of the abovementioned co-factors were separately quantified, and the total and relative oxidised fractions calculated from the measurements.

The assay-concepts and detailed methods are in the Supp. Methods XIV.

Assessing clonogenic survival upon ascorbate treatment.

To assess the recovery from ascorbate treatment, cells were seeded in 6 well-plate at a density of 500000 or 300000 cell/well and allowed to grow and form a tissue like structure for 48 h. Subsequently, cells were treated with 3 mM ascorbate in DMEM vs DMEM with mock treatment. Upon 24 h of treatment, both ascorbate and mock treated cells were dissociated, counted, and seeded at 1500 cells/well. Subsequently, cells were given a recovery time of 3 or 7 days and then fixed and stained using the abovementioned SRB protocol **(Supp. Methods II)**.

The magnitude of recovery and growth upon treatment was quantified by measuring the SRB absorbance and compared to non-treated cells.

Declaration of competing interestCOI

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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