# Influence of environmental distinctions on prooxidant properties of ascorbic acid and utilization of optical imaging tools for assessment

Ph.D. thesis

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List of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
$Asc^{\bullet-}$	Ascorbate (ascorbyl) radical, semidehydroascorbate
Asc^{2-}	Ascorbate dianion
$AscH^{\bullet}$	Neutral ascorbyl radical
$AscH^{-}$	Ascorbate monoanion
$AscH_2$	Ascorbic acid
ATP	Adenosine triphosphate
BCC	Basal cell carcinoma
BCNS	Basal-cell nevus syndrome
CFU	Colony forming units
DHA	Dehydroascorbic acid
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
FAD	Flavin adenine dinucleotide (oxidized form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FDG	2-deoxy-2-[18F]fluoro-D-glucose
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Facilitative glucose transporters
GSH	Glutathione (reduced form)
GSSG	Glutathione disulfide (oxidized form)
H_2O_2	Hydrogen peroxide
HIF	Hypoxia-inducible transcription factor
$HO^{\bullet}$	Hydroxyl radical
HPF	3′-(p-Hydroxyphenyl)-fluorescein
Hsp90	Heat shock protein
IVA	Intravenous pharmacologic ascorbic acid (as prooxidant)
KRAS	Kirsten rat sarcoma viral oncogene
MITF	Melanocyte lineage-specification transcription factor
mTOR	Mammalian target of rapamycin
$NAD(P)^{+}$	Nicotinamide adenine dinucleotide (phosphate) (oxidized form)

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NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) (reduced form)
Nd:YLF	Neodymium-doped yttrium lithium fluoride
NNT	Nicotinamide nucleotide transhydrogenase
NO	Nitric oxide
NTBI	Non-transferrin bound iron
$O_2^{\bullet-}$	Superoxide
$^1O_2$	Singlet oxygen
P-Asc	Pharmacologic ascorbic acid (as prooxidant)
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PGC1 α	Proliferator-activated receptor-gamma coactivator-1alpha
pKa	Cologarithm logarithm of the dissociation constant (K) of an acid
PTCH	Patched
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SHG	Second harmonic generation microscopy
Shh	Sonic hedgehog
Smo	Smoothened
SVCT	Na^+-dependent Vitamin C transporters
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TfR1	Transferrin receptor protein 1
TGF-β	Transforming growth factor-β
TPEFM	Two-photon excitation fluorescence microscopy
UV	Ultraviolet
VEGF	Vascular endothelial cell growth factor
VHL	von Hippel-Lindau
YPD	Yeast extract-peptone-dextrose
YPG	Yeast extract-peptone-glycerol

1. Introduction

1.1. Ascorbic acid – an overview

1.1.1. A brief history

During the Age of Discovery, which corresponds to years between 1500 and 1800, sailors spent at least 3 months continuously at the sea. Due to lack of access to fruits and vegetables for such long periods of time, more than 2 million sailors died of nutritional diseases such as beriberi, pellagra and scurvy, caused by thiamine (Vitamin B1), niacin (Vitamin B3) and ascorbic acid (Vitamin C) deficiency, respectively (1-3). Among these diseases, scurvy was the most frequently encountered one that an English sea captain Sir Richard Hawkins described as the ''plague of the sea'' (1). Although captains and naval surgeons were highly convinced that citrus fruits could cure scurvy, most physicians denied this theory for several years (4).

In 1907, two Norwegian physicians named Axel Holst and Theodor Frolich developed an interest in investigating the factors that led to a ship-related dietary disease then called ''shipboard beriberi'' (5). In an attempt to accomplish this, they fed guinea pigs with a diet based on various types of grains. Although their attempts to develop beriberi failed; to their surprise, they observed the classical features of scurvy (5). Moreover, symptoms of scurvy were resolved upon addition of ''anti-scorbutics'' such as fresh cabbage or lemon juice into their diet (5). This serendipitous discovery would open new horizons in Vitamin C research together with long lasting controversies over who deserved to be nominated for the Nobel Prize (6). In 1912, Casmir Funk, a Polish biochemist proposed that scurvy, beriberi, pellagra and rickets were due to dietary deficiencies of factors which he referred to as 'vitamines', a term that derived from 'vita': life and 'amine': nitrogen containing compound (7, 8). More than a decade later, in 1927, a Hungarian physician named Albert Szent-Gyorgyi discovered and isolated a substance called ''hexuronic acid'' first from the plants at Cambridge University, then from the adrenal gland at Mayo Clinic in Rochester, USA (9-11). Upon his return to Hungary, he asked Joseph Svirbely, an American-born Hungarian post-doctoral fellow in his lab at the University of Szeged, to test whether this crystalline compound protected guinea pigs from scurvy (9, 12). Svirbely's experiments demonstrated that

hexuronic acid was indeed an antiscorbutic factor, today known as Vitamin C. Around the same time, former mentor of Svirbely, Charles Glen King and William A. Waugh from the University of Pittsburgh, were working on isolation and crystallization of Vitamin C from the lemon juice. They have subsequently demonstrated that its daily administration protected guinea pigs from scurvy (13, 14). This generated a bitter dispute over priority, which would escalate in 1937 when Szent-Gyorgyi became the recipient of the Nobel Prize in Physiology or Medicine, in part, for isolating Vitamin C (9). Not long after, Szent-Gyorgyi succeeded to extract Vitamin C samples from paprika peppers and sent them to Walter Norman Haworth, who was at the time a Professor of Chemistry at the University of Birmingham. Haworth and his group determined the chemical structure of Vitamin C and due to its anti-scorbutic properties, together with Szent-Gyorgyi, they decided to rename it as L-ascorbic acid (15-20). Subsequently, Vitamin C was synthesized independently by both Tadeus Reichstein in Switzerland and Haworth's group in England and from that point, it could be produced on a large scale for medical use (16, 21). This work earned Haworth the Nobel Prize for Chemistry the same year with Szent-Gyorgyi.

1.1.2. Chemistry and Biochemistry

Ascorbic acid (L-ascorbic acid, $AscH_2$, Vitamin C) is a ketolactone with a chemical formula of $C_6H_8O_6$ and molecular mass of 176.12 g/mol. As a hexose sugar derivative, it shares a structural similarity to glucose. It is a water soluble, weak organic acid and has two pKa's: pK1=4.2 and pK2=11.6 (22, 23). It is a strong reducing agent (antioxidant) by virtue of two enolic hydrogen atoms that it entails (24). Ascorbate monoanion ($AscH^-$) is the most abundant form at physiological pH (22, 23). When $AscH^-$ donates a hydrogen atom (H^+) or electron (e^-) it forms ascorbate dianion (Asc^{2-}) or neutral ascorbyl radical ($AscH^\bullet$), respectively (25). $AscH^\bullet$ has a pKa of -0.86, and for this reason, it is present as an ascorbate radical (semidehydroascorbate, ascorbyl radical, $Asc^{\bullet-}$) at physiological conditions where pH is around 7.4 (25). Asc^{2-} can also be transformed into $Asc^{\bullet-}$ upon losing an e^-. $Asc^{\bullet-}$ is a relatively unreactive free radical and it can be converted back to $AscH^-$ by utilization of certain enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase or nicotinamide adenine dinucleotide (NADH) dependent-cytochrome b5 reductase. (26-30). Alternatively, two $Asc^{\bullet-}$ molecules form a dimmer that undergoes a

series of reactions, including a reversible disproportionation (31). This results in generation of AscH⁻ and dehydroascorbic acid (DHA) (26, 27, 31) (Figure 1) (Net reacion: $2Asc^{\bullet-}+H^+ \longleftrightarrow AscH^-+DHA$). DHA can be irreversibly hydrolysed to 2,3-diketogulonic acid but can also be recycled back to AscH⁻ either directly by glutathione (GSH) itself or, by GSH or NADPH dependent enzymes such as thioredoxin reductase (26, 27, 32-35).
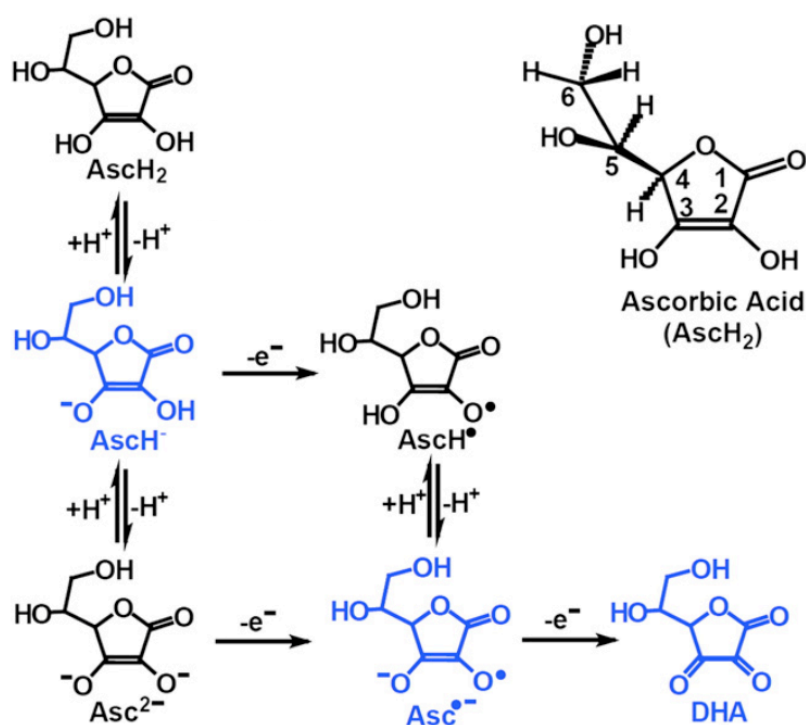
Figure 1: Ascorbic acid oxidation and recycling. Adapted and modified from (23).

Although oxidation of ascorbic acid is a slow process, its anionic forms have variable rates of oxidation (36). In general, rate of oxidation depends on the pH and the presence of catalytic metals (37, 38). At physiological pH, $AscH_2$ and Asc^{2-} coexist with AscH⁻, but the total concentration of $AscH_2$ and Asc^{2-} is less than 0.2% (23). An increase in pH, leads to an increase in the amount of Asc^{2-}, which is in turn accompanied by an increased rate of oxidation resulting in generaton of $Asc^{\bullet-}$ and superoxide ($O_2^{\bullet-}$) (38). Literature suggests that true autoxidation of AscH⁻ is indeed very slow and according to

Williams and Yandell, Asc^{2-} is the only Vitamin C species, which would go through a significant autoxidation (36, 38).

1.1.3. Absorption, Cellular Uptake and Excretion

Through evolution, humans lost their capacity to synthesize ascorbic acid de novo. This is due to a mutation in L-gulono-γ-lactone oxidase gene, which encodes L-gulono-γ-lactone oxidase enzyme that is required for the last step of ascorbic acid synthesis (39). Hence, we are dependent on the dietary intake for obtaining adequate amounts of this essential vitamin. Upon ingestion, ascorbic acid is absorbed through the brush border cells of the small intestine. This takes place via two major mechanisms; such that ascorbate is taken up via Na^+-dependent Vitamin C transporters (SVCT) and its two-electron-oxidized form DHA is taken up via facilitative glucose transporters (GLUT), mainly GLUT1 and GLUT 3 (40-43).

Two isoforms of SVCT mediate the transport of ascorbate; SVCT1 and SVCT2. SVCT1, commonly called the ''bulk transporter'', resides largely in apical brush border membranes of enterocytes and renal tubular cells, which are responsible for absorption and re-absorption, respectively (42, 44, 45). SVCT2 has a broader distribution and can be found in osteoblasts, platelets, cardiac, neural, neuroendocrine, exocrine and endothelial tissues (42, 45, 46). SVCT2's activity seems to be crucial for protection against oxidative injury (47, 48). Unlike most tissues, epidermal cells express both SVCT1 and SVCT2 while dermal cells express SVCT2 only (49). Bioavailability of ascorbate is tightly controlled via regulation of SVCT expression. For example, when its levels are elevated in the intestinal lumen, SVCT1 is downregulated in enterocytes (50). Similarly, when intracellular ascorbate levels are reduced in lung epithelial cells, SVCT2 expression is increased (51). In vitro and in vivo studies have shown that hormones, paracrine factors, oxidative stress and intracellular signaling molecules also play a role in regulation of expression of SVCT (47, 52-54).

Affinity of GLUT to DHA is relatively low when compared to SVCT's affinity for ascorbate (41, 55). Once DHA enters the cells, it is then immediately converted to ascorbate (41). However, there are exceptions to this case; for instance oxidative stress may hamper the accumulation of intracellular ascorbate from DHA (56). Presence of glucose may influence DHA uptake, as they compete for the same GLUT transporters, but this phenomenon varies between cell types (41, 56-60). Although through a

different mechanism, Na$^+$ dependent ascorbate transport was also shown to be modulated by glucose (55, 61). In addition to an endocrine regulated DHA transport, modulatory effects of intracellular signaling molecules and oxidative stress on DHA uptake have also been reported (53, 59, 62). However, it must be noted that SVCT and GLUT transporters may respond differently to the same hormone or cytokine. An intriguing example is the regulatory effects of transforming growth factor-β (TGF-β) on osteoblastic cells (63). While an increased rate of Na$^+$-ascorbate cotransport activity is induced by TGF-β, no changes in DHA levels were observed in the same cells (63).

Amount of Vitamin C that is absorbed and excreted depends mainly on the intake, bioavailability, metabolism and the route of administration. In a study by Levine et al., a steep curve was observed between 30-100 mg daily doses whereas complete plasma saturation was reached at 1000 mg (64). Likewise, urinary excretion was only observed at and above 100 mg and, at a single dose of 500 mg or higher, although partially absorbed, Vitamin C was substantially excreted (64). Nevertheless, Padayatty et al. demonstrated that, one can achieve a higher Vitamin C concentration in plasma, by delivering it intravenously rather than orally (65). In healthy adults, plasma concentration of ascorbic acid is usually in the range of 15-90 μmol (65-68). On the other hand, tissue concentrations vary depending on the tissue and cell type (68-72). For instance, average ascorbic acid contents of adrenal and pituitary gland were reported as 30-40 mg/100g and 40-50 mg/100g, respectively, while that of skeletal muscle was found to be only 3 mg/100g (69, 72).

Excretion of Vitamin C occurs via filtration and tubular reabsorption (73). However, when Vitamin C is presented to the tubules at a rate that is above the maximal rate of tubular reabsorption, excess Vitamin C is excreted in the urine (73). Pathologic conditions may alter stored and excreted levels of ascorbic acid (67, 74, 75). Spellberg and Keeton compared the levels of ascorbic acid excreted in healthy persons and cancer patients (75). They found that after a 400 mg daily ascorbic acid administration, first group excreted 56 to 80% of intake, whereas the latter excreted only 34 to 48% (75). This may be due to metabolic differences in healthy and cancer tissues, which seem to be reflected by lower plasma levels of ascorbic acid in cancer patients (67, 76, 77). Klimant and collegues also propose that a high level of Vitamin C consumption in certain pathological conditions is in part compensated by reduced rate of excretion by the kidneys (67).

As mentioned in the previous chapter, a fraction of DHA is irreversibly converted to 2,3-diketogulonic acid (27, 78). This metabolite can further be degraded into l-erythrulose and oxalate (79). It has been shown that oxalate excretion increases with high ascorbic acid intake (64). When further epidemiological evidence presented in the literature is taken into consideration, high levels of ascorbic acid seem to constitute a risk for oxalate nephropathy, especially when the renal function have been compromised (80-82).

1.1.4. Functions

Ascorbic acid is involved in several fundamental physiological and biochemical processes. Its major and probably the most important role lies in its property as an antioxidant (83). $AscH^-$ readily gives an electron to free radicals such as hydroxyl radical ($HO^\bullet$), $O_2^{\bullet-}$, peroxyl radical, thiol radical, sulphur radicals and tocopheroxyl radical at the expense of generating an $Asc^{\bullet-}$.

$$AscH^- + X^\bullet \rightarrow Asc^{\bullet-} + XH$$
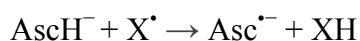
Aside from its antioxidant activities, it is required as a co-factor in synthesis of norepinephrine, serotonin, tyrosine, homogentisic acid, carnitine, hydroxylysine and hydroxyproline. Moreover, it amidates peptides for hormone activation, mediates nitric oxide synthase and hypoxia-inducible transcription factor (HIF) activity, and assists iron absorption in the small intestine (26, 44, 78).

Two amino acids, proline and lysine are among the key components of collagen formation process. Proline needs to be hydroxylated to generate a more stable triple-helical structure of collagen (84). On the other hand, hydroxylysine not only acts as a precursor of the intra- and inter-molecular crosslinking process which gives collagen its tensile strength, but also facilitates the glycosylation process by serving as an attachment site for galactose and glucosylgalactose (85, 86). Hydroxylation of selective proline residues occurs by collagen prolyl-4-hydroxylase and prolyl-3-hydroxylase while lysine residues are hydroxylated by lysyl-hydroxylase (84). These three enzymes and γ-butyrobetaine dioxygenase and trimethylhydroxylase which catalyze the formation of L-carnitine, together with HIF prolyl-4- and asparaginyl- hydroxylases which suppress HIF-1 activity, belong to the family of 2-oxoglutarate and Fe^{2+}-

dependent dioxygenases and require ascorbate either as a co-substrate or to recycle Fe^{3+} back to Fe^{2+} (23, 78, 87-90). Likewise, norepinephrine is synthesized by a copper-containing oxygenase, so called dopamine β-hydroxylase and it does require ascorbate as a co-factor (91).

Hormones and hormone-releasing factors such as gastrin, oxytocin, vasopressin, corticotropin, thyrotropin are initially synthesized as larger, inactive precursor molecules. They need to go through series of post-transitional modifications, to be converted to their active forms. The last step in this process is carboxyl-terminal α-amidation, which utilizes peptidyl glycine α-hydroxylating monooxygenase, an enzyme that is also dependent on O_2, Cu^+ and ascorbate (92, 93).

