PALMITOYL ASCORBATE-MODIFIED LIPOSOMES FOR THE
TREATMENT OF RENAL CELL CARCINOMA

Doctoral Dissertation Presented

By

Madhura Sanjay Deshpande

Advisor: Dr. Vladimir P. Torchilin

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ABSTRACT

Cancer is one of the leading causes of death worldwide. Very limited treatment options exist at present. Nanotechnology has played a major role in cancer therapy and diagnosis by tackling the problem of targeting the site of tumor without harming the normal cells, thus avoiding unwanted side effects. Drug delivery by pharmaceutical nanocarriers such as liposomes has been used successfully to specifically target tumor sites, improve efficacy of the encapsulated drug, modify and control drug pharmacokinetics, increase drug accumulation in the tumor area and reduce undesirable toxic side effects at off-target sites. In this project, liposomes were used as a delivery system for palmitoyl ascorbate to target renal cell carcinoma.

Palmitoyl ascorbate is a hydrophobized derivative of ascorbic acid and a stable alternative to free ascorbate, which itself has strong anticancer properties. Ascorbate is an anti-oxidant at low concentrations, a pro-oxidant at high concentrations, and has a role in hypoxia-inducible factor regulation. Ascorbate can kill tumor cells through peroxide damage and increased production of reactive oxygen species, without harming normal cells. Although ascorbate has shown marked cytotoxicity against tumor cells in vitro and can enhance the activity of many anticancer drugs in vitro, its use in vivo has been limited by the need for very high plasma concentrations to achieve a cytotoxic effect in the tumor cell environment. Encapsulation of palmitoyl ascorbate in liposomes will help generate accumulation of the nanocarrier at the tumor site by virtue of the enhanced permeability and retention effect and thereby effectively deliver ascorbate to the tumor site, thus reducing the need to administer high doses of free ascorbate.

Renal cell carcinomas are highly vascular and exhibit hypoxic gene expression patterns due to abnormal regulation of the hypoxia-inducible factor pathway. Von Hippel Lindau syndrome, in which the tumor suppressor Von Hippel Lindau gene is mutated, leads to development of renal cell carcinoma and demonstrates abnormal hypoxia-inducible factor regulation, leading to generation of reactive oxygen species. These characteristics in renal cancer cells could be
favorable for the use of toxic action of ascorbate and could enhance its activity. The chemical and cellular properties of ascorbate may thus be utilized to improve its anti-tumor delivery and effectiveness in renal cell carcinoma with liposomes incorporating palmitoyl ascorbate.

With this objective, liposomes encapsulating 30 mol% palmitoyl ascorbate were prepared and characterized for their size, zeta potential and palmitoyl ascorbate encapsulation efficiency. Palmitoyl ascorbate liposomes showed a higher association with 786-O human renal carcinoma cells than with non-cancerous human kidney HK-2 cells. Cytotoxicity studies showed higher cytotoxicity of palmitoyl ascorbate liposomes than ascorbic acid in both murine and human renal cancer cell lines as compared to non-cancerous cell lines. The mechanism of action of palmitoyl ascorbate was shown to be by the extracellular generation of reactive oxygen species by observation of the effect of the reactive oxygen species scavenger catalase and antioxidants glutathione and N-acetyl cysteine on palmitoyl ascorbate liposome-mediated cytotoxicity.

Palmitoyl ascorbate liposomes generated increased amounts of reactive oxygen species as compared to ascorbic acid in renal carcinoma cells, thus confirming the role of reactive oxygen species and the resulting oxidative stress in palmitoyl ascorbate liposomal activity. Annexin V staining of renal carcinoma cells showed that palmitoyl ascorbate liposomes induced greater apoptosis than ascorbic acid in the tumor cells indicating apoptosis to be a mechanism involved in palmitoyl ascorbate liposome-mediated cell death. Palmitoyl ascorbate liposomes also induced changes in the cell cycle distribution causing cell arrest in the G2/M phase of the cell cycle.

Ascorbic acid is known to enter cells via dehydroascorbic acid, an oxidized form of ascorbic acid, using glucose transporter receptors due to the structural similarity of dehydroascorbic acid to glucose. So the role of these glucose transporters with respect to palmitoyl ascorbate liposomes was analyzed using 2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose, a glucose analog, and glucose transporters were found to be involved to some extent in the association of palmitoyl ascorbate similar to that of ascorbic acid.
Spheroids, 3D assemblies of cancer cells, mimic in vitro the actual tumor architecture and permeability for various drugs and dosage forms. Spheroids artificially formed in vitro may represent better tumor models compared to monolayers. Hence we performed in vitro studies in both types of cell culture models- monolayers and spheroids. Spheroids of renal carcinoma cells were prepared and characterized to better study anticancer effects. Palmitoyl ascorbate liposomal treatment showed a stronger cytotoxic effect in renal carcinoma cell spheroids than ascorbic acid, and palmitoyl ascorbate liposomes were well distributed in the spheroids- mainly towards the periphery. Although palmitoyl ascorbate liposomes showed good efficacy by themselves, their combination with another chemotherapeutic drug may be expected to improve anticancer effect. Sorafenib is a drug already approved by U.S. Food and Drug Administration for the treatment of advanced renal cell carcinoma. Sorafenib is known to induce production of reactive oxygen species through which it exerts its cytotoxic effects during the inhibition of serine/threonine kinase and tyrosine kinase pathways in both tumor cells and the tumor vasculature. Due to this effect of sorafenib similar with ascorbate, palmitoyl ascorbate liposomal and sorafenib treatments were combined to test for an improved cytotoxic action of sorafenib to achieve an enhanced effect in the treatment of renal cell carcinoma. Sorafenib demonstrated strong cytotoxicity in both renal carcinoma cells and spheroids. A synergistic cytotoxic effect compared to the cytotoxicity of sorafenib or palmitoyl ascorbate liposomal treatment alone was observed for the combination of sorafenib and palmitoyl ascorbate liposomes at and below their IC\textsubscript{50} molar ratios in both monolayers and spheroids. Sorafenib exhibited increased generation of reactive oxygen species. Combination of sorafenib and palmitoyl ascorbate liposomes resulted in higher formation of reactive oxygen species compared to the individual treatments of sorafenib or palmitoyl ascorbate liposomes. In vivo, palmitoyl ascorbate liposomes significantly decreased the tumor growth rate in mice bearing tumors of renal cell carcinoma. Combination of palmitoyl ascorbate liposomes and sorafenib further increased tumor growth inhibition and led to tumor
stabilization compared to palmitoyl ascorbate liposomal or sorafenib treatment alone. These results indicate that palmitoyl ascorbate liposomes can serve as an efficient drug delivery system and provide enhanced anticancer efficacy when used alone and in combination with sorafenib in the treatment of renal cell carcinoma.
Dedicated to Aai-Baba, Mayur and Apoorva
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<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AT</td>
<td>3-amino-1,2,4-triazole</td>
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<tr>
<td>BSO</td>
<td>Buthionine sulfoximine</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CI</td>
<td>Combination Index</td>
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<tr>
<td>c-Kit</td>
<td>Proto-oncogene or tyrosine-proteinkinase Kit</td>
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<tr>
<td>CM-H2DCFDA</td>
<td>5-(and-6)-chloromethyl -2’7’dichlorodihydrofluroscein diacetate acetyester</td>
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<tr>
<td>DHAA</td>
<td>Dehydroascorbic acid</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>Egg PC</td>
<td>Egg phosphatidylcholine</td>
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<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>FLT3</td>
<td>FMS-related tyrosine kinase 3</td>
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<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<tr>
<td>HK-2</td>
<td>Human kidney 2 cells</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>HRE</td>
<td>HIF-responsive elements</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vascular endothelial factor</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>MCL1</td>
<td>Myeloid cell leukemia-1 gene</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<tr>
<td>PA</td>
<td>Palmitoyl ascorbate</td>
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<tr>
<td>PAL</td>
<td>Palmitoyl ascorbate liposomes</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PEG$_{2000}$-PE</td>
<td>1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N[methoxy(poly(polyethylene glycol))]-2000</td>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PL</td>
<td>Plain liposomes</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
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<td>RAG</td>
<td>Murine renal adenocarcinoma cells</td>
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<td>RCC</td>
<td>Renal cell carcinoma</td>
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<tr>
<td>RENCA</td>
<td>Murine renal cortical adenocarcinoma cells</td>
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<td>Rh-PE</td>
<td>Rhodamine-phosphatidylethanolamine</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SFB</td>
<td>Sorafenib</td>
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<td>TGF-α</td>
<td>Transforming growth factor- α</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>Von Hippel-Lindau</td>
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<td>7-Aminoactinomycin D</td>
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1. STATEMENT OF HYPOTHESES

1. Liposomes incorporating palmitoyl ascorbate will associate with renal carcinoma cells and show enhanced selective cytotoxicity towards the tumor cells.

2. Liposomes modified with ascorbate residues will show an increase of reactive oxygen species production and apoptosis, thereby providing an efficient palmitoyl ascorbate-based nanodelivery system to achieve high anticancer efficacy \textit{in vitro}.

3. Palmitoyl ascorbate liposomes will demonstrate an increased penetration and cytotoxicity in spheroids of renal cell carcinoma.

4. Combination of liposomes containing palmitoyl ascorbate with the chemotherapeutic drug, sorafenib, will improve the cytotoxic action of sorafenib \textit{in vitro} in cells and spheroids of renal cell carcinoma and will exhibit greater tumor growth inhibition \textit{in vivo} to achieve an enhanced anticancer effect in the treatment of renal cell carcinoma.
2. OBJECTIVE AND SPECIFIC AIMS

2.1. OBJECTIVE

The goal of this project is to use liposomes modified with palmitoyl ascorbate as a nanocarrier for the utilization of chemical and cellular properties of ascorbate to improve the delivery and antitumor activity of ascorbate *in vitro* in both cells and spheroids of renal cell carcinoma, and *in vivo* in mice bearing tumors of renal cell carcinoma. Our aim is also to increase the effectiveness of a chemotherapeutic drug, sorafenib, when used in combination with palmitoyl ascorbate liposomes to enhance efficacy in the treatment of renal cell carcinoma.

2.2. SPECIFIC AIMS

1. To prepare and characterize liposomes modified with palmitoyl ascorbate and test their cytotoxic effect and cell association towards renal carcinoma cells.

2. To study the mechanisms by which palmitoyl ascorbate exerts its effects on tumor cells, including generation of reactive oxygen species and apoptosis in renal carcinoma cells.

3. To determine the cytotoxicity and generation of reactive oxygen species by sorafenib alone and in combination with palmitoyl ascorbate liposomes in renal carcinoma cells.

4. To prepare and characterize spheroids of renal cell carcinoma and evaluate cytotoxicity and distribution of palmitoyl ascorbate liposomes in spheroids.

5. To determine the cytotoxicity of sorafenib alone and in combination with palmitoyl ascorbate liposomes in spheroids of renal cell carcinoma.

6. To evaluate tumor growth inhibition to determine the effectiveness of palmitoyl ascorbate liposomes, sorafenib and their combination therapy in mice bearing tumors of renal cell carcinoma.
3. INTRODUCTION

3.1. STATEMENT OF THE PROBLEM

3.1.1 RENAL CELL CARCINOMA

Cancer can originate almost anywhere in the body and arises due to a loss of normal cell growth control. The gradual increase in uncontrolled cell growth produces a growing mass of tissue called a tumor or neoplasm. Cancers can spread throughout the body by direct migration and penetration by cancer cells into adjacent tissues (invasion) and by penetration of cancer cells into lymphatic and blood vessels, circulation through the bloodstream, and then invasion of normal tissues elsewhere in the body (metastasis) [1].