Tetrahydrobiopterin, a folic acid derivative, is a co-factor of several enzymes, including nitric oxide synthase, phenylalanine, tyrosine and tryptophan hydroxylase (94-97). However, its plays a slightly different role for nitric oxide synthase in comparison with other enzymes (97). Binding of tetrahydrobiopterin to nitric oxide synthase, enables synthesis of nitric oxide (NO) (98). On the other hand, it gets rapidly oxidized to a short-lived intermediate, quinoid dihydrobiopterin, which then rearranges to dihydrobiopterin (98). As opposed to tetrahydrobiopterin, dihydrobiopterin inhibits NO formation and instead leads to $O_2^{\bullet-}$ generation (98). Ascorbate as a reducing agent and an antioxidant is able to maintain tetrahydrobiopterin in its reduced state (98, 99). In case of tyrosine, ascorbate is required for its catabolism (100). On the other hand, phenylalanine hydroxylase, an iron containing enzyme that catalyses the conversion of L-phenylalanine to L-tyrosine, requires, O_2 and tetrahydrobiopterin as an electron carrier (101). During this process, tetrahydrobiopterin gets oxidized and an NADPH dependent enzyme so called dihydrobiopterin reductase recycles the oxidized form back to tetrahydrobiopterin. Stone and Townsley suggested that presence of ascorbate could also contribute to this recycling process (96).

Iron ingested from food presents in two forms; heme and nonheme iron. Heme, contains iron in ferrous (Fe^{2+}) form, and it is derived from hemoglobin and myoglobin, found in meat, poultry and fish. Nonheme iron, which exists in ferric (Fe^{3+}) state, is present in plant-based foods such as fruits and vegetables. It is known that dissociation of ferric compounds (eg. hydroxide, phosphates, complexes such as iron tannate) are much less than those of ferrous ones (102, 103). One of the key roles of ascorbate in iron

metabolism is that it promotes dietary nonheme iron absorption by reducing Fe^{3+} to Fe^{2+} together with duodenal cytochrome b reductase (103). An iron binding plasma glycoprotein, called transferrin, facilitates transport of iron through the bloodstream. Although to a lesser extent, non-transferrin bound iron (NTBI) can also occur in the circulation (104). In order to bind transferrin, iron in ferrous form needs to be oxidized to Fe^{3+} by hephaestin (104).

Almost all cells acquire most of their iron from the serum iron-carrier protein transferrin, but they are also capable of importing it in the form of NTBI (104). The latter occurs through divalent metal transporter 1 (DMT1) and requires reduction of Fe^{3+} to Fe^{2+} (105, 106). This reduction occurs via release of ascorbate from the cytoplasm into the extracellular space (104-106). However, in case of transferrin dependent iron uptake, ascorbate can facilitate the uptake via an intracellular reductive mechanism, which follows a transferrin receptor dependent endocytosis of di-ferric transferrin complexes (104, 107). Once this complex is located inside the endosome, the endosome becomes acidified and enables release of Fe^{3+} from transferrin. A subsequent ferrireduction is followed by the release of iron, which then gets transported by DMT1 and/or Zip14 (104, 107). In addition to these properties, studies show that ascorbate is likely to further modulate iron metabolism by increasing the expression of the gene for the iron storage protein, ferritin, enhancing iron deposition, inhibiting lysosomal ferritin degradation and reducing iron efflux (104, 107).

Concentration of ascorbic acid in skin is relatively high when compared to other tissues (70, 108-110). In addition to dual expression of SVCT (SVCT1 and SVCT2) in the skin epidermis, there is also a 2 to 5 fold difference between the ascorbic acid content of the epidermis and dermis (49, 108-109). These findings suggest a high dependency on ascorbic acid, especially in the epidermis. There is growing evidence showing that ascorbic acid may play a role in differentiation of keratinocytes and formation of stratum corneum barrier lipids (111-113). In an in vitro study, Pasonen-Seppanen and collegues demonstrated that ascorbic acid improved stratum corneum structure, increased keratohyalin granules and the intercellular lipid lamellae present in the interstices of the stratum corneum (111). Extracellular matrix (ECM), which is an important component of connective tissue, entails two groups of biomolecules; glycosaminoglycans and fibrous proteins such as collagen, elastin, fibronectin and laminin. In this context, ascorbic acid and its derivatives were shown to increase

glycosaminoglycan synthesis, its deposition into the ECM and stimulate elastin (114, 115). Duarte et al. assessed the effect of ascorbic acid 2-phosphate, a more stable derivative of ascorbic acid, on gene expression in primary dermal fibroblasts and found an increase in expression of various genes that are involved in cell motility, matrix remodeling during wound healing, deoxyribonucleic acid (DNA) replication and repair (116). In agreement with these findings, several in vivo and clinical studies demonstrated that ascorbic acid plays a key role in wound healing (117-119). Protein and DNA damage induced by ultraviolet (UV) radiation is one of the leading causes of photoaging and photocarcinogenesis. Although cutaneous damage caused by UV radiation is a complex process, one of the proposed mechanisms of action for generation of UV damage is a possible reaction between UV induced hydrogen peroxide (H_2O_2) and metal ions that are already bound to DNA and, a subsequent generation of $HO^{\bullet}$ (120, 121). A second proposed pathway is the lipid peroxidation of membranes caused by UV induced free radicals, which in turn may cause mutagenesis and cell death (121, 122). Ascorbic acid seems to ameliorate the damaging effects of UV both as a free radical scavenger and as an inducer of DNA repair and regeneration genes (122-128). Increased consumption of ascorbic acid in such cases is likely to be compensated by an increased uptake by keratinocytes in an irradiation time and dose dependent manner (129). However, according to the current literature, in the context of modulation of UV induced skin damage, benefits of ascorbic acid alone is limited and satisfactory results can be achieved only when it is combined with two or more antioxidants (130-132).

1.2. Oxidative stress, antioxidants and prooxidants

1.2.1. Oxidative stress

A free radical is a reactive molecule that has an unpaired electron in its outer orbit. It may derive from endogenous metabolic processes, enzymatic systems or from external sources such as ionizing radiation, pathogens, drugs and chemicals. Free radicals tend to steal an electron from a surrounding molecule, which leaves the other molecule as a free radical with an unpaired electron. This may lead to a chain of reactions and result in cellular damage. Free radicals can bind to DNA bases and modify nucleotides, or cause DNA strand breaks via reacting with the 5-carbon sugar deoxyribose (121, 133). They can also oxidize thiol groups in cysteine residues and cause formation of a disulfide bond, which can alter the protein structure and function (134). Two hydrogen atoms (H^+) that exist between the two double bonds of polyunsaturated fatty acids are highly prone to being targets of free radicals. Lipid peroxidation that is initiated by a reaction between one of these hydrogen atoms and an oxidizing agent such as $HO^•$ or iron-oxygen complex (perferryl or ferryl ion), leads to a cascade of events, resulting in formation of new radicals and a number of compounds such as; lipid alkyl radical, lipid peroxyl radical, lipid hydroperoxide, malondialdehyde and 4-hydroxy-2-noneal, that can in turn cause cellular damage (135, 136). Availablity of sufficient O_2 is crucial during the propagation stage, as the newly generated lipid alkyl radical needs to react with O_2 to form a lipid peroxyl radical, which will then steal a hydrogen atom to generate lipid hydroperoxide and a new lipid alkyl radical to continue the process. Among other enzymes, glutathione peroxidase seems to be the key enzyme that is shown to protect cells from the lipid peroxidation process and their effects (137, 138).

Molecular oxygen (O_2) has two unpaired electrons and therefore it is a biradical. It readily accepts unpaired electrons and gives rise to a variety of partially reduced species named as reactive oxygen species (ROS). These include $O_2^{•-}$, $HO^•$, peroxyl radical and alkoxyl radical. The cellular damage caused by ROS is termed oxidative stress. One-electron reduction of O_2 to $O_2^{•-}$ can occur by various processes such as mitochondrial electron transport by complex I and complex III, UVB induced type I photosensitizing reactions with internal chromophores, by NADPH oxidase, which gets activated by UVB exposure and found mainly in neutrophils, and by nitric oxide synthase as well as xanthine oxidase activity (139-142). $O_2^{•-}$ can react with another free radical, NO to form

peroxynitrite, a highly reactive intermediate which can damage a wide range of molecules in cells (143, 144). In an attempt to convert $O_2^{\bullet-}$ into less damaging species, superoxide dismutase catalyzes the dismutation of two molecules of $O_2^{\bullet-}$ into O_2 and H_2O_2. Although H_2O_2 is not a free radical, it is a potent oxidant and it does contribute to generation of free radicals. This can occur via formation of hypochlorous acid (HOCl), a precursor of free radicals, by an enzyme called myeloperoxidase, by Fenton and Haber-Weiss reactions (Figure 2) or by various cyclical transition metal ion catalyzed redox reactions which all gives rise to $HO^\bullet$, the foremost reactive ROS (139, 145-147). In an attempt to diminish potential harmful effects of H_2O_2, the heme-containing enzyme catalase and glutathione peroxidase participate in decomposition of H_2O_2 to H_2O (148). In addition to $HO^\bullet$ generation from H_2O_2, H_2O exposed to ionizing radiation may also lead to formation of $HO^\bullet$ and GSH plays an important role in its elimination (146).

$$2\,O_2^{\bullet-} + 2\,H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{3+} + O_2^{\bullet-} \rightleftharpoons Fe^{2+} + O_2$$

$$\underline{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\bullet + OH^-} \qquad \text{Fenton Reaction}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^\bullet + OH^- \qquad \text{Haber Weiss Reaction}$$
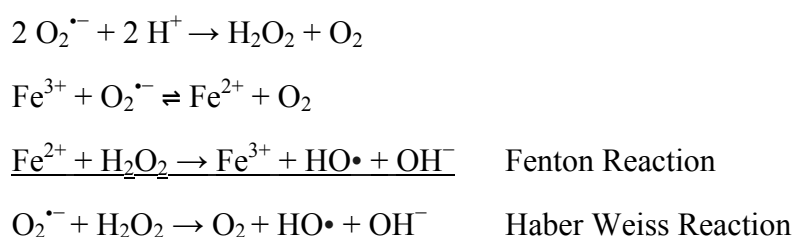
Figure 2: Fenton and Haber Weiss reactions (149-151).

1.2.2. Antioxidants

Normal physiological processes such as aerobic respiration continuously generate free radicals and oxidants and, when produced in moderate amounts, they play important roles in the regulation of intracellular signal transduction pathways, host defense system and immunity (152-157). Additionally, they are essential for a variety of catabolic and anabolic processes to take place. However, each cell maintains a homeostasis between prooxidant and antioxidant species and when there is an imbalance between the two, pathological processes ensue (139, 152, 158, 159).

Halliwell and Gutteridge first defined antioxidants in 1995 as ''any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate'' (160) but this definition

was later simplified and re-defined as ''any substance that delays, prevents or removes oxidative damage to a target molecule'' (161, 162). Recently, Apak and collegues came up with a slightly different and more detailed definition and defined antioxidants as ''natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants having distinctly positive reduction potentials, covering ROS/reactive nitrogen species (RNS) and free radicals (i.e. unstable molecules or ions having unpaired electrons)'' (163). However, a property that was not emphasized in these definitions is the ability of an antioxidant, which scavengers the radical, to generate a new radical which is stable through intramolecular hydrogen bonding upon further oxidation (152, 164). Moreover, substances which up-regulate antioxidant defenses may also qualify as antioxidants.

Antioxidant system is classified into two major categories; enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase prevent formation of ROS or reduce ones that have already been generated. For instance, glutathione peroxidase catalyses the reduction of H_2O_2 by two reduced glutathione (GSH) which leads to generation of oxidized glutathione (glutathione disulfide, GSSG) and two H_2O (165). Regeneration of GSH from GSSG requires glutathione reductase as an enzyme and NADPH as an electron donor (165). In fact, many enzymes of the antioxidant system depend on NADPH for proper function and glucose-6-phosphate dehydrogenase acts as a major supplier for this intracellular reductant (166, 167). Therefore, both GSH/GSSG as well as $NAD(P)H/NAD(P)^+$ ratios are considered to be important indicators of redox status of cells (166, 168, 169).

Non-enzymatic antioxidants are also categorized into two groups; endogenous antioxidants such as Vitamin A, Vitamin C and E, ubiquinol, carotenoids, urate, GSH, flavonoids, NAD(P)H, and synthetic antioxidants, which include butylated hydroxytoluene and butylated hydroxyanisole (152, 170).

α- and β-carotene, cryptoxanthin and lycopene, are the main carotenoids. Among these, β-carotene has a major role in Vitamin A formation by virtue of an enzyme called β, β-carotene-15, 15′ monooxygenase which catalyzes the centric cleavage of β-carotene to yield all-trans-retinal (171). All-trans-retinal can then be reduced to all-trans-retinol (Vitamin A) by retinol dehydrogenase (172, 173). Vitamin A and carotenoids' antioxidant properties lie in their ability to quench singlet oxygen ($^1O_2$), neutralize thiyl

radicals, and combine with peroxyl radicals to protect cells from lipid peroxidation (173, 174). α-Tocopherol, the most dominant isoform of Vitamin E, exerts its main antioxidant effect by donating phenolic hydrogen to the peroxyl radicals, which in turn generate tocopheroxyl radicals (152, 175). This process protects cells from lipid peroxidation and consecutively maintains membrane integrity. In order to provide continuous supply and eliminate the newly generated radical, Vitamin E is recycled from its tocopheroxyl radical either by enzymes such as NADH-cytochrome b_5 reductase or by nonenzymatic pathways, which utilize compounds such as $AscH^-$ and ubiquinol (176-180).

Glutathione is a tripeptide (cysteine, glycine, and glutamic acid) with a redox-active thiol group that generally exists in cells in its reduced state (GSH) (165). When GSH donates a hydrogen atom to a free radical intermediate, it is converted into a glutathiyl radical, which may react with a variety of species depending on the circumstances (181-183). For instance, glutathiyl radical can enter an electron transfer reaction with $AscH^-$ to generate GS^- and $Asc^{\bullet -}$ (183). Glutathione also contributes to the detoxification process by conjugating with a plethora of reactive metabolites and reacting with electrophiles that are generated as a result of metabolic processes (165). Additionally, it facilitates recycling of Vitamins C and E from their oxidized forms and in turn increases availability of antioxidants (169).

Phenols are aromatic compounds that contain an –OH group attached to a benzene ring. Phenols, which have more than two aromatic –OH groups, are termed as polyphenols. Almost all phenols exert a degree of antioxidant activity as scavengers of reactive species such as peroxyl radical, $HO^{\bullet}$ and HOCl. Some also serve as chelating agents by binding transition metal ions, which further reduces oxidative stress (184, 185). Polyphenols are further classified according to the number of phenol rings they accommodate and the structures that bind these rings to one another. According to these properties, they are divided into four major categories; Phenolic acids, flavonoids, stilbenes, and lignans (186). Among these, more attention has been given to flavonoids. Flavonoids consist of a fifteen-carbon skeleton that entails two benzene rings (A and B) linked by three carbon atoms that usually form a third oxygenated heterocyclic ring (C) (187). In majority of cases, B ring is attached to C ring in the 2-position but this may differ among different types of flavonoids such as isoflavones and neoflavonoids. Those in which B ring is linked in position 2 are further subclassified according to the

structural features of the C ring and include flavonols, flavones, flavanones, flavanols, flavanonols, catechins, anthocyanins (186). Flavonoids owe some of their antioxidant properties to the phenolic hydroxyl groups attached to ring structures, which can serve as reducing agents, hydrogen donors, $O_2^{\bullet-}$ scavengers and $^1O_2$ quenchers (188-190). Furthermore, specific ones serve as chelators of iron and copper, inhibitors of oxidases such as xhanthine oxidase and NADH oxidase and activators of detoxifying enzymes such as glutathione S-transferase (190, 191). Some can also replace antioxidant activity of α-tocopherol in the membrane, reduce α-tocopheryl radicals and regenerate α-tocopherol (190, 192-194).

Coenzyme Q is an endogenously synthesized lipid soluble substance that participates in the mitochondrial respiratory chain as an electron carrier (195). Ubiquinone and ubiquinol are the predominant oxidized and reduced forms of Coenzyme Q, respectively (196). Reduction of ubiquinone to ubiquinol occurs by a variety of oxidoreductases such as Complex I, Complex II, electron transfer flavoprotein-ubiquinone oxidoreductase, and non-proton pumping NADH dehydrogenases (in yeast) (196). In mammalian cells, reoxidation of ubiquinol to ubiquinone occurs only by Complex III, whereas in case of yeast, alternative oxidases also take part in the process (196). Ubiquinol is an effective antioxidant. Studies show that it prevents lipid peroxidation, takes part it regeneration of Vitamin E from the α-tocopheroxyl radical and halts oxidation of membrane proteins (180, 195-199).