Renal cell carcinoma (RCC) is cancer that forms in the lining of renal tubules of the kidney that filter the blood, reabsorb nutrients and excrete waste products [2]. RCC is an increasing health risk and is responsible for approximately 3% of adult malignancies and 90-95% of neoplasms arising from the kidney, with 13000 deaths of the 57000 cases of RCC in the U.S. every year [3]. This disease does not exhibit any early warning symptoms, is resistant to chemotherapy and radiation and has varied clinical manifestations [4-6]. In RCC cases, surgical removal of the tumor, or nephrectomy, is the primary standard of treatment, but the results vary depending on the stage of cancer when it is diagnosed. The treatment also becomes difficult in cases of metastasized tumors and advanced renal cell cancers. So, development of advanced therapies is essential.
3.1.2. LIPOSOMES AS NANOCARRIERS FOR DRUG DELIVERY

Even though scientific understanding of cancer biology has increased tremendously over years, the demand to covert this knowledge into effective diagnostic and therapeutic options remains. The primary treatment choices for cancer include surgery, radiation and chemotherapy. Several chemotherapeutic drugs to be administered orally or by intravenous (i.v.) injection have been approved by the U.S. Food and Drug Administration (FDA), but their use has been hindered by dose-restricted toxicity and adverse side effects. In cases of the oral route of drug delivery, the drugs are rendered to the metabolic pathways in the body that lead to altered pharmacokinetics of the drugs. In systemic drug delivery, most chemotherapeutic drugs kill normal cells along with cancerous cells causing uneven and non-specific biodistribution of the drug and undesired off-site effects [7]. Due to this, larger quantities of drugs are required to achieve beneficial therapeutic effect since limited dose of drug can be allowed, and a large part of these may be deposited in the normal tissues resulting in undesirable toxicity [8]. A potential solution to these problems in cancer therapy is to target the tumor site specifically after systemic administration by nanocarriers to avoid the unwanted adverse side effects at off-target sites.

Pharmaceutical nanocarrier-associated drug delivery has been used effectively to improve the delivery of a wide range of compounds such as small molecule drugs, peptides or proteins and genes [9, 10]. An ideal delivery system has several properties including an enhanced therapeutic effect or in vivo efficiency, good biocompatibility and biodegradability, increased stability against drug leakage during transit to target and degradation early drug clearance, increased drug localization at the tumor site, decreased drug accumulation at non-target sites and controlled drug release at the target site [10, 11]. Several nanoparticulate pharmaceutical carriers including liposomes, polymeric micelles, nanospheres, nanocapsules, nanoconjugates, carbon nanotubes and nanorods, lipoproteins, dendrimers and cell ghosts have been investigated and used for therapy and diagnosis, both experimentally and clinically [8, 10, 12-16].
Of all these nanocarrier approaches, the most simple and basic system for drug delivery in cancer includes the lipid/polymer-based drug carriers [17] such as liposomes.

![Figure 1. Schematic representation of the structure of a liposome. Soluble hydrophilic drugs are entrapped into the aqueous interior of the liposome, while poorly soluble hydrophobic drugs are localized in the liposomal membrane [18].](image)

Liposomes are lipid-bilayered vesicular nanostructures which self-assemble from phospholipid and cholesterol molecules (which make up cell membranes) [19]. The sizes of single bilayer, pharmaceutically acceptable liposomes typically vary from 80 to 200 nm. Various water-soluble drugs can be incorporated within the inner hydrophilic core and water-insoluble drugs can be loaded within the hydrophobic compartment of the phospholipid bilayer of the liposomes [20] (Fig. 1). Liposomes are biologically inert, biocompatible and typically cause no toxic or immunologic reactions. They have several useful characteristics such as simple preparation and scale-up, easily controllable composition and size, good drug loading efficiency, long circulation in blood and enhanced in vivo stability. Liposomes protect the drugs incorporated in them from
the damaging external media and show slow and sustained drug release [8, 20]. The association of pharmaceutical nanocarriers such as liposomes with drugs can significantly modify the properties of the drugs and can help improve the drug biodistribution and pharmacokinetics by delayed drug absorption, decreased drug biodistribution volume, limited drug biodistribution, retarded drug metabolism, delayed drug clearance, increased fraction of drug accumulated at the pathological target site and less unwanted, off-target side effects [20].

Pharmaceutical nanocarriers such as liposomes can enter the site of action, such as a solid tumor by a phenomenon known as the enhanced permeability and retention (EPR) effect. EPR effect occurs due to various factors related to the high level of angiogenesis required by the cancer cells for survival and the resulting hypervascularity, formation of a defective vascular architecture in tumor tissue, presence of a defective lymphatic drainage system and the action of several permeability enhancing factors like vascular endothelial growth factor (VEGF), bradykinin, prostaglandins, nitric oxide/peroxynitrite and tumor necrosis factor. The normal vasculature presents relatively tight endothelial gaps that are less leaky. However, the tumor vasculature shows excessive leakiness due to improper blood capillary system formation owing to increased demand of oxygen and nutrients and can allow the penetration of large molecules or small particles, leading to their extravasation and accumulation in the interstitial tumor space [8, 20]. Additionally, the defective lymphatic drainage system in tumor tissue further enables the retention of the extravasated components including nanocarriers with drugs encapsulated in them at the tumor site. Even so, the blood vessels comprising the leaky vasculature in peripheral tumors have a restricted cutoff pore size ranging from 200 nm to 700 nm in diameter [20]. The EPR effect thus allows for passive targeting and excessive extravasation of the drugs encapsulated in nanocarriers such as liposomes into tumors.
Since liposomes have been studied widely as efficient nanocarriers, they can be used successfully to deliver drugs to tumors for effective anticancer therapy. Hence we have used liposomes as carriers for delivery of palmitoyl ascorbate (PA), which possesses anticancer properties, for the treatment of RCC.
3.2. REVIEW OF THE LITERATURE

3.2.1. ASCORBATE IN CANCER THERAPY

Ascorbic acid (AA) is a water-soluble antioxidant and enzyme cofactor. It is a ketolactone with two ionizable hydroxyl groups. It is produced because of the activity of the enzyme gluconolactone oxidase in many species by the hexuronic acid pathway in the liver or kidney [21]. Humans do not possess this enzyme and hence do not generate ascorbic acid. Ascorbic acid, which is the reduced form and dehydroascorbic acid (DHAA), which is the oxidized form are the two chemical states in which vitamin C may be present. The reduced form of vitamin C (i.e. ascorbic acid) is the primary form present in the human body. It is an important micronutrient and is involved in many biological and biochemical pathways essential in normal function of the body [22].

3.2.1.1. HISTORY

Ascorbate treatment has a controversial history in cancer therapy [23]. The anticancer activity of ascorbate was based on two hypotheses, but had no supporting data. About 50 years ago, McCormick hypothesized that ascorbate-induced collagen synthesis led to its anticancer action [24, 25]. In 1972, Cameron and Rotman hypothesized that inhibition of hyaluronidase activity by ascorbate was responsible for its anticancer effects [26, 27]. Ascorbate has long been accepted as safe and well tolerated. However its application in cancer therapy has been controversial due to the contradictory results in several key clinical trials [23]. In the study by Cameron and Pauling in 1976 and 1978, administration of an intravenous infusion of high doses of ascorbate was found to increase the survival period of late-stage cancer patients by up to 20 times compared to control patients [28]. Similar results were found from Scottish and Japanese trials [23, 29].
However, the results of a double-blind placebo-controlled clinical trial at the Mayo clinic reported no anticancer effects of ascorbate [30, 31]. Recent pharmacological studies indicate that the contradictory results in the earlier trials stem from a critical difference between the trials which was not understood then. There was a difference in the route of administration of ascorbate to the patients in those studies [29]. The Cameron-Pauling study used a combination of oral and intravenous ascorbate, while the Mayo clinic trial used only oral ascorbate. The ascorbate administered orally generates far lower ascorbate blood levels than ascorbate administered intravenously. In fact, intravenous ascorbate administration generates 25 times higher blood concentrations when same dose of ascorbate is administered orally [32]. High blood concentrations of ascorbate obtained by intravenous administration, but not by oral delivery, have shown anticancer activity in vivo and in clinical studies. But lower blood concentrations of ascorbate attained by oral supplementation have not shown any significant anticancer activity in vitro or in clinical trials, although it showed some anticancer effect in some animal models. These findings and a better understanding of ascorbate pharmacology has renewed the interest in research on intravenous ascorbate therapy alone or in combination with other therapies for the treatment of solid tumors.

3.2.1.2. PROPERTIES OF ASCORBATE

Ascorbic acid is a water-soluble antioxidant and a ketolactone with two ionizable hydroxyl groups. The ascorbate monoanion, Asc$^-$, is the form predominantly present at the physiological pH (Figure 1) [33]. Ascorbate monoanion and ascorbate radical, Asc$^-$, have a one-electron oxidized state and have low one-electron reduction potentials and can thus reduce most biologically relevant radicals. It can also scavenge oxidants like superoxide anion, hydroxyl
radical or singlet oxygen generated in several extracellular and intracellular biological phenomena [21, 22].

Figure 3. Structures of the redox states associated with vitamin C (ascorbic acid) [33].

Ascorbate is a very strong reducing agent and readily goes through two consecutive, one-electron oxidations to form ascorbate radical (Asc•−) and DHAA. The resonance stabilization of the unpaired electron makes the ascorbate radical quite unreactive and readily dismutates into ascorbate (AscH−) and DHAA [33, 34].

\[
2 \text{Asc}^{•−} + \text{H}^+ \leftrightarrow \text{AscH}^{-} + \text{DHAA}
\]

Ascorbate is an effective donor antioxidant due to these properties [35].
Ascorbate oxidizes readily. The oxidation rate is pH-dependent and is accelerated by catalytic metals. In the absence of catalytic metals, the spontaneous oxidation, or auto-oxidation, of ascorbate via the ascorbate dianion, \( \text{Asc}^{2-} \), is very slow at pH 7 [33, 36].

\[
\text{Asc}^{2-} + \text{O}_2 \rightarrow \text{Asc}^{-} + \text{O}_2^{-}
\]

At pH 7, ascorbate is the predominant form of vitamin C (99.9%) with low concentrations of AA (0.1%) and ascorbate dianion (0.005%).

When present at high concentrations, ascorbate acts as a pro-oxidant. Ascorbate acts as a pro-oxidant via the reduction of transition catalytic metal ions such as iron and copper which generates highly reactive free hydroxyl radicals [21, 33]. These hydroxyl radicals induce oxidative stress on the cells and have been reported to interact with DNA, in turn, damaging it [37].

\[
\text{AscH}^- + \text{Fe}^{3+}/\text{Cu}^{2+} \rightarrow \text{Asc}^- + \text{Fe}^{2+}/\text{Cu}^+ + \text{H}^+
\]

\[
2\text{Fe}^{3+}/\text{Cu}^+ + 2\text{H}_2\text{O}_2 \rightarrow 2\text{Fe}^{2+}/\text{Cu}^+ + 2\text{OH}^- + 2\text{OH}^-
\]

Ascorbate cannot be synthesized in the human body and hence diet is the primary source of ascorbate in humans. Generally, the human diet consists of both ascorbate and DHAA and its absorption occurs in the enterocytes of the small intestine. The bioavailability of ascorbate in the body is governed by internal absorption and renal re-absorption. The highly controlled plasma concentrations of ascorbate at oral doses of more than 100 µM are due to these two processes [38]. The steady-state plasma concentrations are about 80 µM at oral doses of 200 mg. In case of higher doses above 200 mg, the fraction of bioavailable ascorbate is decreased because the intestinal absorption decreases and renal excretion increases in such cases [39]. Intravenous delivery of ascorbate circumvents the intestinal absorption thereby leading to an increased ascorbate plasma concentration. Padayatty et al. found that the peak plasma concentration of ascorbate is significantly higher when administered intravenously than that attained by oral delivery. Peak vitamin C plasma concentrations of only about 0.2 mM for a maximum tolerated
oral dose of 3 g every 4 hours and approximately 15 mM for a 100 g intravenous dose were forecasted by pharmacokinetic modeling [32]. An intravenous dose of 10 g of ascorbate is expected to achieve a plasma concentrations of about 6 mM, which is more than 25-fold greater than the plasma concentration obtained by the same oral dose, was reported in a recent pharmacokinetics study [40, 41]. Thus, the plasma levels and pharmacological effects of ascorbate are highly dependent on its route of administration. Since intravenous ascorbate may produce 70-100 fold higher concentrations than the maximum tolerable dose [42], there is an increased interest in intravenous ascorbate as an anticancer drug. Several clinical trials are being carried out to improve the activity of ascorbate while maintaining its safety profile and use it in combination with other agents in anticancer therapy.

The need for high doses of ascorbate for anticancer toxicity is due largely to the strict physiologic regulation of ascorbate combined with the limited chemical stability of native ascorbate. As a result, several hydrophobized derivatives of ascorbate have been explored as stable alternatives. We have used such a hydrophobized acyl derivative of AA- palmitoyl ascorbate (PA) to make it more lipophilic and stable. AA has a log P value of -3.36 and PA has a log P value of 2.95 indicating a greater lipophilicity of PA as compared to AA. Moreover, PA offers the potential for the formulation of ascorbate-bearing pharmaceutical nanocarriers to improve the delivery of ascorbate to the tumor, thereby reducing the need for high doses of ascorbate.