1.2.3. Prooxidant properties of antioxidants

There is already substantial evidence that antioxidants play a major role in metabolic pathways and protect cells from the harmful effects of reactive species. However, some of the antioxidants can also exhibit prooxidant effects (200, 201). Whether an antioxidant behaves as a prooxidant seems to depend on multiple factors such as its concentration, cellular redox state, availability of other antioxidants and, the presence and amount of O_2, catalytic metal ions and radicals. For instance, green tea polyphenol (-)-epigallocatechin-3-gallate was shown to generate $HO^{\bullet}$ and $O_2^{\bullet-}$ and cause cleavage of DNA in the presence of Cu^{2+} (202). In a study by Palozza et al., β-carotene when tested at high concentrations, increased generation of ROS in leukemia cells (203). Moreover, retardation of cell cycle progression and proapoptotic activity highly coincided with increased ROS levels (203). These effects were reversed when low

concentrations were applied. The authors further assessed the variations in response to β-carotene in differentiated and undifferentiated leukemia cells and found that increase in ROS generation was observed at lower concentrations in undifferentiated cells when compared to differentiated ones. Likewise, post-treatment levels of GSH were higher in differentiated cells than those that are undifferentiated (203). Certain flavonoids, which act as powerful antioxidants, when tested at high concentrations, were shown to generate ROS, induce DNA strand breaks, oligonucleosomal fragmentation as well as caspase-3 activation (201, 204-206). However, sometimes it is challenging to distinguish an antioxidant dose range from a prooxidant dose range. For example, while fisetin, a plant polyphenol from the flavonoid group, at 22 μmol/L could protect cells from DNA strand breaks caused by H_2O_2, it could itself induce DNA strand breaks when H_2O_2 is absent (206). There is also some uncertainty about how flavonoids exert their cytotoxic and DNA damaging effects. Watjen et al. (206) found no indication for metal-catalysed oxidation, lipid peroxidation and ROS involvement whereas Sahu et al. (207) demonstrated that in the presence of transition metal ions, ROS and mainly $HO^{\bullet}$ was responsible for lipid peroxidation and the DNA damage caused by quercetin. Duthie and colleagues observed DNA strand breaks as well as a decrease in GSH levels, however they found no evidence for oxidative DNA base damage (208). It might very well be possible that nucleus was protected from ROS at the expense of intracellular GSH (208). Agullo and co-workers argued that degree of quercetin cytotoxicity was dependent on cellular proliferative activity, and this selective cytotoxicity could be utilized to inhibit growth in tumor cells (209). The group observed a diminished lactate release in dividing cells, which was likely to be caused by its inhibitory action on lactate transporter (209, 210). Nevertheless, a dramatic reduction in levels of adenosine triphosphate (ATP) was observed for both exponentially growing and stationary growing human colonic carcinoma cells (209). DNA topoisomerases play a role in DNA replication, transcription, and recombination by introducing transient breaks in the DNA. These enzymes also act to regulate DNA supercoiling generated during transcriptional elongation (211). Evidence suggests that some flavonoids such as myricetin, quercetin and fisetin can inhibit topoisomerase II (212, 213). This may arrest cell cycle and induce apoptosis via a p53 dependent or independent pathway (201, 213, 214). Selected flavonoids can also exhibit antimicrobial activity (215). One example is quercetin, which was shown to demonstrate antibacterial activity by inhibiting gyrase catalyzed DNA isomerization (216). Some studies also proposed that specific

antioxidants exert their prooxidant effects through inhibition of cellular respiration. Pavani et al. reported that nordihydroguaiaretic acid, a polyhydroxyphenolic antioxidant, acts as an inhibitor of mitochondrial electron transport, by interrupting the flow of electrons at the NADH-dehydrogenase-ubiquinone compartment of ascites tumor cells (217). A consequential decrease in ATP synthesis led to decreased cell viability and growth rates in the same cells (217). Several other phenols were also shown to inhibit mitochondrial electron flow at different sites and/or uncouple redox reactions from that of adenosine diphosphate (ADP) phosphorylation (218-221). Capsinoids, which possess antioxidant properties mainly via forming complexes with the reduced metals and donating H^+ to radical intermediates, can protect cells from iron-mediated lipid peroxidation and copper-dependent oxidation of low-density lipoproteins (222-224). Studies showed that some of its analogues may also exert selective prooxidant effects via preferentially inhibiting NADH oxidase activity in the plasma membrane of cancer cells. This inhibition was accompanied by an inhibition of growth and, apoptosis (225, 226). Furthermore, when HL-60 human promyelocytic leukemia cells were induced to differentiate, capsaicin's effect on the plasma membrane NADH oxidase activity was much less (225). Morre and colleagues further suggested that in capsaicin resistant cases, selective inhibition of NADH oxidase activity and subsequent inhibition of growth could be achieved with co-administration of mild oxidizing agents such as H_2O_2 and t-butyl hydroperoxide (226). These findings indicate that modification of redox state may be considered as an alternative approach for cancer types that do not respond to therapy.

1.2.3.4. Vitamin C as a prooxidant

Ascorbic acid, as an electron donor, gained most of its popularity through its antioxidant effects (83). However it may also act as a prooxidant, at pharmacologic doses (P-Asc) when specific conditions [eg. Kirsten rat sarcoma viral oncogene (KRAS) positivity] are met (23, 227). Literature suggests that P-Asc can exert selective toxicity against some tumor cells and infectious microorganisms (23, 227-229). Nevertheless, these effects remain controversial for various reasons such as fundamental differences between in vitro and in vivo conditions, lack of understanding of the exact mechanism of action and scarce clinical data that supports its efficacy.

The most well accepted mechanism of action of P-Asc has been linked to its ability to generate H_2O_2 (228, 230, 231). It is already well known that $AscH^-$ reduces Fe^{3+} to Fe^{2+} at the expense of producing an $Asc^{\bullet-}$ (229). A subsequent reaction of Fe^{2+} with O_2 generates Fe^{3+} and $O_2^{\bullet-}$ (229). H_2O_2 is then formed via dismutation of two molecules of $O_2^{\bullet-}$. A reaction between the newly generated H_2O_2 and Fe^{2+} leads to formation of $HO^{\bullet}$ and Fe^{3+} (Fenton reaction) (Figure 1). $AscH^-$ can further reduce Fe^{3+} back to Fe^{2+} for the cycle to continue. Studies have demonstrated that levels of antioxidant enzymes differ across tissues and among certain cancer cells versus normal ones (232, 233). Low levels of catalase and glutathione peroxidase detected in a variety of cancer cells renders them especially vulnerable to H_2O_2, and indirectly to P-Asc (233). According to Doskey and colleagues, this vulnerability also varies among different tumor cell types because not all cell types possess same degree of catalase activity (230). Nevertheless, mechanisms for how H_2O_2 elicited by P-Asc, induces toxicity to tumor cells are still under investigation. Some studies suggest that poly (ADP-ribose) polymerase (PARP) activation through H_2O_2 induced DNA damage may lead to catabolization of NAD^+, which could in turn deplete the substrate for NADH formation and hinder ATP synthesis (234-236). In the process of disposing H_2O_2 by an NADPH-dependent glutathione reductase/peroxidase system, NADPH is utilized to reduce GSSG back to GSH. In order to replenish NADPH that is consumed during this process, some of the glucose that is used for glycolysis could be diverted to the pentose phosphate pathway that in turn would result in reduction of ATP synthesis (234, 236). H_2O_2 induced inhibition of glycolysis by inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is assumed to further decrease NADH production and generation of ATP (236, 237). Also, at the level of mitochondria, inhibition of ATP synthase by H_2O_2 exposure appears to interrupt ADP phosphorylation and in turn ATP production (237, 238). However, findings by Du et al. were not in full alignment with some of these hypotheses (231). Although the group agrees that decreased cell viability caused by P-Asc is via a H_2O_2 mediated mechanism, they also indicate that PARP activation and depletion of ATP may not be involved in P-Asc induced cytotoxicity. Their experiments rather suggest that P-Asc's effects are through a caspase-independent cell death mechanism that is associated with autophagy (231).

''Warburg Effect'' first described by Otto Warburg refers to the phenomenon that, in tumor and other proliferating cells, there is an increased rate of glucose uptake and,

even in aerobic conditions, fermentation and subsequent production of lactate is preferred over oxidative phosphorylation (239-243). This preference towards a less efficient pathway for ATP synthesis was initially attributed to defective mitochondria (241). On the other hand, some more recent studies disputed this hypothesis and suggested that cancer cells heavily engage in both glycolysis and oxidative phosphorylation in order to be able to generate sufficient levels of ATP and NADPH but also to synthesize nucleotides and amino acids, which are all crucial for cell proliferation (244, 245). Lately, it has been further elaborated that under certain circumstances tumors can exert a metabolic plasticity to maintain growth and survival (246). One example is melanocyte lineage-specification transcription factor (MITF) upregulated proliferator-activated receptor-gamma coactivator-1alpha (PGC1α) positive melanomas, which are highly dependent on oxidative phosphorylation rather than glycolysis (247). Contrarily, an activated BRAF mutation leads to suppression of oxidative phosphorylation and induces a glycolytic phenotype (248). Moreover, evidence suggests that tumor microenvironment is heterogeneous (249). For instance, centers of solid tumors are generally poorly perfused lacking sufficient glucose and O_2 supply. Therefore, metabolic activity may also vary within the tumor itself. On the other hand, some authors argue that when tumor microenvironment is hypoxic, HIF-1 gets activated which in turn induces enzymes involved in glycolysis, upregulate GLUT transporters, reduce mitochondrial function to save O_2 and in turn reliance on glycolysis becomes more pronounced (239). This process is further enhanced via a positive feedback loop between glucose metabolites and HIF-1 (239, 250). Apart from hypoxia, HIF-1 can also be activated by other factors such as activation of Ras oncogene or loss of tumor suppressor von Hippel-Lindau (VHL) which all lead to a tendency of tumor cells to shift energy production towards glycolysis, even under normoxic conditions (239). Taken together, it seems that tumor cells tend to heavily depend on glycolysis despite the presence of a functional mitochondria but when the circumstances change, a metabolic switch is likely to occur. Consistent with this phenomenon, studies show that majority of tumors significantly upregulate GLUT to meet the glucose demands for increased glycolysis (251-253). Considering that specific GLUT channels are also responsible for transporting DHA across cellular membranes, one would expect a higher uptake of DHA by the tumor cells. Based on this assumption, Yun and collegues tested the effect of P-Asc on KRAS and BRAF mutant colorectal cancer cells that are associated with glycolytic phenotype. The group reported an increased DHA uptake

associated with upregulation of GLUT-1 transporter and elevated levels of oxidative stress caused by depletion of GSH during the conversion of DHA to its reduced form (236). Their study further revealed that GAPDH activity and consequently glycolysis were inhibited by ROS induced pentose posphate pathway activity as well as PARP activation, which leads to diminished level of NAD^+, the substrate required for GAPDH. These chain of events resulted in depletion of ATP, cellular energy crisis and cell death (236). Spielholz et al. demonstrated that melanoma cell lines take up DHA at a rate that is at least 10 times greater than normal melanocytes (254). Subsequently, Corti and colleagues (255) documented that, in the presence of iron, gamma-glutamyltranspeptidase, a plasma membrane enzyme that is often highly expressed in human malignancies (256), could facilitate the oxidation of $AscH^-$ to DHA and promote its uptake through upregulated GLUT transporters in melanoma cells (254). Studies have shown that genetic variations in SVCT-2 are associated with risk of certain cancers such as lypmhoma, human papillomavirus type 16-associated head and neck cancer and gastric cancer (257, 258). On the other hand, Lv and collegues investigated whether level of expression of SVCT-2 would play a role in selective cytotoxicity of P-Asc in cancer stem cells of hepatocellular carcinoma (259). The results suggested that SVCT-2 expression was inversely associated with P-Asc concentrations needed to decrease hepatocellular carcinoma cells by 50%. Moreover, SVCT-2 expression was positively correlated with intracellular P-Asc concentrations and response to P-Asc. Wang et al.'s study further showed that P-Asc treatment of knockdown of SVCT-2 in cholangiocarcinoma cells resulted in less DNA damage, ATP depletion and mammalian target of rapamycin (mTOR) inhibition (260).

HIF-1 is a heterodimeric complex that plays an integral role in adaptive responses of the tumor cells to changes in O_2 (261, 262). This involves not only a metabolic adaptation via channeling cells to a glycolytic pathway, but also transcriptional activation of various pro-angiogenic factors to increase O_2 delivery (262). There is growing body of evidence, which suggests that $AscH^-$ by virtue of its role as a cofactor for HIF hydroxylases, may limit activation of HIF-1 (263). Two retrospective studies identified an inverse association between ascorbate levels in human endometrial (264) and colorectal tumor (265) tissues and the activation of HIF-1 pathway. Higher ascorbate content in tumor tissue was also associated with longer post-surgical disease free period (265). Another study demonstrated that in tumor-bearing Vitamin C dependent $Gulo^{-/-}$

mice, increase in ascorbate intake alleviated levels of HIF-1α expression (266). On the other hand, in VHL-defective renal cancer cells, that already entail high levels of HIF-1α activity, a higher uptake of DHA was observed through the HIF-1α upregulated GLUT-1 transporters (267). Given that these cells already rely on glycolysis through Warburg effect, P-Asc induced ROS generation and subsequent PARP activation led to NAD^+ consumption and left very small amounts of NAD^+ for glycolysis to proceed (267, 268). Consequently, significant reduction in cellular reserves of ATP promoted cell death (267). Whether P-Asc can selectively control tumor cell growth via inhibition of HIF-1 signaling or whether overexpression of HIF-1 serves, as an advantage for P-Asc's selective cytotoxicity towards tumor cells, seems to depend on the tumor type, concentration of administered P-Asc, amount of O_2 available in the tumor microenvironment and the factors that influence the activation of HIF-1 (267). For instance, HIF-1α in VHL-defective renal cancer cells are constitutively stabilized and hypoxia or HIF prolyl hydroxylase activity has little influence on their activation (267, 269). In such cases, inhibition of HIF via prolyl hydroxylase may not play a significant role in P-Asc's anti-tumor activity. Vascular endothelial cell growth factor (VEGF) is a downstream gene product of HIF-1α and has been the focus of various targeted therapies (261, 270). Since HIF-1 activation could be potentially suppressed by P-Asc treatment, a possible concomitant decrease in VEGF was also investigated (264). Human endometrial tumor samples revealed a strong inverse correlation between level of VEGF protein and ascorbate content (264). In a separate in vitro and in vivo study, P-Asc treated sarcoma 180 cancer cells had lower levels of VEGF and other two angiogenesis related proteins, but the authors did not elaborate on the mechanism of action (271). Wilkes and collegues noted that P-Asc treatment did reduce VEGF secretion which correlated with a decrease in HIF-1α expression but these effects were through a H_2O_2 mediated pathway rather than a O_2 or prolyl hydroxylase-dependent inhibition of HIF-1α (272).

Iron plays a critical role for proliferation and metabolism of cancer cells and infectious microorganisms such as bacteria and fungi (273, 274). These rapidly dividing cells are highly dependent on presence of iron to carry out various cellular processes such as DNA synthesis, cell cycle regulation and oxidative phosphorylation (274, 275). In order to meet the increased iron demands, many type of cancer cells upregulate proteins that are involved in its uptake. Transferrin receptor 1 (TfR1) is one of these proteins that

became the target of antibody-mediated chemotherapeutic agents (276). Iron chelators such as desferrioxamine and 3-aminopyridine-2-carboxaldehyde thiosemicarbazone are also being considered as potential anti-cancer agents (277). On the other hand, iron's ability to gain and lose electrons also enables it to participate in Haber-Weiss reaction, which leads to generation of free radicals. Therefore, in cells, which are rich in iron, a complementary strategy for anti-infective or anti-cancer therapy is to focus on agents that foster free radical generation. Cysteine, GSH and $AscH^-$ can slowly release iron from the iron storage protein ferritin (278). However, P-Asc by triggering an uncontrolled release of iron can generate a surplus, which in presence of O_2, would lead to production of $O2^{\bullet-}$, $HO^{\bullet}$ and H_2O_2 (229, 279, 280). ROS generated by P-Asc, can further increase the labile iron pool in part via H_2O_2 mediated disruption of iron-sulfur cluster proteins (280). This labile iron pool redox cycling can contribute to the P-Asc induced selective cytotoxicity.

Schoenfeld and collegues reported that P-Asc treatment exerted preferential killing against of glioblastoma and advanced-stage-non-small-cell lung cancer cells in vitro and in vivo (280). In their study, GLUT mediated DHA uptake did not play a role in P-Asc's selective cytotoxicity but they argued that the effect was rather dependent on the increased redox-active labile iron present in cancer cells (280). Vilcheze et al. assessed the efficacy of P-Asc in treatment of Mycobacterium tuberculosis and their results also demonstrated that presence of high iron concentration played a critical role in its bactericidal effects (229). Kang et al. took a different view and proposed that P-Asc mediated death of melanoma cells were caused by P-Asc induced downregulation of TfR, which resulted in diminished iron uptake and subsequent apoptosis (281).

P-Asc has been the subject of various studies, which assessed its antibacterial, antifungal and antiviral properties (229, 282). Its inhibitory effects on the growth of various microorganisms such as Staphylococcus aureus, Helicobacter pylori, Mycobacterium tuberculosis, Bacillus cereus, and Candida have already been demonstrated (229, 283-287). However, studies, which elucidate its mode of action are limited and, the specific conditions required for P-Asc to exert its anti-microbial effects seem to vary depending on the infectious agent. For instance, while P-Asc can inhibit Helicobacter pylori in microaerophilic conditions, similar concentrations of P-Asc were shown to increase survival under aerophilic conditions (286). On the contrary, P-Asc's effect on Mycobacterium tuberculosis was abolished when the environment lacked O_2

(229). Earlier studies suggested that the bactericidal effect of P-Asc was due to lowering of the pH (288, 289). In 1950, Slade and Knox challenged this hypothesis when they found a bacteriostatic effect for group A hemolytic streptococcus at near neutral pH (290). According to Ehrismann, while P-Asc stimulated growth in anaerobes, its effect was inhibitory in case of aerobes (288). In the process of exploring the role of O_2 in bactericidal effects of P-Asc, Lwoff and Morel found that inhibition of Proteus vulgaris was halted by the presence of reducing agents and substances that catalyzed degradation of H_2O_2 (291). In 1954, Myrvik and Volk's short-term growth experiments on Escheria coli (E. coli), was an attempt to reveal the chemical group that is responsible for the antibacterial properties of P-Asc. They reported that while enediol group of P-Asc had no antibacterial effect on E. coli, oxidized enediol (diketone) could exert immediate and strong antibacterial effects (284). Peloux and collegues proposed that P-Asc had little or no anti-viral activity in the absence of metal ions (282). Polio virus was completely inactivated when P-Asc was combined with 5 μM Cu^{2+} whereas, in the presence of ethylenediaminetetra-acetic acid (EDTA), P-Asc had no effect (282).

Interestingly, presence of various microorganisms such as Acinetobacter baumanii and Candida albicans (C. albicans) induce human neutrophils to uptake DHA rapidly and recycle it to $AscH^-$, such that $AscH^-$ concentrations of activated neutrophils in vitro could increase up to 30 fold above the concentrations present in resting neutrophils (292, 293). Whether accumulation of $AscH^-$ in such high amounts is an attempt of the phagoctytotic cells' to enhance ROS generation or the cells want to take the advantage of its antioxidant properties while undergoing an oxidative burst is still an enigma (294-297).