3.2.1.3. ASCORBATE AND CANCER

Ascorbate has several properties that may improve the treatment of solid tumors and thus has potential as a cancer targeting agent to selectively treat the solid tumors and being relatively non-toxic to normal tissues. Ascorbate is known to accumulate in cells by two modes: sodium-
dependent direct transport of AA or entry of AA via DHAA. Sodium-dependent vitamin C transporter (SVCT) has been identified in the uptake of AA with two sodium cations transported for each ascorbate anion. Two sub-types of SVCT, SVCT-1 and SVCT-2, have been cloned and characterized in rats and humans. Of these, SVCT-2 has been reported to be important for uptake of AA in metabolically active and specialized tissues [43, 44]. The glucose transporter 1 (GLUT-1) and glucose transporter 3 (GLUT-3) are over-expressed on many tumor cells due to the high glucose demand and hypoxic conditions common in solid tumors [45]. DHAA is structurally similar to glucose and hence GLUT-1 has a similar affinity for DHAA and glucose. Stromal tissues surrounding the tumors or the tumors themselves generate superoxide anion, which converts AA to DHAA. This DHAA is transported into the cells by GLUT-1 and GLUT-3 [46]. Once inside the cell, DHAA is reduced back to AA, which then cannot be transported out by the GLUTs. Thus, once the nanocarrier is within the interstitial fluids of solid tumor, the interaction of the carrier with the transporters allows the entry and accumulation of AA in the tumor cells.

Glutathione (GSH) is an important antioxidant and reducing agent and plays a part in several metabolic functions of the cell. GSH is a co-factor for glutathione peroxidase and other enzymes which help reduce oxidative stress. It scavenges hydroxyl radical and singlet oxygen and regulates the redox-sensitive pathways [47, 48]. GSH can react directly with DHAA to regenerate ascorbate. Thus, a sudden influx of ascorbate from the nanocarrier can cause depletion of GSH as the process of conversion of DHAA to AA in the cells utilizes GSH [49]. GSH helps the tumor cells to reduce oxidative stress and evade damage by anticancer drugs, such as by detoxification reactions and some multi-drug resistance related pumps, which use GSH to expel drug molecules out of the cell [50]. GSH depletion in tumor cells can thus help increase the overall effectiveness of the drug carried in the nanocarriers.

Several studies have reported that ascorbate treatment at high doses leads to considerable extracellular generation of hydrogen peroxide (H$_2$O$_2$) in the interstitial fluid of tumor tissues,
which causes oxidative stress leading to death of cancer cells [25, 51-58]. Chen et al. found that high ascorbate concentrations are preferentially toxic to tumor cells, causing H$_2$O$_2$-induced cell death, as compared to normal cells. They found in vitro that 100 to 150 µM peroxide is produced by 2 to 5 mM ascorbate in tumor cells, while even 20 mM ascorbate treatment did not harm normal cells [58]. H$_2$O$_2$ is membrane permeable, so it can have its targets present intracellularly or extracellularly and plays a role in the death of tumor cells by several mechanisms. It can activate apoptotic pathways which in turn are related to the pathways of metabolic regulation, DNA damage detection and repair, structural integrity and cell division. Chen et al. suggested that membrane lipids forming hydroperoxides or reactive intermediates that are quenched or repaired in normal cells, but not in sensitive cancer cells, might be targeted by extracellular H$_2$O$_2$, while DNA, DNA repair proteins, or mitochondria could be targeted by intracellular H$_2$O$_2$ due to reduced superoxide dismutase activity [58, 59]. Delivery of ascorbate in a nanocarrier to tumor tissues produces high local concentrations of ascorbate, causing it to act as pro-oxidant and generate large amounts of H$_2$O$_2$, which accumulate locally due to poor tumor tissue perfusion and directly kill the tumor cells. Erythrocytes in the blood scavenge the H$_2$O$_2$ formed as they have catalase and glutathione peroxidase activities [57]. Access of the nanocarriers is often prevented in the interstitial fluid of normal tissues because of the particle size of the nanocarriers. Thus, normal tissues and blood can generate and accumulate only limited H$_2$O$_2$ from the nanocarrier. On the other hand, tumor tissues are more susceptible to nanoparticle-induced buildup of H$_2$O$_2$ because the increased tumor vasculature permeability and altered fluid dynamics that allow entry of nanoparticles in the tumor tissue [60-62]. Also, H$_2$O$_2$ generated from the AA molecules in the nanocarrier into the tumor environment along with the slow flow of interstitial fluids in tumors causes local accumulation of H$_2$O$_2$. Since tumors exhibit an altered redox status, ascorbate incorporating nanoparticles can show tumor-specific drug release and can be used as an anticancer therapy.
As discussed above, production of \( \text{H}_2\text{O}_2 \) is a mechanism of ascorbate-induced cytotoxicity. \( \text{H}_2\text{O}_2 \) may in turn produce additional reactive oxygen species (ROS) like hydroxyl radical and other secondary products of oxidation such as aldehydes [29]. Poor perfusion of tumor tissues leads to regions of low glucose, acidic extracellular fluid, high metabolic waste levels and hypoxic regions [63, 64]. Molecular oxygen acts as an electron acceptor and low oxygen levels in tumors make them highly reducing. All these factors lead to accumulation of greater than normal levels of intracellular and extracellular ROS in tumor tissues and make them more sensitive to oxidative stress [65]. A low antioxidant status due to an imbalance of antioxidant enzyme levels coupled with decreased levels of superoxide dismutase as well as of catalase and glutathione peroxidase in tumors can further promote sensitivity to ROS [66-68]. The production of peroxide by ascorbate groups encapsulated in nanoparticles serves as a source of ROS and hence can selectively kill cancer cells since they exhibit high endogenous levels of ROS. Thus, ascorbate carrying nanoparticles can be used as anticancer agents to target tumor-specific drug release by means of ROS generation.

We have used liposomes modified with PA (PAL) for delivery of ascorbate to the tumors. PAL will have the hydrophobic palmitoyl group embedded in the lipid bilayer of the liposomes and the polar ascorbate residues on the surface of the liposomes. PAL may act by a dual mechanism for delivery of ascorbate to the tumors. In one possible mechanism, ascorbate residues on the liposomal surface may produce increased ROS extracellularly in the tumor microenvironment leading to oxidative stress in the tumor cells, thereby killing them. On the other hand, few of the PA molecules may hydrolyze in the tumor microenvironment to form AA, which may oxidize to DHAA and then enter the cells via GLUTs. However the way PA may be hydrolyzed is not clear as yet. Such action of PA may leave behind palmitic acid, which is non-toxic and may undergo fatty acid metabolism.
Several anti-tumor properties of ascorbate may improve the solid tumor treatment and various studies have reported ascorbate to increase the efficacy of many chemotherapeutic drugs in vitro or in vivo. Many chemotherapeutic drugs have production of oxidative stress as a mechanism of their anticancer activity. For example, radiotherapy generates ROS (i.e. free hydroxyl radicals) in irradiated tissues by the ionization of cellular water [21, 69]. Studies indicate oxidative stress causes toxicity of some drugs, such as paclitaxel [69-71]. Peroxide generation by ascorbate could enhance the anticancer activity of certain drugs. Ascorbate-induced GSH depletion could enhance the efficacy of several anticancer drugs that can be expelled out of the tumor cells by multi-drug resistant proteins (MRPs) such as paclitaxel, doxorubicin and vincristine [50, 72]. Ascorbate has been reported to increase the efficacy of paclitaxel, doxorubicin and cisplatin in breast cancer cells in vitro [73] and of vincristine in non-small cell lung cancer cells in vitro by reversing their resistance to vincristine [74]. GSH depletion can also improve the effects of anticancer drugs like arsenic trioxide which are detoxified in cancer cells by GSH [75]. GSH depletion of the treated and co-treated cells by ascorbate improved the efficacy of motexafin gadolinium in vitro [76]. Various studies suggest that ascorbate has the ability to normalize the effects of angiogenic factors which are secreted by the tumors on the stromal cells. These factors help in the functioning of the tumor cells by hiring endothelial cells, vascular progenitor cells and immune cells in the tumor tissues. Ascorbate can decrease the formation of these factors by cancer cells with abnormal hypoxia-inducible factor (HIF) activity. Such anti-angiogenic properties of ascorbate can be used in therapy alone or in combination with other anti-angiogenic treatments such as sorafenib (nexavar), avastin and sutent, or with erlotinib and serolimus, which are inhibitors of tyrosine kinase pathway.
3.2.2. RENAL CELL CARCINOMA AND ASCORBATE

Von Hippel-Lindau (VHL) syndrome is one of the genetic precursors associated with the development of RCC. It is an autosomal dominant hereditary cancer syndrome caused by germline mutations or deletions in the VHL gene [77-81]. During the evolution of RCC, an early event is the loss of VHL gene function which predisposes the patients to RCC. This gene is located on chromosome 3p and encodes a 213-amino acid protein. Loss of VHL function plays an important role in RCC pathogenesis because inheritance of a defective copy of the VHL gene leading to VHL disease is the most common cause for inherited clear cell RCC. Up to 75% of patients with sporadic clear cell RCC have aberrant VHL (e.g., chromosome 3p deletion, suppressed expression, or loss-of-function base substitutions) [79, 82]. The VHL gene plays a key role in the oxygen-sensing HIF pathway for proteosomal degradation. HIF is a critical transcriptional activator of a number of genes involved in response to hypoxia [80, 82]. HIF-α and HIF-β subunits bind to hypoxia-response elements (HRE) in gene promoters and they regulate the gene expression of various physiological processes such as angiogenesis, cell metabolism, cell proliferation and apoptosis. HIF-1α and HIF-2α take part in the transcription of a several genes such as those for VEGF A, platelet derived growth factor (PDGF) and transforming growth factor alpha (TGF-α) which are important in tumor biology [80]. The VHL protein is a component of an E3 ubiquitin-protein ligase complex composed of elongin B, elongin C, and cullin 2 [79, 80, 83]. During normoxia, this VHL complex binds with and polyubiquinates HIF-α, thus marking it for proteosomal degradation [77, 84, 85]. HIF-α is enzymatically hydroxylated on a proline residue by HIF prolyl hydroxylase (PHD). During hypoxia, HIF-α is not hydroxylated, hence it cannot bind with the VHL complex and escapes the ubiquitin-mediated proteosomal degradation. Similarly, in the case of loss of VHL function, the mutant VHL gene cannot bind to HIF-α, causing accumulation of HIF-α in the cells [80, 82, 86]. This HIF-α binds with HIF- β, which is constitutively expressed, and this HIF-α /β complex
translocates to the nucleus, binds to HRE and brings about the transcription of genes such as VEGF A (causing angiogenesis) [87], epidermal growth factor receptor type 1 (EGFR1, leading to cell growth and proliferation), PDGF B chain, glucose transporters (for example, GLUT-1), TGF-α (ligand for EGFR1) and erythropoietin [82, 88] (Fig. 4). Studies indicate an overlap in the genes that are transcriptionally regulated by HIF-1α and HIF-2α. However in vitro and in vivo experimental results suggest that HIF-2α is the important HIF for tumorigenesis in RCC [80, 89-91]. Thus HIF-α accumulation in VHL disease and hypoxic tumors facilitate growth of tumor tissue by increasing angiogenesis, cell proliferation, cell survival in hypoxic, low-pH and low nutrient conditions and metastasis [82, 92, 93].

![Figure 4. Schematic representation of the role of VHL protein in HIF pathway [86].](image)


Several drugs which act by disruption of the HIF-α pathway have been approved by U.S. FDA in recent times for the treatment of RCC (Fig. 5). These include compounds which target the downstream agents of HIF-α pathway such as bevacizumab which is a monoclonal antibody
targeting VEGF A, sorafenib which is a multikinase inhibitor which targets VEGF and PDGF receptors and RAF pathway, sunitinib which inhibits VEGF and PDGF receptors, axitinib and pazopanib which inhibit VEGF receptors. Drugs like temsirolimus and everolimus which inhibit mammalian target of rapamycin (mTOR) which translationally controls HIF-1α are also clinically effective [80, 82].