Literature on P-Asc's antifungal effects on C. albicans is contradicting. P-Asc was shown in vitro to reduce virulence of C. albicans cells by lowering proteinase secretion (285). The same study also demonstrated that P-Asc could arrest cell growth and, induce concentration dependent cytotoxicity, which was potentiated when the cells were treated with H_2O_2 (285). In a separate study by the same group, P-Asc induced oxidative stress related enzyme activities in C. albicans were examined and reduced levels of GSH, decreased enzyme activity of catalase, glutathione reductase, glutathione peroxidase and glutathione S-transferase were reported (298). These effects are similar to those observed upon continuous exposure of yeast cells to H_2O_2, where cells accumulated GSSG but the total amounts of GSH and GSSG were substantially

diminished, indicating inhibition of GSH synthesis (299). Surprisingly, some antioxidant effects were also demonstrated, such that with increasing concentrations of P-Asc, superoxide dismutase activity was increased and lipid peroxidation decreased (298). On the other hand, locally applied P-Asc in human subjects showed no antifungal activity against vaginal candidiasis or other antifungal infections (300). Only when P-Asc was applied upon a successful treatment of fungal infection, it was able to prevent reinfection (300). Brajtburg and collegues suggested that P-Asc potentiates the lethal action of amphotericin B on C. albicans and Cryptococcus neoformans cells (301). Although in case of amphotericin B, P-Asc had such enhancing effects, P-Asc antagonized the effects of fluconazole both in vitro and in systemic murine candidiasis model (302). This inhibition was attributed to P-Asc's reducing property which could diminish the oxidative effect of fluconazole induced ROS (302). Moreover, neither intragastrically nor intraperitoneally administered P-Asc had any effect on the survival of mice with systemic candidiasis (302). When Van Hauwenhuyse and colleagues further investigated the antagonistic effect of P-Asc on fluconazole, they found that only in the presence of Upc2 (a transcriptional regulator of Erg11 gene, which encodes an enzyme that is the target of azole antifungal drugs and is involved in ergosterol biosynthesis (303, 304)) P-Asc could exert its antagonistic effects (305). Their analysis also revealed that, P-Asc could restore the ergosterol levels and reverse elongated cell growth caused by inhibition or depletion of heat shock protein (Hsp90), and this activity was Upc2 dependent (305). Nevertheless, it is worth to mention that in normal cells, P-Asc lowered ergosterol levels and did not initiate elongated growth (305, 306).

In the 1970s, Ewan Cameron and colleagues conducted the initial clinical trials to test the effect of P-Asc on improving the survival of patients with terminal cancer (307-309). Although their results were promising, subsequent double-blinded randomized studies in the Mayo Clinic demonstrated that orally administered P-Asc had no effect on patient survival (310, 311). It was later recognized that the route of administration might account for some of the discrepancy observed in, in vivo studies. Data suggested that intravenously administered P-Asc produced much higher plasma concentrations than the orally administered P-Asc due to the tightly controlled absorption process (64, 65, 312, 313). For instance, when 10 g of P-Asc was delivered via infusion, it was possible to achieve plasma concentrations up to 5 mM, whereas predicted peak plasma concentration for P-Asc given at the maximum tolerable oral dose, 3 g every 4 hours, is

not more than 220 μM (65, 312, 313). It is also of importance to note that although several in vitro studies showed P-Asc to be highly effective as a single agent to selectively induce cell death in tumor cells and various microorganisms, many in vivo studies could achieve similar effects only when P-Asc was used as an adjuvant therapy and sometimes even as an adjuvant it indeed antagonized the effects of the chemotherapeutic agents due to drug-drug interactions (229, 285, 300, 302, 314-318). This discrepancy is presumably due to the inability of the in vitro conditions to precisely mimic the tissue environment with hypoxia and relevant metabolic changes. Moreover, tumor tissues are heterogenic and display distinct intra- and inter-tumoral variations in morphological and physiological features, such as diverse gene expression patterns, motility and metabolic profiles (319). The same heterogeneity and relevant cell-to-cell variation is also present in infections (320). A systemic review by Fritz and colleagues concluded that literature still lacks data from a well designed, controlled study with a large sample size, which suggests that P-Asc can be effectively used as a stand-alone cancer treatment (314). Nevertheless, results of current studies, mainly ones where P-Asc was used as an adjuvant therapy seem to be promising (314, 321, 322). An equally important finding of the authors from existing preliminary evidence is that P-Asc is generally well tolerated and has a good safety profile (314).

1.3. Candida albicans

C. albicans is a eukaryotic organism. As the member of a commensal microbiota, C. albicans can be isolated from the oral cavity, vaginal mucosa and gastrointestinal and urogenital tracts of most healthy individuals. However, as an opportunistic pathogen it is also the most common cause of fungal infections (323-327). Although majority of infections are confined superficially to the mucosa, skin and nails, treatment can still be challenging (328-330). Moreover, in immunocompromised patients, invasive candidiasis (eg. candidemia or deep-seated infections) generally caused by direct inoculation to a sterile site, dissemination through blood, or less frequently as a result of ascending pyelonephritis, can lead to significant morbidity and mortality (325, 331).

1.3.1. Physiological, morphological and metabolic profile

One of the distinct features of C. albicans is its ability to grow in a number of different morphological forms such as yeast, pseudohyphae and true hyphae (332, 333). Unicellular yeast cells (blastospore, blastoconidium) generally have a round or oval shape and they divide through budding. Pseudohyphae and hyphae are the two filamentous forms that occur in response to alterations in the environmental conditions such as changes in temperature, pH or nutritional sources (333, 334). Hypha originates from a single elongated yeast cell termed as germ tube, which grows by apical extension and differentiates into tubular structures that are separated by septa (333, 334). Pseudohyphae are intermediate between the yeast and hyphae forms. In case of pseudohypha, daughter bud elongates but does not separate from the mother cell (333, 334). One of the distinct features that distinguish pseudohyphae from hyphae is the presence of constrictions at the septal junctions. In hyphae such constrictions do not exist and walls lie in parallel throughout the entire structure (333, 334).

Most microorganisms including yeasts grow in three main phases; lag phase, exponential (log) phase and stationary phase. Duration of each phase depends on various conditions such as temperature, pH, O_2 level and availability of nutrients (335). Lag phase is the adaptation and preparation phase (335, 336). During this period, cells are metabolically active (337-339). They start to upregulate relevant genes and synthesize the enzymes needed for cell division (335). At this stage number of cells remains relatively constant (335). Once they enter the exponential growth phase, their

number increases rapidly and the cells reach their peak metabolic activity (335). In general, this continues until they exhaust the available nutrients and accumulate metabolic end products (340-342). In stationary phase, there is a dramatic decrease in overall growth rate and cells try to maintain their viability by going through a variety of biochemical and morphological changes (335, 341-343). For instance, Uppuluri and Chaffin demonstrated that during stationary phase (which they defined as cells cultured for a minimum for 5 days), C. albicans showed increased expression of genes that are involved in processes such as cell wall biosynthesis, adherence, DNA repair and stress resistance (341). While majority of the genes involved in glycolysis pathway, and glucose transport were highly expressed in the exponential phase, such high expression was not observed in the cells that were in the stationary phase (341). However, the exact timeline regarding when yeast cells including C. albicans enter stationary phase and whether it is indeed considered as a distinct phase of growth still varies from one study to another (298, 341, 343-349). These variations may also account for some of the contradicting results regarding the biochemical changes that occur in stationary phase.

C. albicans can efficiently adapt to a wide range of environmental conditions, by a rapid metabolic switch, which is usually accompanied by a phenotypic switch (341, 343, 349, 350). This turns metabolism of C. albicans into a highly complicated process. C. albicans contains both conventional and alternative respiratory pathways and their level of expression alternate depending on the growth conditions (351, 352). In mammals, NADH-ubiquinone oxidoreductase (Complex I), cytochrome bc_1 (Complex III) and cytochrome oxidase (Complex IV) are the proton translocating oxoreductases in the respiratory chain (353). On the other hand, besides Complex I, C. albicans also contains other forms of NADH-oxidoreductases (351, 352, 354). They catalyze rotenone (inhibitor of Complex I) insensitive oxidation of matrix NADH or enable direct use of cytoplasmic NADH (351, 352). In addition, C. albicans can also express an alternative cyanide (inhibitor of Complex IV) and antimycin A (inhibitor of Complex III) insensitive oxidase, which is reduced directly by the electrons of the ubiquinol pool (351, 352, 355-357). Both oxidative stress and, respiratory chain inhibitors acting downstream from coenzyme Q was shown to induce anternative oxidase, which suggests that it supposedly protects fungi from oxidative damage (351, 358, 359). It may also be possible that alternative oxidase enables ATP synthesis to continue when the conventional pathway is inhibited (352).

C. albicans grows best under aerobic conditions, but studies suggested that it can also exhibit a limited degree of anaerobic growth (360, 361). This is important for C. albicans infections and especially biofilms that colonize tissues, foreign bodies, prosthetic devices and tissues in regions with insufficient amounts of O_2 like for example gastrointestinal tract and wounds covered with dressings. Studies regarding the respiratory activity of C. albicans in relation to its growth and morphology are still contradicting (345, 356, 362). Aoki and Ito-Kuwa's observed that cells increased their O_2 uptake during lag phase and initial stage of log phase (337). Ogasawara et al. reported that C. albicans cells in lag phase do not use O_2 and instead they generate ATP via fermentative metabolism whereas cells in exponential phase do use O_2 and utilize oxidative phosphorylation (339). They have further suggested that the cells employed glycolysis pathway to generate energy required for proliferation only in anaerobic conditions (339). On the other hand, Uppuluri and Chaffin's study indicated that C. albicans prefers aerobic respiration during exponential growth but they have also demonstrated that expression of genes involved in glycolysis pathway and glucose transport were increased during the same period (341). Land et al. documented that hyphal growth is associated with suppression of mitochondrial activity, diminished O_2 consumption and reduced activity of tricarboxylic acid (TCA) cycle enzymes (362). Majority of mature 'hard to treat' biofilms contain 95% hyphae and interiors of biofilms in general have very limited access to O_2 (363, 364). The commonly encountered difficulty in treating biofilms may be in part explained by the study conducted by Dumitru et al. in which the authors showed that anaerobically grown cells exhibited minimum fourfold more resistance to antifungals like miconazole, fluconazole, amphotericin B and terbinafine compared to the aerobically grown ones (360).

As mentioned above, C. albicans inhabits in diverse niches from the oral cavity and urogenital tract to the bloodstream and internal organs and only few of these niches are rich in glucose, the preferred carbon source for C. albicans (365-367). However, most tissues have sufficient supply of alternative carbon sources, such as lactate, fatty acids, and amino acids. C. albicans possesses the ability to assimilate these less favorable alternative carbon sources when the environment lacks glucose or possibly even for some time after glucose becomes available (368). At the same time, according to Rodaki and colleagues, C. albicans is highly sensitive to glucose, such that upon exposure to glucose at concentrations as low as 0.01%, glycolytic genes were shown to

be up-regulated, and gluconeogenic, glyoxylate cycle, TCA cycle and fatty acid β-oxidation genes were shown to be down-regulated (369). Conversely, when glucose is depleted, expression of genes that are involved in β-oxidation were shown to be elevated (341). Nevertheless, Uppuluri and Chaffin also showed that even at high glucose levels, C. albicans never completely shuts down its respiratory metabolism and indeed mitochondrial respiration is the preferred pathway in all growth phases (341). Glucose also plays a crucial role in response of C. albicans to oxidative stress. For example, it significantly increases the resistance of C. albicans to high doses of H_2O_2 (>10 mM) (369). This phenomenon may justify the increased risk of Candida infections observed in diabetic patients or enhanced colonization and invasion in tissues that are rich in glucose (370, 371). Similarly, it may also explain the increased resistance of glucose treated cells to an azole antifungal drug, miconazole (369).

Lastly, C. albicans cells' response to new environmental conditions differs depending on various factors such as the growth phase, presence or absence of certain quorum sensing molecules and/or the amount of time spent in the previous growth conditions (345). For instance, when cells were grown overnight at 37°C to stationary phase and then diluted into fresh culture medium under the same conditions, this change in the growth environment triggered a transient but substantial induction of hyphae formation (345). However it must be noted that, in the described experimental setting, while the cells grown to exponential phase had no capacity to form hyphae, the number of hyphae generated 3 h after the dilution increased as the cells approached to stationary phase (345). Moreover, H_2O_2 sensitivity of C. albicans was shown to be growth phase dependent, such that incubation of cells with H_2O_2 for 60 min in fresh minimal media resulted in a dramatic reduction in viability of early exponential phase yeast cells whereas stationary phase cells were highly resistant to H_2O_2 exposure (15% survival in early exponential phase cells vs 112% survival in stationary phase cells) (349). Furthermore, when early-exponential-phase cells were resuspended in fresh medium or conditioned medium (supernatant obtained from the overnight stationary phase culture) for 90 min and then exposed to H_2O_2 in minimal fresh media, percentage of cells that survived in pretreated conditioned medium were significantly higher than those pretreated in fresh media (101% vs 11% respectively) (349). Authors of this study suggested that farnesol, a quorum-sensing molecule excreted into the medium was partly responsible for the oxidative resistance generated by the conditioned medium,

and the conditioned medium induced transcription of antioxidant-encoding genes might have played a role in protection of cells against oxidative stress (349).

As discussed in earlier chapters, when organisms perform aerobic respiration, ROS is generated and various antioxidation mechanisms are employed for protection against possible oxidative damage. For fungal pathogens, interaction with the phagocytic cells in particular, causes them to encounter extreme levels of oxidative stress (372). In order to thrive in such environments, fungi utilize various enzymatic and non-enzymatic mechanisms that include catalase, superoxide dismutase and GSH (299, 372). Although, some studies suggest that ascorbic acid in yeast cells is absent (373-375), several studies also show that they can synthesize D-erythroascorbic acid, a five-carbon analogue, which possesses chemical properties, very similar to those of ascorbic acid (376, 377). Studies confirming D-erythroascorbic acid's role as an antioxidant do exist, but whether it has a significant role in protecting cells from stress is still under debate (376, 378, 379). Of note is that, when Branduardi and colleagues constructed recombinant Saccharomyces cerevisiae (S. cerevisiae) cells, producing endogenous L-ascorbic acid, they observed increased resistance to oxidative stress (373). Likeweise, S. cerevisiae cells exposed to L-ascorbic acid at 10 mM exhibited higher tolerance against heath shock and lower levels of ROS accumulation (380).

1.3.2. Treatment

There are five main classes of antifungal agents; polyenes, azoles, allylamines, nucleoside analogues and echinocandins. Azoles such as ketoconazole, fluconazole and itraconazole inhibit the enzyme 14α-lanosterol demethylase and consequently block biosynthesis of ergosterol - the sterol component of the cell membrane (381). Amphotericin B, which belongs to the group of polyenes binds irreversibly to ergosterol, forms pores that cause leakage of intracellular ions and lead to subsequent cell death (382). The allylamine terbinafine exerts its antifungal effects by inhibiting squalene epoxidase (383). Flucytosine is a nucleoside analog and impairs fungal DNA and ribonucleic acid (RNA) synthesis (384). Echinocandins are a relatively new class of antifungal agents and they inhibit beta-1,3-glucan-synthase, an essential component of the fungal cell wall (385). Studies suggest that under specific conditions some of these antifungals may also induce generation of ROS (381, 386). Despite the availability of numerous antifungal agents with broad and narrow activity spectra; emerging antifungal

resistance, detected and anticipated side effects and host toxicity, partly due to extensive similarities between fungal and human cells, require and pinpoint the development of new therapeutic strategies (387-391).

1.4. Basal Cell Carcinoma

Basal cell carcinoma (BCC) is a keratinocyte-derived neoplasm of the skin (392). It is the most common type of cancer among Caucasians and its incidence is on the rise (393-395). There is considerable phenotypic diversity among patients in terms of number of lesions they develop, anatomic locations of these lesions and the patterns of presentation. For example, BCC usually occurs on sun exposed body sites such as the head and trunk but lesions on non-sun exposed areas like the axilla, nipple and genital area have also been reported (396-398). Genetic and environmental factors both play a role on predisposition to BCC. The most significant risk factor is believed to be exposure to solar UVB radiation, which ranges from 280 to 315 nm (399). UVB induces formation of strand breaks and formation of covalent bonds between adjacent pyrimidine bases that leads to generation of cyclodipyrimidine dimers (T/T) and (6-4) pyrimidine-pyrimidone photoadducts in the DNA (121, 399-401). When left unrepaired these photoproducts can cause mutations, which can in turn result in abnormal cell growth. Especially, the mutations that activate the Hedgehog signaling pathway genes, such as patched (PTCH), Sonic hedgehog (Shh) and Smoothened (Smo) play an important role in development of BCC lesions (399, 402-406). Other important risk factors include, UV exposure, drug induced immunosuppression, arsenic exposure, fair skin color and advanced age (407-415).

BCC generally presents as a slow growing, translucent or pearly, papule or nodule with telangiectasias and a central ulceration. It has various subtypes, based on clinical presentation, growth pattern and histology (416-419). Clinical subtypes include nodular, ulcerative, superficial, pigmented and morpheiform BCC and fibroepithelioma of Pinkus (416, 417). A separate classification system identifies histological subtypes, which include but are not limited to nodular, micronodular, superficial, infiltrating, morpheiform and fibroepithelial BCC (417, 420). Growth pattern assists identification of aggressive and high-risk subtypes with potential tumor recurrence and based on growth pattern BCC is further classified as nodular, superficial, infiltrating, morpheiform, micronodular and basosquamous (417, 421). Among these, nodular and superficial are considered as less aggressive and low risk subtypes. However, it must be noted that to date there is no universally agreed classification and variations exist in definition and terminology (418, 421, 422).

BCC occurs sporadically, with some exceptions such as basal-cell nevus syndrome (BCNS, Gorlin syndrome, nevoid basal-cell carcinoma syndrome) - a rare autosomal dominant disorder in which patients inherit a germline mutation in the tumor suppressor gene PTCH (423, 424). Most common features of BCNS include multiple lesions of BCC, palmar and plantar pits, central nervous system abnormalities such as calcification of falx cerebri, skeletal defects and benign odontogenic keratocysts of the jaw (423). However, multiple lesions of BCC are not unique to BCNS. For example, in case of hereditary nonsyndromic multiple BCC, skin lesions exist without any associated anomalies (425). Although rare, some cases of multiple BCC also exist without any associated anomalies and any family history (426-428).

Common methods used in the treatment of BCC include surgical excision, cryosurgery, electrodessication and curettage, radiotherapy, photodynamic therapy, 5-fluorouracil, imiquimod and mohs surgery (429-431). Although most lesions are indolent and respond well to local treatments, some can occasionally progress to an advanced state where they may no longer be suitable for local therapy (431, 432). Moreover, for patients who have multiple BCC, traditional treatment modalities may be impractical, contraindicated, not effective and sometimes grossly disfiguring (428, 430, 433). Vismodegib (Erivedge; Roche) and sonidegib (Odomzo; Novartis), the two small molecule antagonists that target the Hedgehog signaling pathway by binding to and inhibiting Smo, are considered as the therapeutic options for such cases (430, 434, 435). Nevertheless, while using these medications, patients might experience adverse events, which sometimes lead to noncompliance and discontinuation of therapy (435, 436). Moreover, there are cases where patients do not completely or even partially respond or resistance develops (428, 437, 438). Therefore, the development of new BCC therapies is an active field of investigation.