![Figure 5. Schematic representation of the effects of HIF pathway in RCC and targets of clinically effective drugs [82].](image)

In the case of loss of VHL gene function, apart from the increased production of GLUT-1 and GLUT-3 in tumor cells of RCC, an increased production of ROS is also seen [94, 95]. This occurs through an increased generation of NAD(P)H oxidases, which in turn cause continued expression of HIF-1α and so lead to continued HIF-α activity. Additionally, this HIF-induced increase in NAD(P)H oxidases also increases the production of superoxide anion, which renders the tumor cells more sensitive to GSH depletion [95]. In these tumor cells, HIF also induces a
decrease in the number of mitochondria and leads to a lower cellular rate of respiration [96]. Thus, in the cells of RCCs, which lack the tumor suppressor VHL gene and regulate HIF abnormally, ascorbate will not alter the hypoxic response but will act on the genetically normal stromal cells, reducing the effects of angiogenic factors derived from tumors [95]. The abnormal regulation of HIF in VHL-negative tumor cells renders them more sensitive to ascorbate toxicity due to all these factors including increased production of GLUT-1 and GLUT-3, ROS and GSH depletion. Thus ascorbate containing nanoparticles can be used as an effective anticancer therapy to target RCCs that have activated the hypoxia-induced pathways regulated by HIF gene expression.

3.2.3. SORAFENIB IN CANCER THERAPY

Sorafenib is an oral multikinase inhibitor approved by the U.S. FDA for the treatment of advanced RCC and hepatocellular carcinoma [97]. Sorafenib is a biaryl urea, also known as BAY 43-9006 or Nexavar. Sorafenib has the ability to attack the actual tumor and its ability to recruit new blood vessels essential for growth and dissemination via multiple mechanisms. In preclinical studies, sorafenib was shown to target the tumor cell proliferative Ras/Raf/kinase (MEK)/ extracellular signal-regulated kinase (ERK) signaling pathway at the level of the serine/threonine kinase Raf [98, 99]. This pathway plays a pivotal role in controlling tumor cell growth by relaying signals from the cell surface to the nucleus. Sorafenib inhibits Raf-1, wild-type B-Raf and mutant (V600E) B-Raf kinase activities in vitro and diminishes MEK/ERK activation in various tumor cell lines, including those harboring mutant K-Ras or B-Raf [97, 99-101]. In most tumor types, sorafenib inhibited signaling through Raf as evidenced by reduced phosphorylated ERK (pERK) levels [97]. Sorafenib also inhibits several other pro-angiogenic tyrosine kinases involved in tumor progression, including VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, Flt3 and c-Kit [99, 102]. Sorafenib has also been shown to induce apoptosis in
several tumor cell lines primarily by down-regulation of the antiapoptotic protein Mcl-1 possibly by a MEK/ERK-independent mechanism [97]. During the inhibition of serine/threonine kinase and tyrosine kinase pathways in both tumor and vasculature, sorafenib has also been shown to induce ROS production through which it may exert its cytotoxic effects [103]. Sorafenib inhibited tumor growth in various preclinical models of human melanoma, renal, colon, pancreatic, hepatocellular, thyroid, and ovarian carcinomas and non small cell lung cancer [97]. Preclinical studies suggest that sorafenib acts on tumors and tumor vasculature by inhibiting cellular proliferation and angiogenesis and/or by inducing apoptosis.

RCC is characterized by the loss of tumor suppressor VHL protein, resulting in dysregulation of growth factor signaling, including VEGF, PDGF-β, and TGF-α. These factors play key roles in angiogenesis and lymphangiogenesis as well as in dysregulation of Raf pathways that regulate tumor growth and survival [94, 104-107]. As sorafenib acts on these targets, it is an important treatment option for RCC. Sorafenib produced dose-dependent growth inhibition of human RCC 786-O and Renca tumor xenografts [108, 109]. In the 786-O xenograft model, a dose of 15 mg/kg produced a 28% tumor growth inhibition, whereas treatment with 30, 60, or 90 mg/kg dose resulted in 80% tumor growth inhibition. Tumor stabilization occurred at doses of 60 or 90 mg/kg [108]. In clinical trials, sorafenib had an advantage compared with placebo in phase II/III trials in patients with treatment-refractory metastatic RCC, prolonging progression-free survival by 2- to 4-fold [110, 111].

Since sorafenib is an approved therapy for RCC and has the ability to induce ROS generation (similar to ascorbate), it was chosen as the drug to be used in combination with PA therapy to enhance its efficacy in the treatment of RCC.
3.2.4. CANCER CELL SPHEROIDS

Monolayer culture and organ culture have been the most popular models of choice for cancer research. However, monolayers lack most of the tissue characteristics including the altered cell-cell interaction, a hypoxic core and high interstitial fluid pressure that are demonstrated by cancer cells in a 3D microenvironment [112]. Organ culture uses pieces of cancer tissue specimens in 3D, but one of its major disadvantages is the difficulty in obtaining specimens in sufficient quantity. Although, monolayer culture is used more often to study the molecular mechanisms and cytotoxic effects of drugs on cancer cells, it fails to provide a platform where vital mechanisms such as cellular response in 3D as well as tissue penetration of potential molecules or cargoes carrying molecules can be studied. This has often resulted in the lack of correlation between the observed effects in monolayer culture and in vivo data [113, 114]. Multicellular 3D spheroid culture has gained increased interest as a model of choice for cancer research.

A 3D spheroid culture provides a unique opportunity to bridge the gap between monolayer culture assays and in vivo assays [115]. Spheroids are 3D assemblies of cancer cells and have been found in cancer patient tumors [116]. Spheroids are avascular tumor masses with limited penetrability. Also, the limited mass transport in spheroids results in accumulation of metabolic wastes creating conditions similar to in vivo tumors [117-121]. A spheroid above 500µm diameter generally presents characteristics including a necrotic core surrounded by a layer of quiescent cells and an outermost layer of rapidly multiplying cells [122]. This morphology is similar to avascular early stage tumors. Other similarities between the original tumor and the respective spheroids include volume growth kinetics, differentiation characteristics such as the development of specific histological structures or the expression of antigens [123]. Numerous studies have shown that the gene expression in spheroids is more relevant to actual tumor tissue
than a monolayer culture [121, 124]. Various advantages like cell-cell interaction, relevant gene expression, hypoxic core and the presence of a low pH at core, should make a 3D spheroid culture a vital model for cancer research. Spheroids formed with cancer cell lines mimic both the architecture of tumors in vivo and share the limited drug penetration properties since drugs are confined largely to the outer cell layers [125] (Fig. 6). Chemotherapy prolongs survival of patients with solid tumors [126] but is complicated by the tumor drug resistance [127, 128], in particular by the limited drug access to the tumor mass [128, 129]. Resistance to drug penetration is not reproduced in monolayer cultures [125, 130], so spheroids represent a better model for drug resistance compared to monolayer cultures.

Figure 6. Schematic representation of resemblance of architecture of 3D tumor spheroids to that of in vivo tumors [131].

Several studies have been done for the optimization of the protocols of formation of spheroids and all methods have their own advantages and disadvantages [132] (Table 1.).
Table 1. List of various techniques used for spheroid formation.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinner flasks</td>
<td>Easy to perform</td>
<td>Needs high shear force</td>
<td>[133-135]</td>
</tr>
<tr>
<td></td>
<td>Can be cultured over long periods</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large-scale production is possible</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controllable culture conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D Scaffolds</td>
<td>Presence of 3D extracellular support</td>
<td>Scaffold preparation is tedious and requires special equipment</td>
<td>[136, 137]</td>
</tr>
<tr>
<td>Pellet cell culture</td>
<td>Easy to perform</td>
<td>Needs high shear force</td>
<td>[138, 139]</td>
</tr>
<tr>
<td></td>
<td>Pellet is formed rapidly by aggregation of large number of cells</td>
<td>Large-scale production is Difficult</td>
<td></td>
</tr>
<tr>
<td>Hanging drop</td>
<td>Inexpensive</td>
<td>Large scale production is difficult</td>
<td>[140-144]</td>
</tr>
<tr>
<td></td>
<td>Rapid spheroid formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controllable spheroid size</td>
<td>T edious to perform</td>
<td></td>
</tr>
<tr>
<td>Non-adhesive liquid overlay</td>
<td>Easy to perform</td>
<td>Forms only with certain cell types</td>
<td>[145-147]</td>
</tr>
<tr>
<td></td>
<td>Inexpensive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large-scale production is possible</td>
<td></td>
<td></td>
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</tbody>
</table>

The non-adhesive liquid overlay method has been used to prepare spheroids of RCC [148-150]. This method for the formation of spheroids gives uniform spheroids which can be used in additional studies including cytotoxicity and distribution of liposomes in spheroids. Spheroids of RCC, which mimic the in vivo tumor characteristics, can be used to better study the effects of ascorbate-based nanoparticle therapy.
4. MATERIALS AND METHODS

4.1. MATERIALS

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). L-ascorbic acid (AA), palmitoyl ascorbate (PA), catalase (CAT), glutathione (GSH), N-acetyl-L-cysteine (NAC), aminotriazole (AT), buthionine sulfoximine (BSO), 7-Aminoactinomycin D (7-AAD), nocodazole, propidium iodide (PI), Triton X-100, EDTA disodium, RNase A and 2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). 5-(and-6)-chloromethyl-2070-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) was from Invitrogen (Eugene, OR, USA). Sorafenib (BAY-43-9006) was purchased from Cayman Chemical (MI, USA). Protein electrophoresis grade Agarose (with a high gelling temperature) was obtained from Fisher Scientific (NJ, USA). Alexa Fluor 488 annexin V conjugate and Annexin-binding buffer 5x concentrate were purchased from Molecular Probes Inc. (Eugene, OR, USA).

4.2. CELL CULTURES

786-O VHL negative (human renal carcinoma lacking VHL protein) and 786-O VHL positive (786-O VHL negative transformed to contain VHL protein) cell lines were obtained from the Dana Farber Cancer Research Institute (Boston, MA, USA). RAG (murine renal adenocarcinoma), RENCA (murine renal cortical adenocarcinoma), HUVEC (human umbilical vein endothelial cells), Human dermal fibroblast (normal human adult skin) and HK-2 (normal human kidney) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture media, heat-inactivated fetal bovine serum (FBS) and concentrated solution of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from
Cellgro (Herndon, VA, USA) and American Type Culture Collection (Manassas, VA, USA). Puromycin was from Invitrogen (Eugene, OR, USA).

RAG, RENCA and HUVEC cell lines were grown in DMEM cell culture medium supplemented with 10% FBS, 50 U/mL penicillin and 50 µg/mL streptomycin at 37°C, 5% CO₂. 786-O VHL negative and positive cell lines were supplemented additionally with 0.2 µg/mL puromycin. The human dermal fibroblast cell line was grown in Fibroblast Basal medium supplemented with Fibroblast Growth Kit components at 37°C, 5% CO₂. The HK-2 cell line was grown in Keratinocyte Serum Free medium supplemented with 0.05 mg/mL bovine pituitary extract (BPE) and 5 ng/mL human recombinant epidermal growth factor (EGF) at 37°C, 5% CO₂.

4.3. PREPARATION OF LIPOSOMES

PA (30 mol%) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N [methoxy(poly(polyethylene glycol))]2000 (PEG2000-PE) (4 mol%) were incorporated in liposomes (PAL) composed of egg phosphatidylcholine (egg PC)/cholesterol (70:30) by the rehydration of lipid films as reported previously in [151-153]. Plain liposomes (PL) were prepared similarly but without PA. The stock solutions of egg PC, cholesterol and PA were measured in required quantities to form a thin film of lipids in a round bottom flask by solvent removal on a rotary evaporator. The lipid film formed was freeze-dried and then rehydrated with phosphate buffered saline, pH 7.4, to give a final lipid concentration of 10 mg/mL. The solution was bath-sonicated for 30 minutes followed by extrusion through 200 nm polycarbonate membranes.

Wherever applicable, liposomes were fluorescently labeled by the incorporation of 0.5 to 1 mol% rhodamine-PE (Rh-PE) in the lipid film. All formulations were filtered using 0.2 µm membrane syringe filters before use for any experiments.
4.4. CHARACTERIZATION AND STABILITY OF LIPOSOMES

4.4.1. SIZE AND ZETA POTENTIAL

Liposomes were characterized for size (hydrodynamic diameter) by dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). Zeta potentials of the liposomal formulations were determined using a 90 PLUS particle size analyzer with ZETA PALS System, Brookhaven Corp. (Holtsville, NY, USA).

4.4.2. PA CONTENT

The PA content of liposomes was estimated by reverse phase-HPLC. Liposomes were destructed with the mobile phase [phosphate buffer (20 mM, pH 2.5)/acetonitrile /methanol mixture (5:40:55 % v/v)] before injecting into the HPLC column. A D-7000 HPLC system equipped with a diode array and fluorescence detector (Hitachi, Japan) and Spherisorb ODS2 column, 4.6 mm x 250 mm (Waters, Milford, MA, USA) was used. The column was eluted with the mobile phase at 1.0 mL/min. PA was detected at 254 nm. Injection volume was 50 µl.

4.4.3. PA LIPOSOMAL STABILITY

PAL were stored at 4°C in sealed vials purged with argon for one week and monitored for PA content (as determined by HPLC) and size distribution by DLS.
4.5. IN VITRO STUDIES IN RENAL CARCINOMA CELLS

4.5.1. CELL VIABILITY STUDIES

4.5.1.1. CYTOTOXICITY OF PAL, AA AND H_{2}O_{2}

Cells were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 60-70% confluent. Next day, cells were incubated with various formulations for 1 hour and were then washed and incubated in complete medium for 24 hours. For long-term cytotoxicity, cells were incubated with low concentration PAL for 24 hours. After 24 hours, the cells were analyzed for viability using CellTiter-Blue® cell viability assay (Promega, Madison, WI, USA).

4.5.1.2. EFFECT OF VARIATION IN PASSAGE NUMBER AND CELL DENSITY ON CYTOTOXICITY

For effect of variation in passage number in cytotoxicity, cells of varying passage number up to passage number 15 were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 60-70% confluent. On the next day, cells were incubated with various formulations for 1 hour and were then washed and incubated in complete medium for 24 hours. For effect of cell density on cytotoxicity, cells of varying density (3000 or 6000 cells) were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 60-70% confluent. Next day, cells were incubated with various formulations for 1 hour and were then washed and incubated in complete medium for 24 hours.

For all experiments, after incubation of cells with complete medium for 24 hours, the cells were analyzed for viability using CellTiter-Blue® cell viability assay (Promega, Madison, WI, USA).
4.5.2. CELL ASSOCIATION BY FLOW CYTOMETRY

Cells were seeded and allowed to attach overnight in 6-well plates (Corning, Inc., NY, USA) until 60-70% confluent. Next day, cells were incubated with rhodamine-PE labeled PL and PAL (lipid concentration 10 µg/mL) for 1 hour followed by washing, trypsinization and resuspension in 10% paraformaldehyde in PBS. The fixed cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with fluorescence emission at 570 nm. Cells were gated using forward versus side scatter to exclude dead cells and a total of 10,000 events were acquired for each sample.

4.5.3. MECHANISM OF PAL-MEDIATED CYTOTOXICITY

To study effect of various ROS scavengers/ antioxidants or their inhibitors on ascorbate-induced cytotoxicity, cells were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 60-70% confluent. On the next day, cells were pre-incubated for 30 minutes with ROS scavengers/ antioxidants such as CAT (300 U/mL), GSH (1 mM) or NAC (2.5 mM) and with AT (25 mM) or BSO (0.2 mM) which are inhibitors of CAT and GSH/NAC respectively. This was followed by co-incubation of cells with these and different formulations for 1 hour. After 1 hour, cells were washed and incubated for 24 hours with complete medium with or without inhibitors. After 24 hours, the cells were analyzed for viability using CellTiter-Blue® cell viability assay (Promega, Madison, WI, USA).

4.5.4. DETECTION OF ROS GENERATION BY PAL

Cells were seeded in 12-well plates (Corning, Inc., NY, USA) and allowed to attach overnight until 60-70% confluent. On the next day, cells were washed twice with HBSS and incubated with CM-H$_2$DCFDA dye (1 µM) solution in HBSS for 30 minutes. After 30 minutes, the cells were washed twice with HBSS followed by incubation with CAT (300 U/mL) or NAC (2.5 mM) for
another 30 minutes. The cells were then washed twice with HBSS and treated with AA (0.5 mM, 5 mM, 10 mM), PAL (0.5 mM, 5 mM) or PL at concentrations similar to PAL for 1 hour in HBSS. H$_2$O$_2$ (0.5 mM)-treated cells were used as positive controls. After 1 hour of treatments, cells were washed, trypsinised and resuspended in ice-cold sterile PBS, pH 7.4. The cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with fluorescence emission at 530 nm. Cells were gated using forward versus side scatter to exclude dead cells and a total of 10,000 events were acquired for each sample.

**4.5.5. FLOW CYTOMETRIC MEASUREMENT OF APOPTOSIS INDUCED BY PAL**

Flow cytometric analysis of apoptosis was carried out by dual staining with Alexa Fluor® 488 annexin V and 7-AAD according to manufacturer’s instructions. Cells were seeded in 12-well plates (Corning, Inc., NY, USA) and allowed to attach overnight until 60-70% confluent. Next day, cells were washed and treated with AA (5 mM), PAL (5 mM, 2.5 mM, 1.25 mM) and PL at concentrations similar to PAL for 1 hour. After 1 hour of treatment, cells were washed, detached gently using trypsin-EDTA, washed twice with ice-cold sterile PBS, pH 7.4 and re-suspended in Alexa Fluor® 488 annexin V conjugate (2.5 µL) and 7-AAD (1 µL of 50 µg/mL)-added binding buffer (200 µL) for 15 minutes in darkness. The stained cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with fluorescence emission at 530 nm and 670 nm. A total of 10,000 events were acquired for each sample.

**4.5.6. CELL CYCLE ANALYSIS**

786-O VHL negative cells were seeded in 60 mm dishes and allowed to attach overnight until 60-70% confluent. Next day, cells were washed and incubated with PL and PAL (0.5 mM, 1 mM) for 1 hour. After 1 hour, cells were washed twice and incubated for an additional 24 hours in complete medium. Nocodazole (0.1 µg/mL) was used as a positive control and was incubated
with cells for about 18-20 hours in complete medium. The cells were washed with PBS, fixed in 70% v/v cold ethanol for 1 hour and stained with PI staining solution (0.1% Triton X-100, 0.1 mM EDTA disodium, 50 µg/mL RNAse A and 50 µg/mL PI in PBS, pH 7.4). Cells were then incubated at 37°C for 30 minutes in the dark and finally washed twice with PBS and resuspended in 100 µL ice-cold PBS, pH 7.4. The stained cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A total of 10,000 events were acquired for each sample.

4.5.7. ROLE OF GLUT IN ASSOCIATION OF PAL AND AA IN RENAL CARCINOMA CELLS

Cells were seeded in 12-well plates (Corning, Inc., NY, USA) and allowed to attach overnight until 60-70% confluent. Next day, the cells were suspended in glucose-free, serum-free medium for 2 hours. After 2 hours, cells were treated with PL and PAL (1 and 2 mM) and AA (10 and 25 mM) for 1 hour in glucose-free, serum-free medium. Cells were then washed and incubated with 2-NBDG (50 µM) for 30 minutes in glucose-free, serum-free medium followed by washing, trypsinization and resuspension in ice-cold sterile PBS, pH 7.4. The cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with fluorescence emission at 530 nm. Cells were gated using forward versus side scatter to exclude dead cells and a total of 10,000 events were acquired for each sample.

4.5.8. CYTOTOXICITY OF SORAFENIB IN RENAL CARCINOMA CELLS

Cells were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 40-50% confluent. Next day, cells were incubated with sorafenib (SFB) for 48 hours. After 48 hours, the cells were analyzed for viability using CellTiter-Glo® Luminescent Cell Viability
assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. IC\textsubscript{50} values were calculated using CompuSyn software.

4.5.9. CYTOTOXICITY OF COMBINATION OF PAL AND SORAFENIB IN RENAL CARCINOMA CELLS

Cytotoxicity studies were done to evaluate the dose-response relationships between sorafenib and PAL either alone or in combination in renal carcinoma cells. A constant-ratio method proposed by Chou and Talalay was used to establish the nature of interaction (synergism, additivity or antagonism) between the two drugs, sorafenib and PAL, in combination [154, 155]. Cells were seeded and allowed to attach overnight in 96-well plates (Corning, Inc., NY, USA) until 40-50\% confluent. Next day, the cells were:

1. Pretreated with PAL for 1 hour followed by treatment with sorafenib for 48 hours, or
2. Co-treated with PAL and sorafenib for 1 hour followed by washing and treatment with sorafenib for 48 hours, or
3. Pre-treated with sorafenib for 48 hours followed by washing and treatment with PAL for 1 hour.

After treatments, cells were analyzed for viability using CellTiter-Glo\textsuperscript{®} Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. Sorafenib was combined with PAL at a given constant concentration molar ratio based on their corresponding IC\textsubscript{50}’s. Molar ratios of sorafenib:PAL were chosen as follows:

For 786-O VHL negative cells, IC\textsubscript{50} of sorafenib: IC\textsubscript{50} of PAL = 1:75. This was considered as the IC\textsubscript{50} molar ratio for the combination treatment. Sorafenib and PAL were also combined at a molar ratio lower than the IC\textsubscript{50} molar ratio of the combination i.e. 1:35 and at a molar ratio higher than the IC\textsubscript{50} molar ratio of the combination i.e. 1:150.
Similarly, for 786-O VHL positive cells, IC$_{50}$ of sorafenib: IC$_{50}$ of PAL = 1:30. This was considered as the IC$_{50}$ molar ratio for the combination treatment. Sorafenib and PAL were also combined at a molar ratio lower than the IC$_{50}$ molar ratio of the combination i.e. 1:10 and at a molar ratio higher than the IC$_{50}$ molar ratio of the combination i.e. 1:75.

Cells were treated with serial dilutions of sorafenib and PAL alone or in combination at the given constant ratios (1:35, 1:75 and 1:150 for 786-O VHL negative cells and 1:10, 1:30 and 1:75 for 786-O VHL positive cells).

IC$_{50}$ values and combination index (CI) for the combination treatment were calculated using CompuSyn software based on the classic isobologram equation of Chou and Talalay for combination of two or more drugs [154, 155]. A CI of less than 1 indicates a synergistic effect.

**4.5.10. DETECTION OF ROS GENERATION BY SORAFENIB ALONE AND IN COMBINATION WITH PAL**

Cells were seeded in 12-well plates (Corning, Inc., NY, USA) and allowed to attach overnight until 60-70% confluent. Next day, cells were washed twice with HBSS and incubated with CM-H$_2$DCFDA dye (1 μM) solution in HBSS for 30 minutes. After 30 minutes, the cells were washed twice with HBSS followed by incubation with PAL (1 mM), sorafenib (12.5 μM) or combination of sorafenib (12.5 μM) and PAL (1 mM) for 1 hour in HBSS. H$_2$O$_2$ (0.5 mM) treated cells were used as positive controls. After 1 hour of treatments, cells were washed, trypsinised and resuspended in ice-cold sterile PBS, pH 7.4. The cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with fluorescence emission at 530 nm. Cells were gated using forward versus side scatter to exclude dead cells and a total of 10,000 events were acquired for each sample.
4.6. IN VITRO STUDIES IN RENAL CARCINOMA CELL SPHEROIDS

4.6.1. PREPARATION OF RENAL CARCINOMA CELL SPHEROIDS

Spheroids were formed from 786-O VHL negative and positive cells in 96-well plates (Corning, Inc., NY, USA) by a non-adhesive liquid overlay method as previously reported in [148-150] with modifications as follows. Approximately 9,000 cells in 100 µL of complete media were added to each well of 96-well plates pre-coated with 50 µL of 1% agarose in serum-free medium. The plates were then centrifuged at 1500 rcf for 15 minutes to form spherical cell aggregates which continued to form dense spheroidal structures when incubated at 37°C with no medium change over the next few days. Spheroid formation was monitored using a Nikon Eclipse E400 microscope (Nikon, Inc.) at 10x magnification and with a Spot Insight 3.2.0 camera with Spot Advanced software (Spot Imaging). 786-O VHL negative spheroids of 400-500 µm size and 786-O VHL positive spheroids of 500-600 µm size were used for experiments.

4.6.2. CHARACTERIZATION OF RENAL CARCINOMA CELL SPHEROIDS

Spheroids were characterized for their size and shape over their growth period using a Nikon Eclipse E400 microscope (Nikon, Inc.) at 10x magnification and with a Spot Insight 3.2.0 camera with Spot Advanced software (Spot Imaging). Growth profile of spheroids was obtained by measuring the surface area of the spheroids over their growth period.

4.6.3. DISTRIBUTION OF LIPOSOMES IN RENAL CARCINOMA CELL SPHEROIDS

Distribution of PL and PAL in 786-O VHL positive and 786-O VHL negative cell spheroids was studied by confocal microscopy. Rh-PE (1 mol%) labeled PL and PAL were incubated with spheroids for 2 hours and penetration of Rh-PE labeled PL and PAL in spheroids was evaluated by confocal microscopy using Z-stack imaging with 10 µm intervals [149]. Fluorescence
intensities of optical sections were quantified using Image J software [156]. For determination of penetration of Rh-PE labeled PL and PAL in spheroids, a constant region in the spheroid area was selected in all the optical sections and its fluorescence intensity was measured using ImageJ software.