It has been postulated that activation of Hedgehog signaling pathway is associated with altered energy metabolism (439, 440). In 2012, Chen and colleagues explored the role of Hedgehog signaling pathway in induction of a metabolic switch to glycolysis, which could in turn result in lactate accumulation, and inhibition of adipogenesis, in quiescent hepatic stellate cells that transform into myofibroblasts (439). Their findings demonstrated that Hedgehog signaling was indeed necessary for increased expression of genes that regulate glycolysis and, Smo upregulated HIF-1α expression was involved in this process (439). Recently, Seleit et al. demonstrated for the first time that nodular

type BCC lesions in humans showed higher HIF-1α expression percentage when compared to the normal dermis (441). Teperino and colleagues further illustrated that activation of Smo could initiate a Warburg-like metabolic reprogramming by modulating proteins such as adenosine monophosphate (AMP)-activated protein kinase, pyruvate kinase M1/M2 and pyruvate dehydrogenase α-1, some of which also play a role in stimulation of GLUT-4 dependent glucose uptake (440). On the other hand, literature regarding GLUT expression in BCC is contradicting. Abdo et al. detected GLUT-1 expression in 62.5% of nodular and adenoid type BCC cases (n=16), and the expression was more likely to be localized at the center of the malignant basaloid nests (442). This type of localization may be associated with presence of hypoxia which is more common in areas that are not in close proximity to blood vessels (443). However, when the same authors investigated the GLUT-1 expression in cutaneous squamous cell carcinoma (SCC), they found that GLUT-1 was expressed in all SCC lesions (n=16) and this expression was related to differentiation status, such that high percentage of GLUT-1 expression was usually associated with poorly differentiated SCC (442). In a subsequent study performed with 20 nodulo-ulcerative type BCC lesions, GLUT-1 expression was found to be downregulated in comparison to normal skin (441). When histological subtypes were analyzed in terms of localization, in a nodular type BCC lesion, GLUT-1 expression was mainly confined to the center of the lesion, whereas in a keratotic type BCC lesion, GLUT-1 was expressed mostly around the areas of keratotic differentiation (441). On the other hand, Baer et al. found no GLUT-1 expression in any of the BCC lesions, and the expression was also absent in areas with focal squamous metaplasia and keratinization (444). Intriguingly, same study also showed that almost all cases of SCC had moderate to intense levels of GLUT-1 expression (444). In addition, in case of invasive SCC, GLUT-1 expression was more prominent in infiltrative and/or less differentiated regions (444). Positron emission tomography (PET) is a commonly used modality in oncology and it detects the tumor cell uptake of the radiolabeled form of glucose so called 2-deoxy-2-[18F]fluoro-D-glucose (FDG). This modality relies on the higher rate of glycolysis commonly observed in the tumor tissue which results in a higher uptake of FDG (445). A positive correlation between FDG uptake and GLUT-1 expression have already been demonstrated in various types of lung cancers (446). Therefore, PET imaging of BCC lesions may further elucidate the GLUT-1 expression in BCC cells. For example, a case series of 6 patients with 4 nodular and 2 invasive BCC lesions demonstrated that 3 of the 4 nodular lesions were

PET-positive while all invasive lesions were found to be PET-negative (447). The authors argued that although negative results could be due to the differences in tumor metabolism among histological subtypes, various factors such as small tumor size or low blood flow could have also affected the sensitivity (447). On the other hand, Ali and colleagues were able to successfully image a small BCC lesion, which demonstrated focal nodulocystic growth and partly basosquamous differentiation (448). Therefore, they suggested that rather than lesion size, it was probably GLUT-1 expression or other factors involved in variations in PET images of BCC (448). A third case report by Beer and Waibel also documented the detection of a large recurrent BCC by a PET scan (449). Likewise, an intense FDG uptake was observed in a primary BCC lesion with nodular and infiltrative patterns and this was accompanied by hypermetabolic lymph nodes, suggestive of metastatic BCC (450). Thacker and colleagues utilized PET to assess the treatment response of advanced and metastatic BCC to vismodegib by monitoring the changes in metabolic activity (451). Authors concluded that metabolic activity decreased in all lesions following therapy although the degree of reduction varied among lesions. As demonstrated by Evans et al. in 2006, O_2 levels in human skin are not uniformly distributed, such that while dermis is well oxygenated, epidermis is mildly hypoxic and the regions where sebaceous glands and hair follicles are located are in part severely hypoxic (452). Consistent with these findings, high levels of HIF-1α were detected particularly in the basal layer of the epidermis of human skin (453-456). Considering that in both healthy skin and certain types of skin cancers, regions of hypoxia and HIF-1α expression generally coincides with regions of GLUT-1 expression, HIF-1α and GLUT-1 in BCC lesions are expected to be disproportionally expressed both intratumorally and among different subtypes (443, 453, 457). As BCC lesions are mostly indolent and GLUT 1 expression was shown to be much higher in ''stem-like'' basal cells in comparison to differentiated keratinocytes, one may also expect variations in expressions among invasive and non-invasive subtypes (457).

Tumor angiogenesis, which is generally associated with microvessel density and VEGF expression, is considered as an important step in acquisition of an aggressive phenotype and metastasis (458-464). Stabiano et al. demonstrated that angiogenic rates were much higher in infiltrating and metastasizing BCC lesions than those in non-aggressive subtypes (463). Consistent with these findings, Loggini and colleagues showed that

mean vascular density was higher in sclerosing subtype – an aggressive form of BCC, than in the nodular and superficial subtypes which are considered to be indolent forms of BCC (461). Likewise, a separate group revealed that both VEGF expression and mean vascular density were higher in morpheaform and, nodular lesions with a deep dermal involvement, than those in superficial BCC lesions (459). However, the expression of VEGF, peritumoral and intratumoral blood vessel areas and their counts, and the mean vascular density in BCC do not seem to be as high as those in SCC (460, 461, 463, 465, 466). Moreover, some studies did not even find any VEGF expression or increase in capillaries, in certain BCC lesions (461, 467, 468). This may be attributed to variations in growth pattern and metabolism in different BCC subtypes (461).

1.5. Two-Photon Excitation Fluorescence Microscopy

Fluorophore is a molecule that can fluoresce by absorbing energy of a particular wavelength light (photon) and subsequently emitting light of a longer wavelength than what was initially absorbed (469, 470). When the electron in the ground state absorbs energy from an incoming photon, it may rise to a higher energy state, so called the ''excited state''. While the electron goes back to its ground state, several processes occur with varying probabilities, but generally the energy that was initially absorbed is partly dissipated as heat and mainly emitted in the form of fluorescence radiation (469). One-photon fluorescence process utilizes only a single photon that is usually in the UV or blue/green spectral range, to excite a fluorophore from the electronic ground state to an excited state (471, 472). On the other hand, in two-photon excitation fluorescence microscopy (TPEFM), the molecule is excited by the simultaneous absorption of two-photons, which are usually in the red or near infrared spectral range (473, 474). Hence, each of these photons carries almost half the energy that is needed to excite the molecule via a one-photon process. As the likelihood of simultaneous absorption of 2 photons is extremely low, a high photon flux needs to be delivered to the sample. This is commonly achieved by tightly focused, high repetition rate (100 MHz), ultrafast (femtosecond or picosecond pulse widths) lasers, such as titanium–sapphire or neodymium-doped yttrium lithium fluoride (Nd:YLF) lasers (473, 474). Consequently, the fluorophore excitation and thus the emission gets restricted to the area near the focal plane where the photon density is highest (474). Unlike UV or blue-green light, excitation wavelengths used in TPEFM scatter less and the molecules that are abundantly found in tissues and cells generally do not absorb these wavelengths. These properties enable deep tissue penetration, minimize photobleaching and photo-damage and, reduce signal loss by eliminating the need for a pinhole aperture (473, 474). Of note, the pinhole aperture is commonly employed in single photon microscopes in order to reject the out-of-focus fluorescence that is indistinguishable from the scattered light emitted from the excited fluorophore. These advantages turn TPEFM into a suitable tool for real time lable free imaging of deep tissues and live cells to assess cellular and subcellular events.

1.5.1. Monitoring intracellular redox status by conventional confocal and two-photon excitation fluorescence microscopy

One of the applications of TPEFM is label free monitoring of cellular metabolic activity and redox status in living cells, by evaluating intracellular levels of the endogenous fluorophore, NAD(P)H (475-478). While NADH is mainly generated by glycolysis and TCA cycle and consumed by electron transport chain, anaerobic glycolysis or fermentation; its phosphorylated analogue NADPH is involved in anabolic reactions such as lipid, amino acid and nucleotide biosynthesis (479). NADPH also has an important role in detoxification and antioxidant defense, for example it is required for reduction of GSSG and subsequent generation of GSH (169, 479-481). Both NADH and NADPH absorb light and emit fluorescence at 340 ± 30 nm and 460 ± 50 nm, respectively, but their oxidized forms NAD^+ and $NADP^+$ are not fluorescent (482-484). While NAD(P)H has a relatively low quantum yield (ratio of emitted photons to the number absorbed) and the fluorescence signal acquired by conventional one photon imaging is usually weak, TPEFM can provide the energy that is required for its transition from ground state to the excited state (168, 485, 486). TPEFM can also reduce the potential photodamage and photobleaching that generally stems from its excitation peak, which falls in the UV range.

In most cells, NAD^+ levels are much higher than those of $NADP^+$, however the difference in intracellular concentrations of NADH and NADPH is generally less (166, 168, 487). One reason is that $NADH/NAD^+$ ratio is usually kept low due to NAD^+'s role as an electron acceptor in catabolic pathways (166, 168, 488). Moreover, in case of hypoxia or oncogenic metabolic transformations where Warburg effect is proposed to take place, in order to keep gycolysis active, cells may restore the NAD^+ pool through regeneration of NAD^+ from NADH by the enzyme lactate dehydrogenase, which facilitates the conversion of pyruvate to lactate (168, 240, 489). On the other hand, in anabolic pathways and antioxidant defense, the primary role of NADPH is as an electron donor and hence the $NADPH/NADP^+$ ratio needs to be kept at relatively high levels (166, 168, 490). In most cells, NADPH is produced and maintained at such high levels mainly through the actions of the two enzymes of the pentose phosphate pathway; glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (491). Nevertheless, there is an intriguing interplay between NAD(H) and NADP(H) that is

particularly pronounced in response to new conditions such as aeration, oxidative stress and UV irradiation. Multiple enzymes have been shown to take part in this interplay and they directly or indirectly regulate the balance between the two (479, 487, 492-494). For example, nicotinamide nucleotide transhydrogenase (NNT), can catalyze the reduction of $NADP^+$ at the expense of NADH oxidation and H^+ reentry to the mitochondrial matrix (487). NADH kinase is another enzyme, which is shown to transfer a phosphate group from ATP to NADH to generate NADPH (495). Recently Singh and colleagues also demonstrated that under conditions of oxidative stress, Pseudomonas fluorescens utilized the enzyme NAD kinase to orchestrate the production of $NADP^+$ at the expense of NAD^+ (494). A concomitant increase in $NADP^+$ in turn promoted the production of NADPH, and enhanced the cell's antioxidant activity. In addition, reduction of the available NAD^+ pool diminished synthesis of NADH, hence limited the potential generation of ROS from its downstream metabolism mediated by complexes I, III, and IV during electron transport chain (494, 496). In a separate study, when ROS generation was induced by menadione, a concomitant decrease of NAD(P)H autofluorescence was recorded (497). Likewise, when C. albicans cells were treated with garlic extract, containing endogenous dialliyl disulphide, increased level of ROS was observed (498). This increase was paralleled by a decreased mitochondrial membrane potential, GSH and attenuated cytoplasmic and mitochondrial NAD(P)H signals detected by TPEFM (498). Taken together, at each pixel of an image, the change in the intensity of NAD(P)H fluorescence might provide insights into the changes in the $NAD(P)H/NAD(P)^+$ ratio and reflect the balance of oxidation and reduction reactions at that region (168). However, it must also be noted that some groups consider the cellular level of NADH to be greater than NADPH and interpret the change in NAD(P)H signal as an alteration of NADH only (484, 499-503). This leads to inconsistencies regarding the interpretation of the signals collected from NADH and NADPH.

In general, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) serve as cofactors for enzymes that participate in oxidation-reduction reactions (504). For instance, during the electron transport chain, similar to NADH, reduced form of FAD ($FADH_2$) transfers its hydrogen atoms to O_2 to drive the synthesis of ATP. Research has demonstrated that endogenous fluorescence signal intensity of FAD can provide further input to better understand the metabolic redox state of the mitochondria (475-477, 484, 505, 506). As only FAD and NAD(P)H are sufficiently fluorescent, and as their redox

states are generally in equilibrium within the mitochondria, signals from these two tend to respond oppositely to changes in mitochondrial metabolic activity and redox state (475, 476, 507). For instance, in breast cancer cells treated with rotenone, an inhibitor of Complex I, a reduced cellular redox state was confirmed by an increased NADH fluorescence, decreased FAD fluorescence and a decreased FAD/NADH ratio (506). On the other hand, Armstrong et al. demonstrated that upon exposure to various concentrations of H_2O_2, a concentration dependent decrease in NADH and an increase in FAD autofluorescence occurred in vitro (508). Kuznetsov and colleagues' study further revealed that doxorubicin treatment of breast cancer cells induced ROS and, resulted in a rapid increase in flavoprotein signal, which was accompanied by a remarkable decrease in NADH autofluorescence in the mitochondria (476). These findins paralleled with reduced membrane potential (476).

Current imaging techniques that utilize TPEFM for imaging of FAD, also have their drawbacks such as difficulty in completely separating the overlapping NAD(P)H and flavoprotein signals, and low quantum efficiency in detecting flavoprotein autofluorescence (475). Therefore, some studies prefer quasi-simultaneous imaging of flavoproteins by conventional confocal microscopy and, NAD(P)H by TPEFM, over simultaneous imaging of the two via TPEFM (507). Moreover, discrepancies related to alterations in redox potential caused by variations in tumor microenvironment, patient's age, substrate availability, tissue processing, imaging parameters and set ups or methods used for quantitative analysis of redox ratios do also exist (501, 509-511). Taken together, detection and monitoring of NAD(P)H and FAD autofluorescence via conventional confocal and TPEFM imaging may have potential future implications for mechanistic studies, diagnostics as well as for monitoring treatment response (501). However, further studies are needed for standardization purposes and further improvement of these techniques.

1.6. Second Harmonic Generation Microscopy

Second harmonic generation microscopy (SHG) is based on two photons interacting simultaneously with a target molecule to emit a new photon with twice the energy, however unlike TPEFM, in which two photons are simultaneously absorbed to produce a single photon of fluorescent emission, SHG specifically involves scattering of photons and the target molecule is non-centrosymmetric (501, 512-514). In biological tissue, structures such as type I and II collagen fibers, microtubules and myosin can produce SHG signals endogenously without the addition of exogenous probes (515-517). Because SHG is a coherent process, the amount, direction, and polarization of emission is not only dependent on the concentration of the scatterers, but it also depends on their spacing, order and orientation (501). This property enables imaging of various fine details of the tissue structure that is not visible by other modalities.

Literature suggests that the cross talk between tumor and tumor stroma has a strong influence on behavior of cancer cells (518-521). Along these lines, many of the in vivo studies utilizing SHG aim to elucidate the role of peritumoral collagen in detection of tumor genesis and prediction of tumor progression (522-525). For instance, Conklin et al. demonstrated that perpendicular alignment of straightened collagen fibers to the tumor boundary was suggestive of decreased survival in human breast cancer (526). However, it must also be noted that collagen alterations differ according to the tumor type, stage and grade (525, 527). A study on ovarian cancer found an association between malignant tissue and dense and highly ordered collagen fibers (528) and when Chen and colleagues compared normal vs cancerous gastric tissues, they observed diminished collagen area in the latter group (529). Moreover, according to Drifka et al. increased collagen width, length, straightness and alignment, especially around malignant ducts, were stromal characteristics of pancreatic ductal adenocarcinoma (530).

In case of skin cancer, studies are yet limited. Lin et al. detected lower SHG signals obtained from collagen in BCC stroma than those obtained from collagen in normal reticular dermis (531). Two other groups described that in BCC, basaloid nests are tightly surrounded by parallel fibers (532, 533) but in healthy skin straight fibers are oriented along different directions (533). Recently, Kiss and colleagues further

demonstrated that in comparison to healthy skin samples, BCC lesions exert increased collagen fiber length and alignment but similar collagen width and straightness (534).

One major concern in evaluation of tumor stroma by SHG is the stability of collagen features under various tissue-processing procedures (eg. frozen samples in embedding medium, frozen samples that have not been embedded, and formalin-fixed paraffin-embedded tissue). Various reports suggest that collagen features and fluorescence properties are stable and well maintained in all these conditions (525, 526, 535). Nevertheless, further work is still needed to clarify any possible effect of tissue processing procedures.

2. Objectives

Literature regarding P-Asc's prooxidant effects on tumor cells and infectious agents is still controversial and, rather scarce for the latter. This controversy stems from various reasons including but not limited to, differences between in vitro and in vivo conditions, tissue heterogeneity and AscH⁻'s tendency to interfere with reagents used in chemical assays. Moreover, its exact mechanism of action is yet to be further explored. In an attempt to clarify some of these problems, we aimed:

1/a. To define specific metabolic and environmental conditions under which P-Asc exerts cytotoxic effects on C. albicans cells

1/b. To investigate production of HO˙ formation and, indirectly the involvement of Fenton reaction in prooxidant effects of P-Asc on C. albicans cells

1/c. To utilize lable free single live cell fluorescence imaging techniques to monitor intracellular redox status of P-Asc treated cells and real time treatment response

1/d. To investigate morphologic and organ specific alterations at the single cell level by utilization of lable free optical imaging tools

2. To exploit lable free single live cell fluorescence imaging techniques to assess fine changes of tissue pathology during intravenous P-Asc (IVA) therapy that may not be apparent in standard hematoxylin and eosin stained histology sections.

3. Materials and methods

3.1. In vitro studies on Candida albicans

3.1.1. Materials

All chemicals used for the experiments were purchased from Sigma-Aldrich (MO, USA) unless otherwise stated. L-ascorbic acid was dissolved in phosphate-buffered saline (PBS) (free of Ca^{2+}/Mg^{2+}), PBS with D-(+)-glucose (20 g/l), YPD medium (yeast extract 10 g/l; peptone 20 g/l; dextrose 20 g/l, [BD-DifcoTM, NJ, USA]) or YPG medium (yeast extract 10 g/l; peptone 20 g/l [ForMedium Ltd., Norfolk, UK]; glycerol 38 g/l). Stock solutions of P-Asc were prepared on the same day of experiment and pH was adjusted to $\cong$ 7 with $NaHCO_3$ (Fisher Scientific, PA, USA). The final concentration of P-Asc was 90 mM in all experiments. Final concentrations of 2,2′-bipyridyl dissolved in dimethylacetamide and 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid [(3′-(p-Hydroxyphenyl)-fluorescein, HPF; Molecular Probes, OR, USA)] were 500 μM and 5 μM, respectively.

3.1.2. Cell culture

The C. albicans strain CEC 749 was used in this study (536). C. albicans was grown at 30°C on YPD agar and subcultured in liquid YPD medium in a rotary shaker incubator at rpm 120 (New Brunswick Scientific, NJ, USA), at 30°C. Log phase cultures obtained by reculturing stationary overnight cultures were used for all experiments except as follows: In one set of experiments, stationary cultures grown overnight or 4 days and a stationary culture grown for 4 days and then refreshed for 4 h were used. The reason for selecting two different time points for stationary phase cultures was because in some studies, C. albicans culture grown overnight or for 48 h was considered to be in stationary phase, while other studies reported the stationary phase to start much later, for example, between 3 and 8 days (298, 341, 343-349). All broth cultures were centrifuged at 3200 rpm for 10 min (centrifuge 5417 C; Eppendorf, Hamburg, Germany) and resuspended in PBS. The concentrations were then adjusted by measuring optical density (OD 570 of 0.65) to give an approximate cell density of 10^7 colony forming units (CFU)/ml. In one experiment, YPG medium and in another PBS with glucose, was used as described above.

3.1.3. Experimental design

The experiments were performed in 35 × 10 mm diameter Petri dishes (BD Falcon NJ, USA) containing approximately 3×10^7 cells in 3 ml growth media, PBS with glucose or PBS. To examine the effects of different media and glucose on P-Asc sensitivity, cells were compared in different growth media; YPD or YPG, or in PBS with glucose. YPD or YPG was used both for initial growth and also for P-Asc treatment. Cells were agitated/shaken at 157 rpm at 37°C. At each time point two aliquots (10 μl each) were withdrawn. One was plated on YPD or YPG agar, the second was added to 90 μl PBS, then four additional tenfold dilutions were carried out in a 96 well plate. Subsequently, 10 μl was transferred from each well on agar plates via drop plate method. Plates were incubated between 24 and 48 h in an incubator at 30°C and cell viability was assessed by colony counting. In the next experiment cells were treated with P-Asc in PBS with agitation at 157 rpm (4 and 37°C); kept at 25 and 37°C under static condition (maintained unshaken for the duration of the experiment), or only in PBS with agitation at 157 rpm (4 and 37°C). Samples were withdrawn at different time points.

To determine whether the effect of P-Asc is dependent on the presence of iron, washed cells were resuspended in PBS solution with P-Asc, and 2,2′-bipyridyl, an iron chelator that predominantly binds Fe^{2+} (537), was added at a final concentration of 500 μM. Subsequently, cells were incubated at 37°C with agitation at 157 rpm, and samples withdrawn at different time points.

3.1.4. Microscopy

3.1.4.1. Detection of hydroxyl radical generation

HPF fluorescent probe was added to washed cells and kept in a rotary shaker incubator with or without P-Asc in YPD or PBS or kept under static condition at 25°C with P-Asc in PBS. Subsequently, cells were spun down (3200 rpm, 3 min), supernatant was removed and they were resuspended in PBS. Images were acquired using a 35 mm 4-chamber glass-bottom petri dish (In Vitro Scientific, CA, USA).
HPF fluorescence images were captured by Olympus FV1000-MPE system with 40X NA0.8 water immersion lens. Fluorescence response of HPF was detected with single-photon excitation at 488 nm using multiline argon laser and the fluorescence emission

were collected with laser scanning spectral detector at bandwidth 500–545 nm. Brightfield images were acquired simultaneously.

3.1.4.2. Assessment of intracellular redox status

Intracellular redox status was measured by autofluorescence imaging of NAD(P)H and FAD, using an Olympus FV1000-MPE confocal microscope system with 40X NA0.8 water immersion lens. The FAD autofluorescence was imaged with single-photon excitation at 488 nm using multiline argon laser and the autofluorescence was collected with laser scanning spectral detector at bandwidth 500–600 nm. The autofluorescence of NAD(P)H was measured with two-photon excitation at 710 nm using MaiTai Deep See Ti:Sapphire laser (femtosecond) (Spectra-Physics, MaiTai HP DS-OL). The autofluorescence from two-photon excitation was collected with external two-channel photo-multiplier detector for NAD(P)H (band-pass filter 420–460 nm). Brightfield images were acquired simultaneously.

3.1.4.3. Transmission electron microscopy

C. albicans cells were spun down (3200 rpm, 3 min) immediately after treatment, supernatant was removed, cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, and stored overnight at 4°C. After spinning down (1200 rpm, 10 min) and decanting the fixative, 0.1 M sodium cacodylate buffer (pH 7.2) was added to the pellets. Following fixation, hot agar was added to each pellet. The solidified cell pellets were then processed routinely for transmission electron microscopy (TEM). The cell pellets were postfixed in 2% OsO_4 in sodium cacodylate, dehydrated in graded alcohol, embedded in Epont812 (Tousimis, MD, USA). Ultrathin sections were cut on a Reichert-Jung Ultracut E microtome (Vienna, Austria), collected on uncoated 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a Philips CM-10TEM (Eindhoven, The Netherlands) at 80 kV.

3.1.4.4. Statistics

Experiments were repeated at least three times. Data points are means and error bars are standard deviations. Means were compared for significance ($p < 0.05$) by one-way ANOVA and Bonferroni post hoc test.

3.2. Ex-vivo studies on basal cell carcinoma

3.2.1. Treatment protocol

We have investigated skin biopsies taken from a 47 year old female patient who participated in a pilot study investigating the efficiency of long-term IVA therapy on locally advanced basal cell carcinoma. This work was conducted at the Department of Dermatology, Venereology and Dermatooncology of the Faculty of Medicine, Semmelweis University Budapest, Hungary, in accordance with the ethical standards as dictated by the Declaration of Helsinki and informed consent was obtained. Compounding of the intravenous vitamin C solution and its off-label use were approved by the Regional Committee of National Science and Research Ethics (TUKEB 80/2010) and the National Institute for Quality and Organizational Development in Healthcare and Medicines (39.798/56/09). Further details regarding patient selection criteria, demographic and clinical data can be found in (538). The infusion solutions were prepared from concentrated ascorbic acid solutions. Each 50 ml vial contained 25 g ascorbic acid (500 mg/ml) and pH was adjusted to 5,5-7 with sodium bicarbonate and edetate disodium, as described before (315). Solutions were diluted in 1000 ml Ringer's lactate infusion and administered for the duration of 3 hours by a Port-A-Cath device. In general IVA dosage was 1.8 g/kg body weight and it was administered for a total of 173 sessions.

3.2.2. Specimen collection and histopathology

Skin biopsy samples were taken from two different micronodular lesions after a two-week drug free interval, and subsequent two weeks of intensive (10 sessions) IVA therapy. Hematoxylin and eosin staining was performed on sections from 10% formalin-fixed and paraffin embedded skin biopsies.

3.2.3. Assessment of tumor collagen environment by second harmonic generation and two-photon excitation fluorescence microscopy

TPEFM and SHG images of deparaffinized tissue samples were acquired by a custom modified Axio Examiner LSM 7 MP laser scanning two-photon microscope (Carl Zeiss AG, Germany) using a 20X water immersion objective (W-Plan – APOCHROMAT 20x/1,0 DIC (UV) VIS-IR, Carl Zeiss AG, Germany). We employed a femtosecond pulse Ti-sapphire laser (FemtoRose 100TUN NoTouch, R & D Ultrafast Lasers Ltd, Hungary) tuned for a 800 nm excitation wavelength and a 395-415 nm band-pass emission filter to separate SHG signal from the TPEFM signal, which was collected at 565-610 nm and intracellularly attributed to mainly FAD (539, 540). The size of each field of view corresponded to 0.42 x 0,42 mm, from which mosaic images of larger areas up to 6,72 x 6,72 mm were constructed by ImageJ software (NIH, USA). The imaging setup is further described in Ref. (534, 541). From the tumor nests and their associated peritumoral stroma, five field of views were selected for quantitative analysis. Alterations in collagen morphology (fiber length and width) were assessed by CT-FIRE (v1.3) (LOCI, USA), a curvelet transform-fiber tracking algorithm in the selected field of views (542).

4. Results

4.1. In vitro studies on Candida albicans

4.1.1. Ascorbate sensitivity of Candida albicans

4.1.1.1. Effect of different media and carbon source

We compared the time-dependent killing of P-Asc when the cells were shaken at 37°C, suspended either in growth medium YPD or in PBS. Moreover to test whether the antifungal activity of P-Asc against C. albicans is altered in different growth media, we compared media containing fermentable carbon source dextrose (YPD) or a nonfermentable carbon source glycerol (YPG) (543). Figure 3 shows that treatment with P-Asc in PBS at 37°C by shaking almost eradicated the cells (>5 logs killing) after 90 min, while in growth media there was no killing, but rather modest proliferation (up to fivefold). Growth media containing fermentable or nonfermentable carbon sources made no difference with no fungicidal activity observed in either case. To further investigate whether presence of glucose in PBS would have an effect on P-Asc sensitivity, we compared cells exposed to P-Asc in PBS with those in PBS-glucose at 37°C with agitation. Almost two logs of inhibition of killing was observed with added glucose at 60 and 90 min (Figure 3).
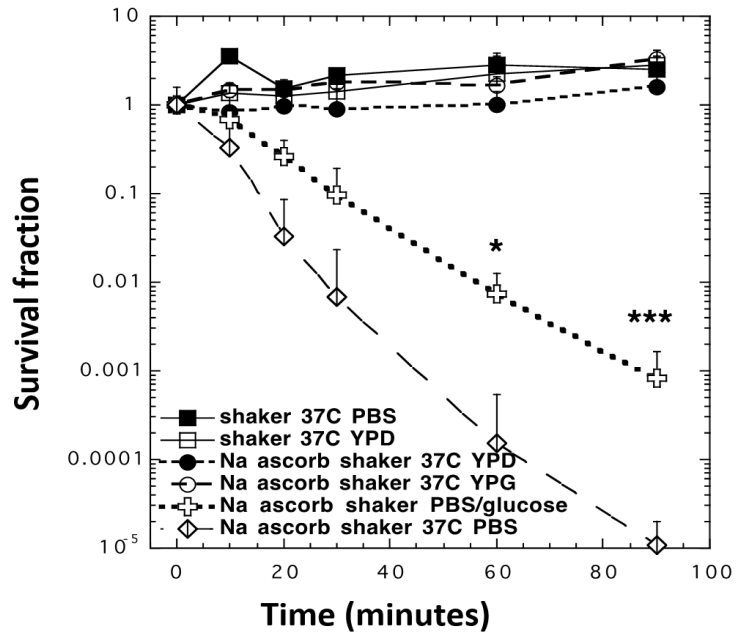
Figure 3: Time-dependent killing of C. albicans with 90 mM P-Asc shaken in phosphate-buffered saline (PBS), yeast-peptone-dextrose (YPD) medium, yeast-peptone-glycerol (YPG) medium or PBS with 20 g/L glucose at 37°C. Controls included shaking in PBS or YPD without P-Asc. * $p < 0.05$, *** $p < 0.001$. Na ascorb: buffered L-ascorbic acid.

4.1.1.2. Effect of oxygenation and temperature

We compared cells that were treated with P-Asc, in PBS with agitation at 4°C and 37°C with those, in PBS under static condition at 25 and 37°C. Control cells were agitated in PBS without P-Asc at 4 and 37°C. Figure 4 shows that all three conditions, completely abrogated the killing with P-Asc in PBS with agitation at 37°C. It is of interest to note that, cells exposed to P-Asc under static conditions formed colonies at a much later time when compared with nonexposed cells (data not shown). These findings show the importance of both temperature and sufficient oxygenation levels, implying possible relevance of active respiration in P-Asc sensitivity.
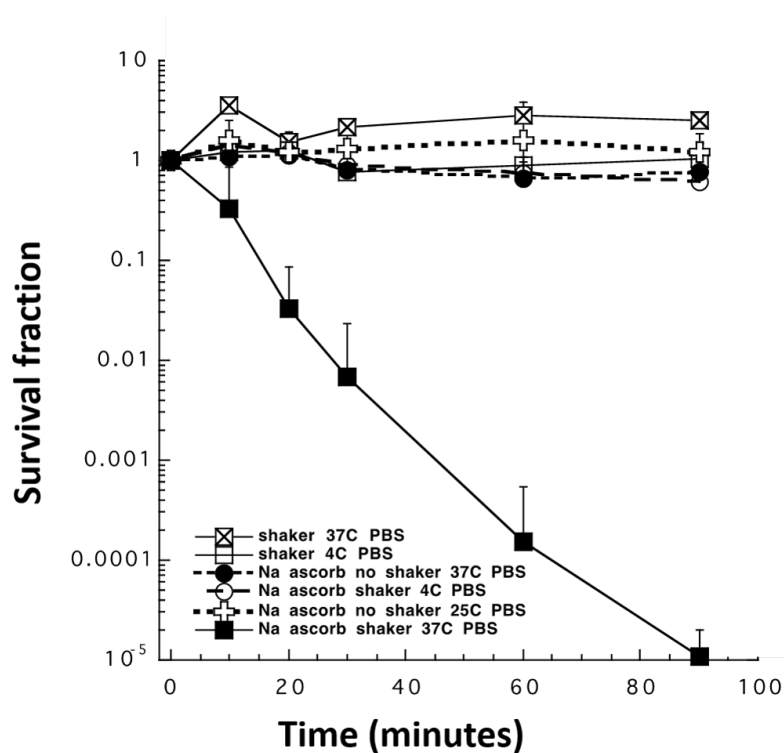

Figure 4: Time-dependent killing of C. albicans with 90 mM P-Asc shaken in phosphate-buffered saline (PBS) at 37°C or 4°C, and not shaken at 37°C or 25°C. Controls included shaking in PBS alone at 37°C or 4°C. Na ascorb: buffered L-ascorbic acid.

4.1.1.3. Involvement of free iron

As the literature suggests that iron-catalyzed Fenton reaction is involved in the toxicity of P-Asc to tumor cells and infectious microorganisms (229, 316, 544,), we tested the effect of a cell-permeable iron chelator 2,2′-bipyridyl, at a final concentration of 500 µM, on the P-Asc killing of C. albicans in PBS with agitation. There was substantial inhibition (>2 logs) of fungicidal activity at time-points between 30 and 90 min (Figure 5).
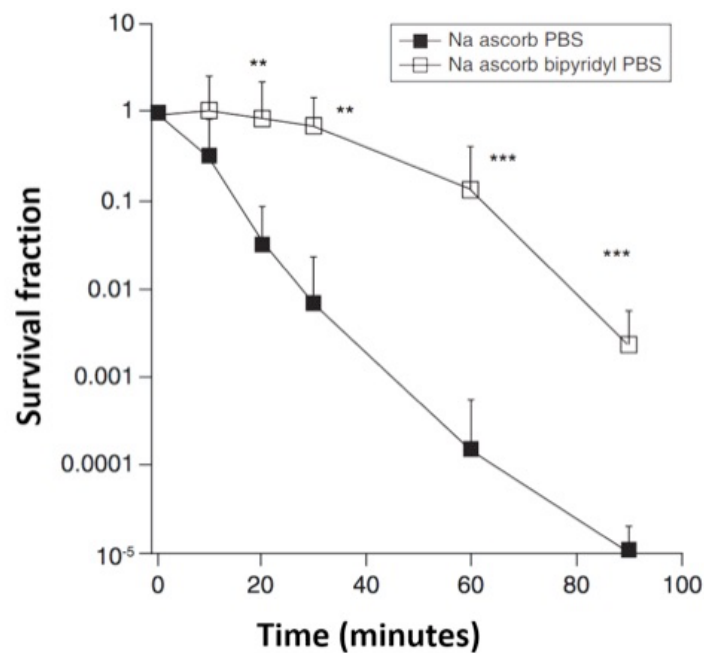

Figure 5: Time-dependent killing of C. albicans shaken in phosphate-buffered saline (PBS) at 37°C with 90 mM P-Asc with or without addition of the iron chelator 2,2-bipyridyl (500 µM). ** $p < 0.01$; ***$p < 0.001$. Na ascorb: buffered L-ascorbic acid.

4.1.1.4. Effect of growth history

We compared the susceptibility of cells coming from early (1 day) and late (4 days) stationary phase inoculum, and in the latter case, from refreshed and not refreshed cultures, to those coming from log phase, by treating them with P-Asc in PBS with agitation at 37°C. Early stationary phase cells (1 day) were the most sensitive, being killed 2–3 logs more than log phase cells (Figure 6). Late stationary phase cells (4 days) were most resistant, and 4 h refreshing provided more killing only during the first 60 min of the treatment but not later.
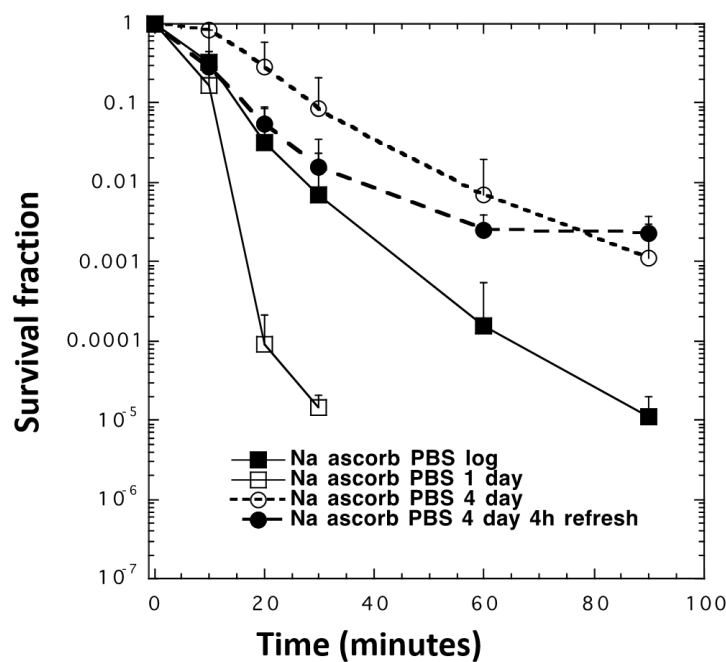

Figure 6: Time-dependent killing of C. albicans taken from cultures at different growth phases shaken in phosphate-buffered saline (PBS) at 37°C containing 90 mM P-Asc. Na ascorb: buffered L-ascorbic acid.