4.6.4. CYTOTOXICITY OF PAL AND AA IN RENAL CARCINOMA CELL SPHEROIDS

After incubation of spheroids with AA, PL and PAL (1.25, 2.5 and 5 mM) for 24 hours, cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. After treatments, the spheroids were dissociated with 0.09 % Triton® X-100 in complete medium. They were then pipette mixed, aspirated and transferred into black-sided, flat-bottomed plates (Corning, Inc., NY, USA). This was followed by incubation with the CellTiter-Glo® reagent for 10 minutes and luminescence was then measured on a Synergy luminescence microplate reader (Biotek, VT, USA).

4.6.5. CYTOTOXICITY OF SORAFENIB IN RENAL CARCINOMA CELL SPHEROIDS

After incubation of spheroids with sorafenib for 48 hours, cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. After treatment, the spheroids were dissociated with 0.09 % Triton® X-100 in complete medium. They were then pipette mixed, aspirated and transferred into black-sided, flat-bottomed plates (Corning, Inc., NY, USA). This was followed by incubation with the CellTiter-Glo® reagent for 10 minutes and luminescence was then measured on a Synergy luminescence microplate reader (Biotek, VT, USA). IC₅₀ values were calculated using CompuSyn software.
4.6.6. CYTOTOXICITY OF COMBINATION OF PAL AND SORAFENIB IN RENAL CARCINOMA CELL SPHEROIDS

Similar to cell viability studies of combination of sorafenib and PAL in monolayers, cytotoxicity studies were done to evaluate the dose-response relationships between sorafenib and PAL either alone or in combination in spheroids of RCC. A constant-ratio method proposed by Chou and Talalay was used to establish the nature of interaction (synergism, additivity or antagonism) between the two drugs, sorafenib and PAL, in combination [154, 155].

Spheroids were co-incubated with sorafenib and PAL for 48 hours followed by determination of cell viability using a CellTiter-Glo® Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to manufacturer’s instructions. After treatment, the spheroids were dissociated with 0.09% Triton® X-100 in complete medium. They were then pipette mixed, aspirated and transferred into black-sided, flat-bottomed plates (Corning, Inc., NY, USA). This was followed by incubation with the CellTiter-Glo® reagent for 10 minutes and luminescence was then measured on a Synergy luminescence microplate reader (Biotek, VT, USA).

Similar to cell viability studies of combination of sorafenib and PAL in monolayers, spheroids were treated with sorafenib and PAL at a given constant concentration molar ratio based on their corresponding IC_{50}’s in spheroids. Molar ratios of sorafenib:PAL were chosen as follows:

For 786-O VHL negative spheroids, IC_{50} of sorafenib: IC_{50} of PAL = 1:10. This was considered as the IC_{50} molar ratio for the combination treatment. Sorafenib and PAL were also combined at a molar ratio lower than the IC_{50} molar ratio of the combination i.e. 1:5 and at a molar ratio higher than the IC_{50} molar ratio of the combination i.e. 1:15.

Similarly, for 786-O VHL positive spheroids, IC_{50} of sorafenib: IC_{50} of PAL = 1:8. This was considered as the IC_{50} molar ratio for the combination treatment. Sorafenib and PAL were also combined at a molar ratio lower than the IC_{50} molar ratio of the combination i.e. 1:4 and at a molar ratio higher than the IC_{50} molar ratio of the combination i.e. 1:12.
Spheroids were treated with serial dilutions of sorafenib and PAL alone or in combination at the
given constant ratios (1:5, 1:10 and 1:15 for 786-O VHL negative spheroids and 1:4, 1:8 and
1:12 for 786-O VHL positive spheroids).
IC\textsubscript{50} values and combination index (CI) for the combination treatment were calculated using
CompuSyn software based on the classic isobologram equation of Chou and Talalay for
combination of two or more drugs [154, 155]. A CI of less than 1 indicates a synergistic effect.

4.7. IN VIVO TUMOR GROWTH INHIBITION STUDY

Tumors were initiated in 6-8 week old female athymic nude mice by a subcutaneous injection
over the right flank of about 5 x 10\textsuperscript{6} 786-0 VHL negative cells in 100 µL sterile PBS. Tumors
were allowed to develop. When the tumors reached a volume of 100 mm\textsuperscript{3}, treatments were
started. Treatments were administered once every 4 days. Treatment groups were as follows:

1. Control/PL (concentration similar to PAL treatment) (n=4)

2. Vehicle (sterile PBS containing 12.5% Cremophor EL and 12.5% ethanol) (n=4)

3. PAL (15 mg/kg of PA i.v. via tail vein) (n=7)

4. SFB: Sorafenib in vehicle (25 mg/kg of sorafenib by oral gavage) (n=7)

5. PAL+SFB (full dose): Combination of PAL (15 mg/kg of PA i.v. via tail vein) and sorafenib
   in vehicle (25 mg/kg of sorafenib by oral gavage). Sorafenib was administered first followed by
   PAL after 3 hours (n=7)

6. PAL+SFB (half dose): Combination of PAL (7.5 mg/kg of PA i.v. via tail vein) and sorafenib
   in vehicle (12.5 mg/kg of sorafenib by oral gavage). Sorafenib was administered first followed by
   PAL after 3 hours (n=4)

7. PAL+SFB (split dose): Combination of PAL (15 mg/kg of PA i.v. via tail vein) and sorafenib
   in vehicle (25 mg/kg of sorafenib by oral gavage). PAL were administered first followed by
   sorafenib on the next day (n=4)
Body weights of mice were measured on alternate days as an indicator for signs of toxicity. Tumor sizes were measured on alternate days with a vernier caliper in two dimensions. Individual tumor volume was estimated using the formula: \( V = (L \times W^2)/2 \), where length (L) is the longer dimension and width (W) is the shorter dimension perpendicular to length.

The experiment was performed as per a protocol approved by the Northeastern University Institutional Animal Care and Use Committee. The animals were allowed free access to food and water.

**4.8. STATISTICAL ANALYSIS**

The data were tested for statistical significance using Student’s t-test. With more than two groups, ANOVA was used to compare results. P-values were calculated with the Graph Pad prism 5.0 software (GraphPad Software, Inc, San Diego, CA, USA). All numerical data are expressed as mean ± standard deviation from 3 different experiments (n=3). Any p-values less than 0.05 were considered statistically significant.
5. RESULTS AND DISCUSSION

RESULTS AND DISCUSSION OF SPECIFIC AIM 1.

To prepare and characterize liposomes modified with palmitoyl ascorbate and test their cytotoxic effect and cell association towards renal carcinoma cells.

5.1. CHARACTERIZATION OF LIPOSOMES

<table>
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<th>Formulation</th>
<th>Particle Size Mean ± S.E. (nm)</th>
<th>Zeta potential mean ± S.D. (mV)</th>
<th>PA content Incorporation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>198.3 ± 2.1</td>
<td>-12.7 ± 2.1</td>
<td>NA</td>
</tr>
<tr>
<td>PAL</td>
<td>174.4 ± 1.5</td>
<td>-37.4 ± 3.4</td>
<td>91 ± 4.3 %</td>
</tr>
</tbody>
</table>

Table 2. Size, zeta potential and PA content of liposomes. (n=3).

PA was stably incorporated in liposomes at 30 mol% as measured by HPLC with an encapsulation efficiency of around 91%. Size of PL and PAL was 198.3 ± 2.1 nm and 174.4 ± 1.5 nm respectively. PAL demonstrated an increased zeta potential of -37.4 ± 3.4 mV compared to PL with zeta value of -12.7 ± 2.1 mV, possibly due to the presence of ascorbate residues. PAL were stable for 1 week at 4°C with no significant change (p > 0.05) in PAL size and PA content of PAL as determined by HPLC.
5.2. CYTOTOXICITY IN MURINE RENAL CANCER AND HUMAN PRIMARY NON-CANCEROUS CELLS

PAL demonstrated increased dose-dependent cytotoxicity towards murine renal cancer cell lines RAG and RENCA at 1 hour (Fig. 7A) and 24 hour (Fig. 7B) incubation periods. PAL did not induce any significant cytotoxic effects in the human primary non-cancerous cell lines HUVEC and dermal fibroblasts at both 1 hour (Fig. 8A) and 24 hour (Fig. 8B) incubation times. AA also showed almost no cytotoxicity at 1 hour (Fig. 8C). Lower concentrations of formulations were used for all 24 hours incubation times. AA did not show any effect at these low concentrations in HUVEC and dermal fibroblast cells (data not shown). Cells treated with PL showed more than 90% viability at all concentrations tested (data not shown).

Figure 7. Cytotoxicity of PAL with RAG and RENCA cells. (A) Cells were treated with PAL for 1 hour followed by washing and incubation in complete media for 24 hours or (B) cells were treated with PAL for 24 hours. Then the cells were analyzed for viability using CellTiter-Blue® cell viability assay. The data represent mean ± standard deviation where n = 3.
Figure 8. Cytotoxicity of ascorbate formulations (PAL and AA) in human primary non-cancerous dermal fibroblast and HUVEC cells. Cells were treated with (A) PAL or (C) AA for 1 hour followed by washing and incubation in complete media for 24 hours or (B) cells were treated with PAL for 24 hours. Then the cells were analyzed for viability using CellTiter-Blue cell viability assay. The data represent mean ± standard deviation where n = 3.

5.3. COMPARISON OF CYTOTOXICITY OF PAL, AA AND H₂O₂ IN HUMAN RENAL CARCINOMA AND NON-CANCEROUS RENAL CELL LINES

PAL demonstrated increased cytotoxicity towards human renal carcinoma 786-O cells as compared to normal human kidney HK-2 cells. PAL exhibited higher cytotoxicity towards 786-O VHL negative cells than 786-O VHL positive cells at concentrations of 0.313 mM, 0.625 mM, 1.25 mM and 2.5 mM (Fig. 9A). AA showed no cytotoxicity towards HK-2 cells and exhibited higher cytotoxicity towards 786-O cells only at higher concentration (12.5 mM). There was no significant difference observed in cell viabilities of 786-O VHL negative and positive cells (Fig. 9B). Cells treated with H₂O₂ were used as positive control. H₂O₂ did not show any cytotoxicity at lower (< 0.5 mM) concentrations in HK-2 cells (Fig. 9C). Cells treated with PL showed more than 90% viability at all concentrations tested (data not shown).
Figure 9. Cytotoxicity of ascorbate formulations (PAL and AA) and H$_2$O$_2$ with 786-O and HK-2 cells. Cells were treated with formulations for 1 hour followed by washing and incubation in complete media for 24h. Then the cells were analyzed for viability using CellTiter-Blue® cell viability assay. The data represent mean ± standard deviation where n = 3. * indicates p-value < 0.05.

5.4. EFFECT OF VARIATION IN PASSAGE NUMBER AND CELL DENSITY ON CYTOTOXICITY OF PAL, AA AND H$_2$O$_2$ IN RENAL CARCINOMA CELLS

The results of in vitro studies can be influenced by cell culture conditions such as cell density, the length of time since cell passage [157], and other factors [151]. Effect of these factors was studied on the cell viabilities of 786-O cells upon treatments with PAL, AA and H$_2$O$_2$ by varying the passage numbers (from passage 1 to 15) or varying the cell densities (3000 or 6000 cells per well of 96-well plates) (data not shown) and the results are summarized in Table 3.

These factors were considered while performing all the cytotoxicity studies.
<table>
<thead>
<tr>
<th>Effect of variation in passage number</th>
<th>Effect of variation in cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage numbers of cells varied from 0 to 15</td>
<td>Cells seeded at varying densities (3000 or 6000 cells/well)</td>
</tr>
<tr>
<td>Variation in passage number had no significant effect on cytotoxic action of formulations in both 786-O cell lines</td>
<td>Cells seeded at higher density (6000 per well) were less sensitive to treatment than the lower density seeding (3000 per well) for both 786-O cell lines</td>
</tr>
</tbody>
</table>

Table 3. Effect of variation in passage number and cell density on cytotoxicity of PAL, AA and H$_2$O$_2$ in 786-O renal carcinoma cells.