4.1.2. Imaging studies on C. albicans

4.1.2.1 Detection of hydroxyl radical generation by conventional confocal microscopy

The literature suggests that HO$^\bullet$ formation could explain the toxicity of P-Asc (229). Thus, we used a highly sensitive and specific fluorescence probe HPF for imaging of intracellular HO$^\bullet$ generation (545). Figure 7A and B showed no HO$^\bullet$ production (green fluorescence) when cells were shaken at 37°C either in YPD alone or with P-Asc in YPD. Minimal green fluorescence was observed when *C. albicans* was shaken in PBS without ascorbate at 37°C (Figure 7C). More pronounced (but still modest) green fluorescence was detected, when *the cells were* treated with P-Asc in PBS at 25°C without shaking (Figure 7D). However, upon 20 min treatment with P-Asc in PBS at 37°C with agitation there was an intense green fluorescence visible in every cell indicating a marked increase in HO$^\bullet$.
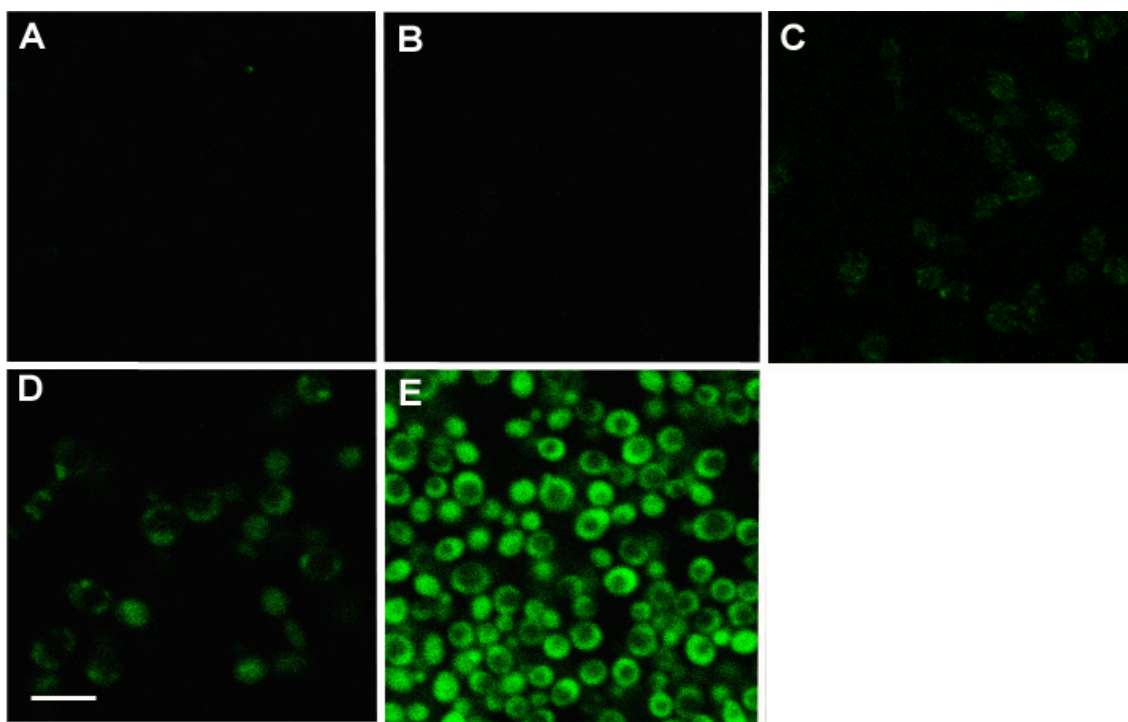

Figure 7: Representative confocal images of HO$^\bullet$ generation in Candida albicans after 20 min treatment with P-Asc in phosphate-buffered saline (PBS) with or without shaking at 37 and 25°C, respectively. Cells were stained with 3′-(p-hydroxyphenyl)-fluorescein and images were acquired at 40X-zm-6, scale bar = 5 μm. Cells incubated in: (A) yeast-peptone-dextrose (YPD) with agitation at 37°C; (B) P-Asc-YPD with agitation at 37°C; (C) PBS with agitation at 37°C; (D) P-Asc-PBS under static condition at 25°C; (E) P-Asc-PBS with agitation at 37°C.

4.1.2.2. Imaging of intracellular changes in redox state by combination of conventional confocal and two-photon excitation fluorescence microscopy

Intracellular redox state can be observed by detecting the blue (420–460 nm) autofluorescence from NAD(P)H, using TPEFM and the green autofluorescence (500–600 nm) from FAD when excited by single photon (540, 546). In general, healthy cells without oxidative stress have high NAD(P)H fluorescence and low FAD fluorescence, whereas cells that have been subjected to oxidative stress have lower NAD(P)H and higher FAD fluorescence (476, 498, 508).

Figure 8A and B shows that cells agitated in YPD for 30 min without P-Asc had significant amounts of NAD(P)H fluorescence and, no visible FAD fluorescence, respectively. Cells in PBS agitated for 30 min without P-Asc, had slightly reduced NAD(P)H (Figure 8C) and slightly increased FAD fluorescence (Figure 8D). Cells treated with P-Asc at 25°C for 30 min in PBS under static condition showed a prominent reduction in NAD(P)H fluorescence (Figure 8E) and a small amount of FAD fluorescence appeared (Figure 8F). Cells stirred in PBS with P-Asc for 20 min also showed a prominent loss of NAD(P)H fluorescence (Figure 8G), while a substantial amount of FAD fluorescence (Figure 8H) was visible. We consider that a slight component of NAD(P)H fluorescence may correspond to FAD fluorescence due to the minor overlap in their two-photon excitation emission wavelengths (475). Thus the NADH signal observed in 20 min is likely to be due to the increased autofluorescence of FAD (540).
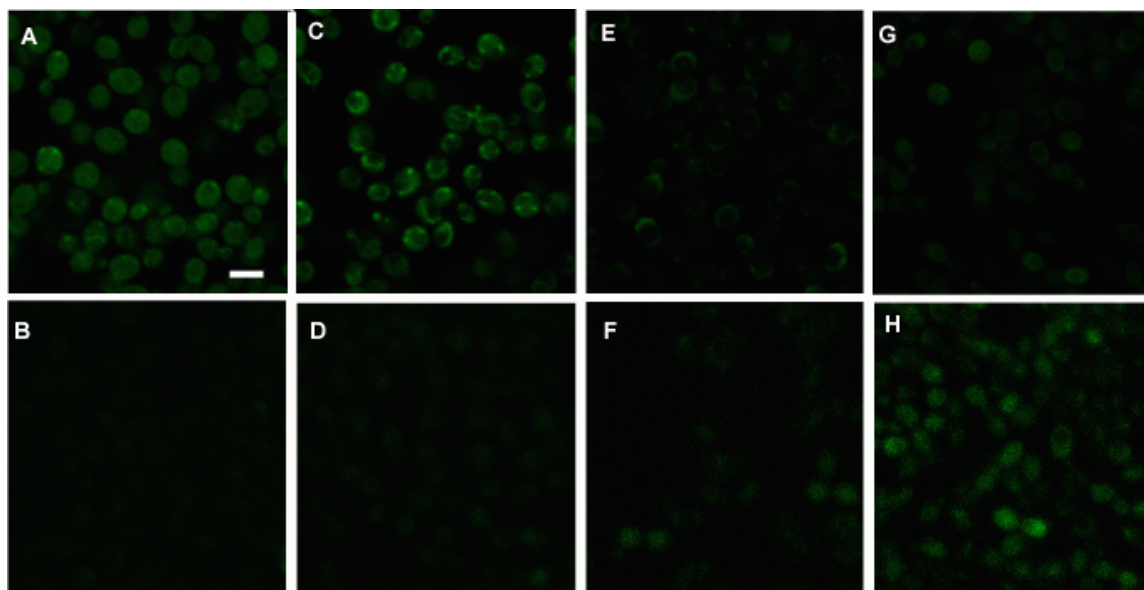
Figure 8: Representative two-photon excitation fluorescence and conventional confocal microscopy images of intracellular cell redox changes in C. albicans cells after incubation in different culture conditions and treatments. Simultaneous imaging of autofluorescence of NAD(P)H (A, C, E & G) and FAD (B, D, F & H). All images were acquired in phosphate-buffered saline (PBS) at 40X. Scale bar = 5 μM. Cells incubated in: (A-B) yeast-peptone-dextrose with agitation at 37°C for 30 min; (C-D) PBS with agitation at 37°C for 30 min; (E–F) P-Asc-PBS under static condition, at 25°C for 30 min; (G–H) P-Asc-PBS with agitation at 37°C for 20 min.

4.1.2.3. Tracking of morphological alterations at the cellular and subcellular level by brightfield and transmission electron microscopy

Fungal vacuoles, resembling mammalian lysosomes are the most acidic compartment of the cell with roles such as protein degradation, ionic transport, metabolite and metal ion storage as well as detoxification (547). Alterations in vacuolar morphology have been reported in response to stress ranging from ionic and acute osmotic shock to long-term nutrient deprivation (547). TEM images of C. albicans treated with P-Asc in PBS for 30 min in a rotary shaker incubator revealed vacuole overgrowth, nuclear condensation as well as loss of organelle identity (Figure 9C-D). Consistent with these findings, bright field microscopy images showed a similar pattern resembling a peripheral ring with a central hole (Figure 9H). Same morphology was also observed in bright field images of P-Asc treated cells in PBS at 25°C under static condition (Figure 9G).
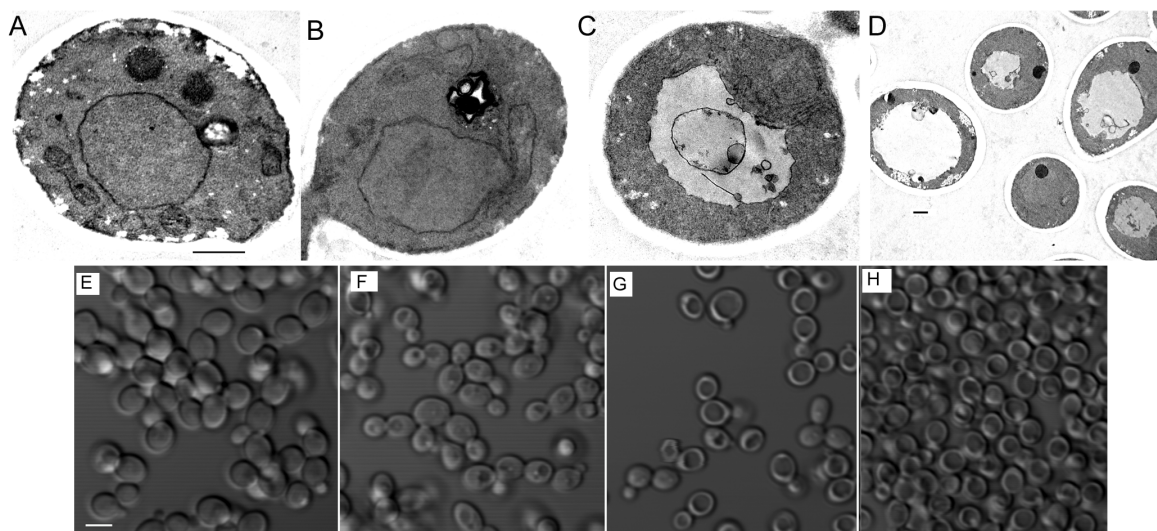

Figure 9: Transmission electron microscopy (TEM) and brightfield microscopy images of C. albicans cells in different incubation and treatment conditions. (A–D) TEM images of C. albicans cells following 30 min incubation at 37°C in: (A) yeast-peptone-dextrose (YPD) with agitation; (B) phosphate-buffered saline (PBS) with agitation; (C–D) PBS with P-Asc with agitation. (A–C) were imaged at 9800X direct magnification, (D) was imaged at 3800X. Scale bar = 500 nm. (E–H) Brightfield microscopy images of C. albicans cells following 30 min incubation in: (E) YPD with agitation at 37°C (F) PBS with agitation at 37°C; (G) PBS with P-Asc under static condition at 25°C; (H) PBS with P-Asc with agitation at 37°C. Scale bar 5 μM.

4.2. Ex-vivo studies on basal cell carcinoma

4.2.1. Label free imaging of tumor collagen environment to assess ascorbate's effect on basal cell carcinoma

Studies suggest that interactions between tumor cells and extracellular matrix can promote cancer progression, and in turn, alterations in peritumoral collagen fibers may reveal prognosis or treatment response (519, 521). We evaluated the fine changes of collagen in peritumoral stroma of two micronodular lesions, which showed a stable and partial response at the end of IVA therapy. In Figure 10c, hematoxylin and eason stained tissue reveal typical histologic features of BCC under brightfield microscopy. These features such as peripheral palisading cells (yellow) and nests of basaloid cells (yellow), are clearly revealed by images acquired by TPEFM (Figure 10a-b). Moreover, parallelly aligned collagen fibers (magenta) surrounding the tumor nests could be identified by SHG (Figure 10a-b). Of note, TPEFM was utilized only to visualize tissue architecture, rather than quantification of tissue characteristics or biochemical alterations in intracellular milieu.

CT-FIRE software is an image processing algorithm that can extract individual collagen fibers from acquired SHG images, to quantitatively assess fiber properties such as length and width (542). Application of CT-FIRE to SHG images of peritumoral stroma revealed that upon intense IVA treatment, thinner and shorter collagen fibers were identified (Figure 10 d).
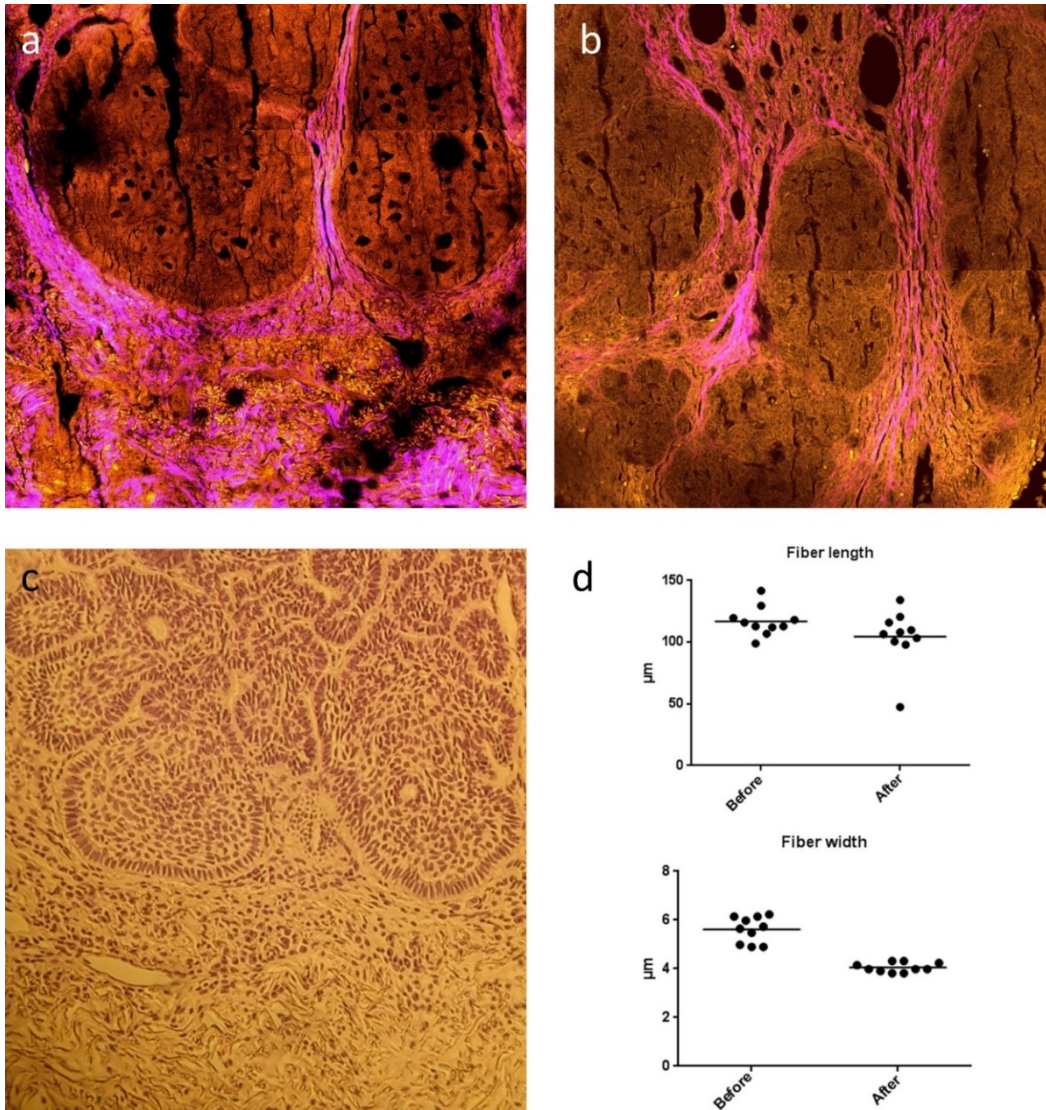
Figure 10: (**a-b**) Two-photon emission fluorescence and second harmonic generation microscopy (TPEFM and SHG, respectively) images from the same micronodular basal cell carcinoma lesion. a: after a two-week drug free interval, b: after two weeks of intensive (10 sessions) intravenous high dose ascorbic acid (IVA) therapy. TPEFM signal of FAD is displayed in yellow, while SHG signal of collagen fibers is shown in magenta color. (**c**) hematoxylin and eosin stained section corresponding to the TPEFM-SHG image shown in panel a. (**d**) evaluation of mean length and width of collagen fibers from the SHG images before and after the abovementioned intensive IVA therapy period assessed by CT-FIRE (LOCI, USA) algorithm.