### 5.5. INCREASED CELL ASSOCIATION BY PAL IN RENAL CARCINOMA CELLS THAN IN NON-CANCEROUS RENAL CELLS

PA-modified nanocarriers have been reported previously to preferentially associate with various cancer cell lines compared to non-cancerous cells [151-153]. Here cell association of PAL was compared with 786-O VHL negative and positive renal carcinoma cells to normal kidney HK-2 cells. As seen in Fig. 10, PAL showed the highest association with 786-O VHL positive cells followed by 786-O VHL negative cells. The lowest cell association was observed with normal HK-2 cells. PL did not show significant association in any cell line. This indicates that ascorbate residues may be responsible for association of liposomes preferentially to cancer cells and may hence possibly enhance an anticancer cytotoxic effect.
Figure 10. Cell association of Rh-PE labeled formulations as analyzed by flow cytometry. Cells were incubated with PAL and PL for 1 hour, washed, trypsinized and fluorescence of the cells was analyzed by flow cytometry. The data represent mean ± standard deviation where n = 3.
RESULTS AND DISCUSSION OF SPECIFIC AIM 2.

To study the mechanisms by which palmitoyl ascorbate exerts its effects on tumor cells, including generation of reactive oxygen species and apoptosis in renal carcinoma cells.

5.6. MECHANISM OF PAL-MEDIATED CELL DEATH IS BY ROS GENERATION

Since AA is reported to produce cytotoxic effects on cancer cells due to generation of ROS [57], we evaluated the role of ROS in the cytotoxic effect. We have previously reported that pretreatment of 4T1 murine breast cancer cells with CAT, a major enzyme involved in the detoxification of H$_2$O$_2$, and superoxide dismutase, an antioxidant enzyme involved in the dismutation of superoxide species, reversed the cytotoxicity of PAL thus indicating generation of ROS to be responsible for the cytotoxic effect of PAL [153]. Similar to these previous results, pretreatment of 786-O cells with CAT protected cells from the cytotoxic effect of PAL. Additionally, treatment of cells with AT, a specific inhibitor of CAT, increased cytotoxic effect of PAL confirming involvement of H$_2$O$_2$ formation as a result of PAL treatment. Similar results were obtained with H$_2$O$_2$ and AA treated cells (Fig. 11A and 11B). Reduced GSH is a major antioxidant present in cells and NAC is also a known thiol antioxidant. Pretreating the cells with GSH or NAC effectively abrogated the PAL, AA and H$_2$O$_2$-mediated cytotoxicity. To determine if depletion of thiol levels would enhance the cytotoxicity of PAL, AA and H$_2$O$_2$, cells were treated with BSO, which is known to decrease cellular free thiol pool by inhibiting γ-glutamylcysteine synthetase, a key enzyme in GSH biosynthesis [158]. Depletion of GSH by BSO increased the cytotoxicity due to PAL, AA and H$_2$O$_2$ (Fig. 11C and 11D). Cells treated with PL, CAT, AT, GSH, NAC and BSO alone showed more than 90% viability (data not shown).

Ascorbate has been reported to induce the production of extracellular H$_2$O$_2$ [57, 58]. Since exogenous CAT and GSH are cell membrane impermeable, protection of cells from PAL-
mediated cytotoxicity by pretreatment with CAT and GSH confirms that PAL also induces the formation of H$_2$O$_2$ extracellularly.

Figure 11. Cytotoxicity of ascorbate formulations and H$_2$O$_2$. 786-O cells were pre-incubated for 30 minutes with CAT (300 U/ml), GSH (1 mM), NAC (2.5 mM), AT (25 mM) or BSO (0.2 mM) followed by co-incubation of cells with these and different formulations (PAL 1mM, AA 1mM, H$_2$O$_2$ 0.25mM) for 1 hour. The cells were then washed and incubated for 24 hours with complete medium with or without inhibitors (AT or BSO). After 24 hours, cells were analyzed for viability using CellTiter-Blue® cell viability assay. The data represent mean ± standard deviation where n = 3.
5.7. PAL ALLOWED FOR INCREASED ROS GENERATION COMPARED TO AA

It has been known that cancer cells are highly susceptible to toxic effects of ROS compared to normal cells. This is due to the elevated levels of ROS in cancer cells which push cancer cells to the brink of their toxic threshold making them highly sensitive to oxidative stress. Thus, if any agent induces additional ROS generation, it could kill cancer cells due to ROS-mediated toxicity. Recently, the selective *in vitro* toxicity of high concentrations (1-10 mM) of AA towards cancer cells has been described, whereas normal cells were resistant [58, 159-161]. AA treatment in high pharmacological concentrations significantly decreased tumor growth *in vivo* without toxicity to normal tissues [28, 162]. The mechanism of cytotoxicity was also reported to be based on the production of ROS [57]. We have previously reported that PAL generated increased amounts of ROS in 4T1 breast cancer cells compared to PL treatment [153]. Here, we analyzed ROS generation by PAL treatment in 786-O cell lines and compared it to AA treatment using a fluorescent ROS-sensitive probe CM-H<sub>2</sub>DCFDA. Cells treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> were used as positive control. Treatment with PAL (0.5 mM and 5 mM) generated significantly increased ROS compared to AA in both cell lines after 1 hour exposure. ROS production was more in 786-O VHL negative cells compared to 786-O VHL positive cells. When cells were pretreated with CAT or NAC, potent scavengers of H<sub>2</sub>O<sub>2</sub>, ROS production by PAL was inhibited significantly. This observation suggested the extracellular generation of ROS (since CAT is cell impermeable) and intracellular accumulation of H<sub>2</sub>O<sub>2</sub> leading to oxidative stress since NAC is cell permeable and CM-H<sub>2</sub>DCFDA is an indicator of intracellular ROS, as reported previously for AA [57]. The effect of these inhibitors, CAT and NAC, on ROS generation by AA was seen only in 786-O VHL negative cells with 10 mM AA. For all other lower AA concentrations tested, this effect was not as pronounced since AA treatment by itself generated very minor increase in ROS after 1 hour of treatment (Fig. 12). This minor increase in ROS generation by AA may be because much higher concentrations of AA may be required to produce large amounts of ROS.
5.8. DETECTION OF APOPTOSIS INDUCED BY PAL

Extracellular \( \text{H}_2\text{O}_2 \) generation may have intracellular targets as \( \text{H}_2\text{O}_2 \) is cell membrane permeable [58] and this may lead to oxidative stress in tumor cells. Such oxidative stress by \( \text{H}_2\text{O}_2 \) and other ROS may induce cell death by apoptosis/necrosis [163, 164]. AA was reported to induce dose-dependent apoptosis or pyknosis/necrosis [58] and pharmacologic concentrations of AA led to necrotic cell death \textit{in vitro} [57]. So the presence of apoptosis or necrosis as the type of cell death induced by PAL was evaluated using Alexa Fluor\textsuperscript{®} 488 annexin V and 7-AAD dual staining. In brief, the phosphatidyl serine (PS) phospholipid in the membrane of apoptotic cells is exposed to the external cellular environment by translocation from the inner leaflet of the plasma membrane to the outer leaflet. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS then binds to PS, thus marking apoptotic cells. 7-AAD is a viability probe used to distinguish viable from non-viable cells. Viable cells with intact plasma membrane
will exclude 7-AAD, whereas it is permeable to the membranes of dead and damaged cells. Cells that stain negative for Alexa Fluor® 488 annexin V and 7-ADD are live healthy cells. Cells that stain positive for Alexa Fluor® 488 annexin V and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for Alexa Fluor® 488 annexin V and 7-AAD are either in late stage of apoptosis, are undergoing necrosis, or are already dead.

Results of apoptosis with representative sample images are shown in Figs. 13, 14, 15. The early apoptotic, late apoptotic and necrotic cells were increased after treatment with PAL at all concentrations tested in 786-O VHL negative cells. For 786-O VHL positive cells there was significant increase in early apoptotic and necrotic cells with all tested PAL concentrations, whereas only 5 mM PAL treated cells showed significant increase in late apoptotic cells. Cells treated with AA (5 mM) did not show any increase in apoptotic or necrotic cells in both cell lines. This may be because higher concentrations or longer incubation period of AA may be required to induce apoptosis/necrosis.

Figure 13. Quantification of apoptosis induced by ascorbate formulations. 786-O cells were treated with PAL, AA or PL for 1 hour followed by washing, trypsinization, and incubation with Annexin V-Alexa Fluor® 488/7-AAD in binding buffer at room temperature as per manufacturer's protocol. Stained cells were analyzed by flow cytometry.
Figure 14. Apoptosis induced by ascorbate formulations in 786-O VHL negative cells. 786-O VHL-negative cells were treated with PAL, AA or PL for 1h followed by washing, trypsinization, and incubation with Annexin V-Alexa Fluor® 488/7-AAD in binding buffer at room temperature as per manufacturer's protocol. Stained cells were analyzed by flow cytometry. LL: Annexin V−/7-AAD− cells (normal); LR: Annexin V+/7-AAD− cells (early apoptosis); UR: Annexin V+/7-AAD+ cells (late apoptosis); UL: Annexin V−/7-AAD+ cells (necrosis).
Figure 15. Apoptosis induced by ascorbate formulations in 786-O VHL positive cells. 786-O VHL positive cells were treated with PAL, AA or PL for 1h followed by washing, trypsinization, and incubation with Annexin V-Alexa488/7-AAD in binding buffer at room temperature as per manufacturer's protocol. Stained cells were analyzed by flow cytometry. LL: Annexin V−/7-AAD− cells (normal); LR: Annexin V+/7-AAD− cells (early apoptosis); UR: Annexin V+/7-AAD+ cells (late apoptosis); UL: Annexin V−/7-AAD+ cells (necrosis).

5.9. PAL ARRESTED RENAL CARCINOMA CELLS IN G2/M PHASE OF THEIR CELL CYCLE

DHAA is reported to affect progression of cell cycle and bring about cell cycle arrest at late G2/M DNA damage checkpoint during oxidative stress [165]. Also, ascorbyl stearate has been shown to accumulate cells in the late S/G2/M phase of cell cycle, thus delaying cell cycle progression [166, 167]. To investigate if PAL-induced cytotoxicity and apoptosis could be due to
Changes in cell cycle, cell cycle analysis was done with propidium iodide staining of 786-O VHL negative cells. Results of cell cycle distribution with representative flow cytometric histogram plots are shown in Figs. 16, 17. Sub G0 indicates resting phase of the cells, G0/G1 indicates period between G0 (Gap 0, resting phase) and G1 (Gap 1 phase) where cells increase in size and keep everything ready for DNA synthesis, S phase indicates DNA synthesis and G2/M is checkpoint between S and mitosis (M) phases which ensures that cells are ready to enter the M phase and divide. Cells treated with nocodazole, which causes a change in the cell cycle distribution, increasing the number of cells in G2/M phase, were used as positive control. Treatment with PAL (0.5 and 1 mM) resulted in increase in the percentage of cells arrested in S and G2/M phases of cell cycle as compared to PL and untreated control cells.

![Figure 16. Effect of PAL on cell cycle. 786-O VHL negative cells were treated with PAL (0.5 and 1 mM) for 1 hour followed by incubation in complete media for 23 hours. Cells were then stained with propidium iodide staining solution and cells in different phases of the cell cycle were analyzed by flow cytometry. The data represent mean ± standard deviation where n=3.](image-url)
Figure 17. Effect of PAL on cell cycle. 786-O VHL negative cells were treated with PL (0.5 mM) and PAL (0.5 and 1 mM) for 1 hour followed by incubation in complete media for 23 hours. Nocodazole (0.1 µg/mL) was used as a positive control and was incubated with cells for about 18-20 hours in complete medium. Cells were then stained with propidium iodide staining solution and cells in different phases of the cell cycle were analyzed by flow cytometry. M1: sub G0 phase, M2: G0/G1 phase, M3: S phase, M4: G2/M phase.

5.10. ROLE OF GLUT IN ASSOCIATION OF PAL AND AA IN RENAL CARCINOMA CELLS

AA is known to enter tumor cells via DHAA using the GLUTs overexpressed on them associated with the high glucose demand and hypoxic tumor conditions. DHAA, the oxidized form of AA, has a structure similar to glucose and hence GLUTs have an affinity similar for DHAA as glucose. GLUTs allow transport of ascorbate in the cells in the form of DHAA which is converted back to AA when inside the cell. So, the possible role of GLUTs in the association of PAL was analyzed using a competitive inhibition assay by flow cytometry. The 2-NBDG is a fluorescent derivative of glucose modified with a 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)] amino group at the C-2 position. This product shows intense fluorescence at 542 nm when
excited at 467 nm. 2-NBDG and D-glucose compete for GLUT to enter cells. Thus in the case of PAL and AA, competitive inhibition of 2-NBDG fluorescence by PAL and AA interaction with GLUTs was examined. 2-NBDG uptake by 786-O cells was significantly decreased in the presence of PAL (1 and 2 mM) or AA (10 and 25 mM) indicating competition between 2-NBDG and PAL or AA for GLUT receptors on tumor cells. Cells treated with PL at concentrations similar to PAL did not inhibit 2-NBDG uptake (data not shown). 2-NBDG fluorescence was significantly higher in 786-O VHL negative cells as compared to 786-O VHL positive cells (Fig. 18). This may be due to lower expression of GLUT receptors in 786-O VHL positive cells. These results correlate well with earlier findings that expression of GLUT1 in the absence of VHL is increased 2-fold over the basal GLUT1 expression in 786-O VHL positive cells [168]. Also, re-expression of the VHL gene as in 786-O VHL positive cells has been found to restore the hypoxia-related instability and rescue the ability of hypoxia to induce GLUT1 expression in renal carcinoma cells [84, 168].