5. Discussion

P-Asc's selective anticancer and antimicrobioal effect, alone or as an adjuvant, has been described in various in vitro and in vivo studies (23, 229, 318, 548). However, these findings are not without controversy. The controversy in part stems from the route of administration (oral vs intravenous) (309, 311, 549) and the discrepancy between in vitro and in vivo studies (229, 317, 318, 550-552). Moreover, P-Asc, due to its reducing properties interferes with several conventional assays that rely on oxidation/reduction processes (553-556). P-Asc's exact mechanism of action is yet to be elucidated. To date, most commonly suggested mechanism for prooxidant effects of P-Asc has been attributed to the formation of Fe^{2+} and $Asc^{\bullet-}$ by reduction of Fe^{3+} with $AscH^{-}$ ($Fe^{3+}+AscH^{-} \rightarrow Fe^{2+}+ Asc^{\bullet-}$). Reaction of O_2 with Fe^{2+} leads to generation of Fe^{3+} and $O_2^{\bullet-}$ (23, 229, 557). A subsequent dismutation of $O_2^{\bullet-}$ results in formation of H_2O_2, which in turn undergoes a Fenton reaction with Fe^{2+} to produce $HO^{\bullet}$ and Fe^{3+}. P-Asc as a reductant, can recycle Fe^{3+} back to Fe^{2+}, thus drive the Fenton reaction (23, 229). A second commonly proposed view is that high levels of DHA, the oxidized form of $AscH^{-}$, enter the cells via GLUT channels and, intracellular reduction of DHA to $AscH^{-}$ leads to depletion of GSH and NADPH and in turn generate oxidative stress (27, 236). Verrax et al. demonstrated that preincubation of tumor cells with deferoxamine mesylate, a cell-permeable metal chelator had a protective effect against P-Asc toxicity and in contrast, two cell-impermeable iron chelators failed to protect, verifying the importance of intracellular metals in P-Asc toxicity (558). Our studies revealed that presence of a cell permeable iron chelator 2,2′-bipyridyl hindered killing, which imply the involvement of Fenton reaction in antifungal effect of P-Asc on C. albicans (Figure 5). Notable amounts of $HO^{\bullet}$ detected by conventional confocal microscopy in P-Asc treated cells in PBS (Figure 7E) further confirmed this hypothesis.

Several studies suggest that respiration as well as availability of molecular O_2 plays a significant role in efficacy of antifungal and antibacterial agents (360, 559, 560). Lobritz and colleagues also described that by accelerating basal rate of respiration, efficacy of bactericidal drugs can be enhanced (560). As discussed by Fenchel and Finlay, because deaeration of liquid media occurs during autoclave sterilization process (561) and the diffusion coefficient for O_2 into water is extremely low in a static culture, cells below ~1 mm grow anaerobically (561, 562). In addition, static yeast cells tend to

sediment rapidly, and in turn uniform oxygenation cannot be maintained. For these reasons, agitation/shaking is necessary both for augmenting O_2 diffusion into liquid media and its equal distribution to cells (561). High level of HO$^•$ generation and fungicidal activity observed in cells treated with P-Asc in PBS with agitation (aeration) (Figure 7E, Figure 4), and the lack of it under static condition (Figure 4) may be attributed to this requirement for oxygenation. Moreover, inhibition of killing observed in cells treated with P-Asc in PBS with shaking at 4°C (Figure 4) is likely to be due to the low metabolic activity, thus lower rate of respiration.

When cells are exposed to different levels of stress, while some may be killed, or damaged, others may show no noticeable phenotypic change (563, 564). In microbiology, dormancy often refers to a state in which cells are not able to form a colony when plated on an agar medium, but at the same time they are not dead such that when conditions are suitable they can return, by resuscitation, to a state of colony-forming (564). Along these lines, it is notable that C. albicans cells exposed to P-Asc in PBS under static condition (Figure 4) may have gone towards a state of early apoptosis considering that there was no loss of viability by CFU determinations, but only a delay in colony formation (data not shown) (565). Oxidative damage, shown by slight generation of HO$^•$ (Figure 7D), attenuated signal of NAD(P)H (Figure 8E) and morphological changes observed by a peripheral ring with a central hole in bright field microscopy (Figure 9G), was probably repaired by the still active antioxidant defense system of these cells.

When glucose as a carbon source was added into PBS in the absence of nitrogen, P-Asc killing was partially inhibited (Figure 3). As previously mentioned, presence of glucose increases resistance to oxidative stress (369). For instance, transient exposure to glucose was shown to protect cells from H_2O_2 and also from miconazole, an azole antifungal drug (369). Apart from upregulation of genes involved in stress response, a metabolic shift to the glycolytic pathway and reduced activity of electron transport chain, may also be involved in this process (369, 566). In addition, generation of sufficient levels of NADPH through pentose phosphate pathway may restore cellular antioxidant capacity eg. by facilitating regeneration of GSH. Yeast cells presumably uptake P-Asc through glucose (hexose) transporters (567-569). An alternative explanation could be the diminished uptake and in turn diminished P-Asc concentration in cells due to saturation of hexose channels by the presence of glucose.

To date, C. albicans stress responses have been mainly studied on cells cultured in rich glucose-containing media, but such environments are significantly different from host microenvironments, which are mostly glucose-limited, heterogenous and complex (366). For instance, while glucose levels in blood and vaginal secretions are around 0,8% and 0,5%, respectively, glucose content in one of the commonly used nutritious media, YPD is 2% (365-367). In an attempt to understand variations in, in vitro and in vivo activity of P-Asc, sensitivity of C. albicans cells to P-Asc was tested in different media containing fermentable carbon source dextrose (YPD), nonfermentable carbon source glycerol (YPG) (543) and PBS, under aeration (shaking). No reduction in number of CFU was observed when the cells were treated in either nutrient rich growth media (Figure 3). Inhibition of killing observed in YPD media is partially due to presence of glucose, because the same amount of glucose introduced in PBS, hindered cytotoxic effects of P-Asc. Moreover, peptone, present in both YPD and YPG, is a source of carbon, nitrogen, vitamins and minerals and as known, the initial two are the major sources for biosynthetic processes and energy. Therefore, depleted cellular metabolic products and enzymes caused by prooxidant effects of P-Asc may be compensated by the abundant supply of substrates present in the nutrient rich growth media. Nevertheless, YPD and YPG are considered as complex mediums with undefined compositions, therefore unknown interactions may have also taken place between the media components and P-Asc that in turn increase its consumption and/or reduce its activity.

Literature suggests that, growth history plays an important role in fungi cells' response to new conditions such as starvation or oxidative stress (345, 570). In general, cells in stationary phase are more tolerant to stress conditions than those in the logarithmic-growth phase (341). Although exact timing of entry into stationary phase is not clearly defined (298, 341, 343-349), several distinct features such as drug resistance, DNA repair, cell wall biosynthesis, virulence gene expression, and gluconeogenesis have been attributed to the stationary phase (341). Whilst some studies consider a C. albicans culture grown overnight or for 48 h to be in stationary phase (348, 349, 571, 572), others report this phase to start at a much later time, e.g. between 3 and 8 days (341, 347, 573-575). Based on these reports, we investigated whether cells exposed to P-Asc in PBS with aeration (agitation) demonstrated growth history and phase dependent sensitivity and, we defined 1 day cultures as early stationary phase and 4 day cultures as

late stationary phase. The cells were more sensitive to P-Asc when they were in early stationary phase or in a log phase inoculum than, those that come from a late stationary-phase inoculum (Figure 6). These findings are in agreement with Uppuluri and Chaffin's study in which the group reported higher expression of oxidative stress resistance genes at 3 days and beyond (341).

Intracellular autofluorescence is usually dominated by NAD(P)H and FAD, both of which are indicators of intracellular redox state and metabolic activity (476, 540, 546, 576). Elevations in FAD autofluorescence were shown to be correlated with markers of apoptosis and oxidative stress (476, 508, 577, 578). Conversely, remarkable reduction in NAD(P)H and GSH signals were observed when cells encountered high levels of ROS (498, 508). Our results demonstrated that, cells exposed to P-Asc in PBS for 30 min under static condition resulted in almost total depletion of NAD(P)H together with a slight increase in FAD autofluorescence intensity (Figure 8E-F), whereas same treatment for 20 min by shaking, led to a similar attenuation in NAD(P)H signal but this time together with a rapid strong increase in FAD autofluorescence (Figure 8G-H). Increased oxidative stress, denoted by increased $HO^\bullet$ is most likely responsible for the shift in overall cellular redox state of C. albicans (Figure 7D-E). In addition to ROS, generated by Fenton reaction, recycling $Asc^{\bullet-}$ and DHA to $AscH^-$ may also consume significant amounts of NAD(P)H and GSH (23, 231, 229, 236). This would in turn result in depletion of cellular antioxidant capacity, enhalt ATP production, and lead to a cellular energy crisis (231).

Fungal vacuoles are acidic organelles with detoxification function and, degradative and storage capacities like mammalian lysosomes (547, 579). They are considered as the major storage compartment for amino acids, phosphate, and many metal ions (547, 580). Vacuoles also have a significant role in responding to various stresses such as nutrient deprivation, ionic and osmotic stress. For instance, they regulate iron homeostasis by altering expression of iron transporters in response to new conditions such as oxidative stress and iron deprivation (547, 581-583). In response to stress, changes in vacuole morphology are observed. Whilst actively metabolizing log phase cells have 2-3 medium sized vacuoles, during stationary phase or with glucose deprivation these vacuoles merge and form a single large vacuole (547). In this study, we compared morphological modifications in C. albicans by brightfield microscopy and TEM to elucidate the effect of P-Asc on intracellular organells. When cells were

exposed to P-Asc in PBS (with or without agitation), the most prominent feature was a deflated ball like morphology observed in majority of the cells (Figure 9G-H). These cells exhibited a large central depression with a peripheral ring. To further investigate and confirm this observation, we compared cells incubated in nutritious growth media, PBS and PBS with P-Asc (all with agitation) by TEM (Figure 9A,B,C,D). In cells treated with P-Asc in PBS, small electron dense vacuoles were replaced with a large electron lucent vacuole and this morphological change was also accompanied by loss of organelle structure (Figure C-D).

Studies suggest that modifications in collagen structure and orientation in tumor stroma can provide insights into tumor development, progression and/or metastasis (501, 526, 539). Sapudom and colleagues showed that breast cancer invasiveness increased with increasing collagen fiber thickness (521). Likewise, Drifka et al. described increased collagen width around malignant ducts of pancreatic ductal adenocarcinoma (530). On the other hand, a recent study by Kiss et al. have demonstrated that tumor stroma of BCC lesions had reduced SHG signal intensity and fiber angle, higher allignment of collagen fibers, increased collagen fiber length but similar collagen width and straightness (534). When majority of these findings are taken into account, reduced peritumoral collagen fiber length and width after intense IVA therapy (Figure 10d) is suggestive of response to therapy, which can be monitored by SHG. Nevertheless, small sample size used in this study warrants further studies with larger sample size and validation.

6. Conclusion

In order to address seemingly contradicting results from various studies investigating prooxidant effects of P-Asc and understand the conditions in which P-Asc shows antifungal activity against C. albicans, we have conducted our studies in different experimental settings and employed various optical imaging tools to assess its effects. According to our results, it is evident that some of the conflicting findings are due to the variations in cell metabolism and environment in vitro and in vivo conditions. P-Asc's efficacy as a prooxidant depends on oxygenation, temperature, access to nutrition, presence of iron and cell growth history. Therefore, inter- and intra-host heterogeneity in infections and, inter- and intra-tumor heterogeneity in cancer cells as well as their types should be taken into account while considering P-Asc as a prooxidant. Moreover, in the light of these findings, therapeutic response may be augmented by addition of vasoactive agents or hyperoxic gas mixtures. In an attempt to further elucidate mechanistic insights into P-Asc's prooxidant effects, we employed various optical imaging modalities, some of which can potentially serve as real time monitoring tools for treatment response. To the best of our knowledge, we demonstrated for the first time that intracellular HO$^{\bullet}$ generation by P-Asc is an active participant in oxidative damage to cells. Consistent with this finding, simultaneous label-free live cell imaging with TPEFM and conventional confocal microscopy revealed a marked reduction in NAD(P)H and elevation in FAD^{+} levels, respectively, the latter mainly in dying cells. Concomitantly, a vacuolar enlargement was captured by TEM, and coincided with the deflated ball appearance seen in brightfield microscopy.

Ex-vivo imaging studies were performed with TPEFM and SHG using limited number of skin biopsy samples taken from basal cell carcinoma lesions. Results indicated that IVA therapy alters tumor collagen environment. These novel label free optical imaging tools may enable real time in vivo evaluation of treatment response and eliminate biopsy as well as routine staining processes.

7. Summary

The discovery of hexanuric acid, later renamed as L-ascorbic acid, by the 1937 Nobel Prize laureate Albert Szent-Györgyi was a milestone in the expanding and multidisciplinary field of antioxidants. The second turning point in the history of ascorbic acid began in 1970s, when first reports appeared describing its potential effects against common cold and cancer. Since then, pharmacologic use of ascorbic acid as a prooxidant (P-Asc), especially at high doses became the subject of a long-standing controversy. Some of this controversy stems from experimental methodology, fundamental differences between in vitro and in vivo conditions and tissue heterogeneity, which in part results from environmental distinctions. Literature regarding antifungal effects of P-Asc against Candida albicans is sparse and inconclusive. In addition, its exact mechanism of action is not yet fully elucidated. In an attempt to gain mechanistic insights, understand the conditions in which P-Asc exerts its prooxidant effects and, circumvent some of the limitations arising from the experimental methodology, we investigated the effect of P-Asc under different experimental conditions and exploited various optical imaging tools. Our results demonstrated that, growth history of cells, their access to nutrition, oxygenation, temperature, presence of transition metal ions are all important factors that need to be taken into consideration. On the other hand, intracellular hydroxyl radical generation seems to play an important role in oxidative stress generated by P-Asc. In parallel with this finding, simultaneous label-free live cell imaging with two-photon emission fluorescence microscopy (TPEFM) and conventional confocal microscopy revealed a marked reduction in NAD(P)H and elevation in FAD levels, respectively, the latter mainly in dying cells.

Combining TPEFM with second harmonic generation microscopy and an advanced image analysis technique enabled us to assess fine changes of tissue pathology during intravenous P-Asc (IVA) therapy of basal cell carcinoma (BCC), which were not apparent in standard hematoxylin and eosin stained histology sections. The results of our ex-vivo study with limited skin biopsies taken from BCC lesions revealed that IVA therapy alters tumor collagen environment. These morphological alterations could be exploited to monitor real time label free in vivo evaluation of treatment response without any need for biopsies.

8. Összefoglalás

Az 1937-ben Nobel-díjat nyert Szent-Györgyi Albert által felfedezett henaxuronsav, későbbi nevén L-aszkorbinsav mérföldkő volt az antioxidánsok egyre jobban fejlődő és később multidiszciplinárissá váló területén. Az aszkorbinsav történetében bekövetkező második fordulópont az 1970-es évekre tehető, ekkor jelentek meg először a megfázás és a daganatok elleni potenciális hatásáról szóló publikációk. A nagy dózisban adott aszkorbinsav mint prooxidáns hatóanyag (P-Asc) gyógyszerként való használata ugyanakkor régóta tartó ellentmondás tárgya. Az ellentmondások részben a kísérletes módszertanok eltéréseiből, az in vitro és in vivo körülmények alapvető különbözőségéből és a szöveti heterogenitásból erednek, melyek részben a környezeti különbségek eredményei. Az irodalmi adatok a P-Asc Candida albicans elleni antifungális hatásáról szegényesek és nem meggyőzőek. Emellett a pontos hatásmechanizmus sem volt ezidáig teljesen tisztázott. A P-Asc prooxidáns hatásának és hatásmechanizmusának jobb megértéséhez, azért hogy kizárjuk a kísérletes módszertanból adódó különbségeket, eltérő kísérletes körülmények között különböző optikai képalkotó eszközök használatával végeztünk vizsgálatokat. Eredményeink azt mutatták, hogy a sejtek növekedésének üteme, a tápanyagokhoz való hozzáférése, az oxigenizáció, a hőmérséklet, valamint az átmeneti fémek ionjainak jelenléte mind jelentős faktorok, melyeket mind fontos figyelembe venni.

Másrészről az intracelluláris hidroxilgyök-termelődésnek is fontos szerepe lehet a P-Asc által generált oxidatív stresszben. Ezzel az eredménnyel egybevágott a festékjelölés-mentes sejtes képalkotás kétfoton fluoreszcencia mikroszkópiával (TPEFM) illetve konvencionális konfokális mikroszkópiával végezett vizsgálatának eredménye, jelentős csökkenést mutattak a NAD(P)H, valamint emelkedést a FAD szintek, utóbbi főként a pusztuló sejtekben volt megfigyelhető.

A TPEFM másodharmonikus keltéses mikroszkópia és a fejlett képfeldolgozó módszerek kombinációja lehetővé tették a szöveti patológia finom változásainak elemzését bazálsejtes carcinoma (BCC) intravénás P-Asc (IVA) terápiája mellett, melyek hagyományos hematoxilin-eozin festéssel nem tehetők láthatóvá. Az ex-vivo tanulmány limitált számú, BCC léziókból származó bőrbiopsziás mintáinak eredménye azt mutatatja, hogy az IVA kezelés befolyásolja a tumor kollagén környezetét. Ezen

morfológiai változások felhasználhatók a terápiás válasz real-time festékjelölés-mentes in vivo monitorozására, így a kontroll bőrbiopsziás mintavétel szükségtelenné válhat.

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10. Acknowledgements

11. List of publications

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Bánvölgyi A, Lőrincz K, Kiss N, **Avci P**, Fésűs L, Szipõcs R, Krenács T, Gyöngyösi N, Wikonkál N, Kárpáti S, Németh K. Efficiency of long-term high-dose intravenous ascorbic acid therapy in locally advanced basal cell carcinoma – a pilot study. **Adv Dermatol Allergol**. 2019, in press, https://doi.org/10.5114/ada.2019.83027

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