![Figure 18. Role of GLUTs in association of PAL and AA with 786-O renal carcinoma cells. Cells were treated with PAL (1 and 2 mM) and AA (10 and 25 mM) for 1 hour followed by incubation with 2-NBDG (50 µM) for 30 minutes in glucose-free, serum-free medium and 2-NBDG fluorescence was then analyzed by flow cytometry. The data represent mean ± standard deviation where n=3.](image)
RESULTS AND DISCUSSION OF SPECIFIC AIM 3.

To determine the cytotoxicity and generation of reactive oxygen species by sorafenib alone and in combination with palmitoyl ascorbate liposomes in renal carcinoma cells.

5.11. CYTOTOXICITY OF SORAFENIB IN RENAL CARCINOMA CELLS

The anticancer activity of chemotherapeutic drugs has been enhanced by their combination with AA. Since PAL generated ROS and showed cytotoxicity towards renal carcinoma cells, they can be used to alter the tumor-redox status and make the cells more sensitive to oxidative stress, which is important in the treatment of cancer. So to assess the ability of PAL to improve the activity of another chemotherapeutic drug, sorafenib (U.S. FDA approved for the treatment of advanced RCC) was chosen to be used in combination with PAL.

For that purpose, cytotoxicity of sorafenib was first evaluated in renal carcinoma cells. Treatment of 786-O VHL negative and positive cells with different concentrations of sorafenib led to a strong dose-dependent cytotoxic effect in both cell lines. Sorafenib had a greater IC<sub>50</sub> value in 786-O VHL positive cells (33.3 µM) as compared to 786-O VHL negative cells (13.3 µM), indicating VHL negative cells to be more sensitive to sorafenib (Fig 19).
Figure 19. Cytotoxicity of sorafenib in 786-O renal carcinoma cells. Cells were treated with varying concentrations of sorafenib for 48 hours followed by measurement of cell viability using CellTiter-Glo® Luminescent Cell Viability assay according to manufacturer’s instructions. The data represent mean ± standard deviation where n = 3.

5.12. DOSE-RESPONSE RELATIONSHIP AND COMBINATION INDEX OF CYTOTOXICITY OF SORAFENIB AND PAL IN COMBINATION IN RENAL CARCINOMA CELLS

A constant-ratio method proposed by Chou and Talalay was used to establish the dose-response relationship for cytotoxic effect of sorafenib and PAL in combination. Combination index (CI) values for the combination treatment were calculated using CompuSyn software based on the classic isobologram equation of Chou and Talalay for combination of two or more drugs [154, 155, 169]. CI indicates whether drug combination may be synergistic (CI<1), additive (CI=1) or antagonistic (CI>1).

Cytotoxic effect of treatment with PAL followed by sorafenib, and concurrent sorafenib and PAL treatment was significantly better than that of treatment with sorafenib followed by PAL (data not shown). Treatment with PAL followed by sorafenib (data not shown) exhibited
cytotoxicity similar to co-treatment with PAL and sorafenib. Cells were treated with serial dilutions of sorafenib and PAL (data not shown) alone or in combination at the given constant molar ratios (1:35, 1:75 and 1:150 for 786-O VHL negative cells and 1:10, 1:30 and 1:75 for 786-O VHL positive cells).

Dose-response curves and CI values for combination treatments in 786-O cells are shown in Fig. 20. Combination data are presented in terms of sorafenib concentration. Shift of the dose-response curves of sorafenib and PAL combination treatments to the left relative to that of sorafenib alone is indicative of greater potency of the combination treatment than sorafenib monotherapy. Alternatively, shift of the dose-response curves of combination treatments to the right means that the combination treatment is less potent than sorafenib treatment alone. For all concentration molar ratios of sorafenib and PAL in combination, in both 786-O VHL negative and positive cells, the dose-response curves showed a shift towards left compared to the curve of sorafenib treatment alone, indicating combination of sorafenib and PAL to be more potent than sorafenib monotherapy. The shift towards left of the dose-response curves for combination treatments became greater as the molar ratio of sorafenib to PAL increased in both 786-O VHL negative and positive cells. In both 786-O VHL negative and positive cells, a synergistic effect (CI at IC₅₀ < 1) was observed for combination of sorafenib and PAL at the IC₅₀ molar ratio (1:75 for VHL negative and 1:30 for VHL positive cells) and at lower than the IC₅₀ molar ratio (1:35 for VHL negative and 1:10 in VHL positive cells) (Fig. 20). Collectively, combination of PAL with sorafenib was shown to improve the cytotoxic effect of sorafenib in renal carcinoma cells.
Figure 20. Dose-response curves for cytotoxicity of sorafenib and PAL in combination in 786-O renal carcinoma cells. Cells were co-treated with serial dilutions of sorafenib and PAL alone or in combination at molar ratios, as indicated, for 1 hour followed by washing and incubation with sorafenib for 48 hours. Cell viability was then measured using CellTiter-Glo® Luminescent Cell Viability assay according to manufacturer’s instructions. For sorafenib and PAL combination treatments, concentration is plotted in terms of sorafenib dose. CI less than 1 indicates synergistic effect. The data represent mean ± standard deviation where n = 3.
5.13. COMBINATION OF PAL AND SORAFENIB GENERATED INCREASED ROS COMPARED TO PAL OR SORAFENIB ALONE

Sorafenib has been shown to induce ROS production through which it may exert its cytotoxic effects during the inhibition of serine/threonine kinase and tyrosine kinase pathways in both tumor cell and tumor vasculature [103]. PAL was shown to generate increased ROS in 786-O cells (Fig. 12). Hence ROS generation by sorafenib alone and in combination with PAL in 786-O cell lines was also analyzed. Cells treated with 0.5 mM H₂O₂ were used as positive control. Cells treated with PL at concentrations similar to PAL did not generate any significant ROS (data not shown). Treatment with sorafenib (12.5 µM) generated ROS in both cell lines after 1 hour exposure. Combination of PAL (1 mM) and sorafenib (12.5 µM) produced significantly increased ROS in both 786-O VHL negative and positive cells compared to PAL or sorafenib alone. ROS production was more in 786-O VHL negative cells compared to 786-O VHL positive cells (Fig. 21).

Figure 21. The effects of sorafenib treatment alone and in combination with PAL on ROS generation in 786-O renal carcinoma cells as analyzed by flow cytometry. Cells were incubated with CMH₂DCFDA dye (1 µM) for 30 minutes followed by treatment with PAL, sorafenib, combination of PAL and sorafenib and H₂O₂ (positive control) for 1 hour and were then analyzed by flow cytometry. The data shown represent % increase in ROS from dye treated control cells. The data represent mean ± standard deviation where n = 3.
RESULTS AND DISCUSSION OF SPECIFIC AIM 4.

To prepare and characterize spheroids of renal cell carcinoma and evaluate cytotoxicity and distribution of palmitoyl ascorbate liposomes in spheroids.

5.14. CHARACTERIZATION OF RENAL CARCINOMA CELL SPHEROIDS

Spheroids are a good \textit{in vitro} tumor model as they mimic the architecture of tumors and the limited drug and drug delivery system penetration more closely as compared to monolayers. Spheroids of 786-O cells were prepared using non-adhesive liquid overlay technique and imaged periodically over their growth period to monitor the size and shape consistency. Cells (9000 cells/well) seeded initially formed a loose aggregate. The cells grew to form a compact, dense mass over the next few days. The dense spheroid structure grew till about day 7-8, and the spheroids then started to shed cells (Fig. 22). The spheroids formed were consistent with respect to shape and size over their growth period. The surface area of the spheroids was measured to monitor their size over the growth period. The spheroids showed a consistent decrease in their surface areas as they became dense and compact over their growth period. 786-O VHL negative and positive cell spheroids showed similarly consistent growth profiles (Fig. 23).
Figure 22. 786-O renal carcinoma cell spheroids prepared by non-adhesive liquid overlay technique. 9,000 cells per well in 96-well plates pre-coated with 50 µL of 1% agarose in serum-free medium were centrifuged at 1500 rcf for 15 minutes to form spherical cell aggregates. The spheroids were imaged periodically over their growth period to check for consistency in shape and size.

Figure 23. Growth profile of 786-O renal carcinoma cell spheroids. Spheroids were imaged over their growth period and their surface area was measured. The data represent mean ± standard deviation where n = 3.
5.15. DISTRIBUTION OF LIPOSOMES IN SPHEROIDS

Z-stack images of spheroids untreated or treated with Rh-PE labeled PL and PAL are shown in Figs. 24, 25 for 786-O VHL negative and positive cell lines respectively. PL did not show any interaction with spheroids. PAL showed enhanced penetration in the spheroids than PL and PAL were located mainly towards the periphery of the spheroids (Figs. 24, 25, 26).

Figure 24. Distribution of Rh-PE labeled formulations throughout 786-O VHL negative cell spheroids. Spheroids were treated with Rh-PE labeled PL or PAL for 2h. The penetration of Rh-PE labeled PL and PAL in spheroids from surface (0 µm) towards interior was analyzed by confocal microscopy using Z-stack imaging with 10 µm intervals. Scale bar represents 100 µm.
Figure 25. Distribution of Rh-PE labeled formulations throughout 786-O VHL positive cell spheroids. Spheroids were treated with Rh-PE labeled PL or PAL for 2h. The penetration of Rh-PE labeled PL and PAL in spheroids from surface (0 µm) towards interior was analyzed by confocal microscopy using Z-stack imaging with 10 µm intervals. Scale bar represents 100 µm.
Quantitation of Rh-PE fluorescence from 786-O spheroid surface after incubation with Rh-PE labeled formulations. Spheroids were treated with Rh-PE labeled PL or PAL for 2h. The penetration of Rh-PE labeled PL and PAL in spheroids from surface (0 µm) towards interior was analyzed by confocal microscopy using Z-stack imaging with 10 µm intervals and quantified by ImageJ software. The data represent mean ± standard deviation where n = 3.

5.16. CYTOTOXICITY OF PAL AND AA IN SPHEROIDS OF RENAL CELL CARCINOMA

Spheroids of 786-O VHL negative and positive renal carcinoma cell lines were used to test the cytotoxic effect of PAL and AA. Higher concentrations of PAL were required to induce cytotoxicity in spheroids than in monolayers- 1.25 mM PAL did not induce cytotoxicity in spheroids unlike in monolayers, 2.5 mM and 5 mM PAL induced lesser cytotoxicity in spheroids as compared to monolayers (Figs. 10A, 27). Also, PAL and AA were required to be incubated for 24 hours with spheroids to induce cytotoxicity as compared to 1 hour exposure period needed in monolayers to have cytotoxic effect. PAL showed increased cytotoxicity compared to AA at higher concentrations (2.5 mM and 5 mM). PAL (2.5 mM and 5 mM) exhibited increased cytotoxicity with 786-O VHL negative spheroids than 786-O VHL positive spheroids (Fig. 27).
Spheroids treated with PL showed more than 90% viability at all concentrations tested (data not shown).

Figure 27. Cytotoxicity of ascorbate formulations in 786-O renal carcinoma cell spheroids. Spheroids were treated with formulations for 24 hours followed by their dissociation with 0.09 % Triton® X-100 and measurement of cell viability using CellTiter-Glo® Luminescent Cell Viability assay according to manufacturer’s instructions. The data represent mean ± standard deviation where n = 3.
RESULTS AND DISCUSSION OF SPECIFIC AIM 5.

To determine the cytotoxicity of sorafenib alone and in combination with palmitoyl ascorbate liposomes in spheroids of renal cell carcinoma.

5.17. CYTOTOXICITY OF SORAFENIB IN SPHEROIDS

Treatment of 786-O VHL negative and positive spheroids with sorafenib led to a strong dose-dependent cytotoxic effect in both cell lines. Higher concentrations of sorafenib were required to induce cytotoxicity in spheroids as compared to monolayers- IC$_{50}$ of sorafenib increased more than 20-fold in 786-O VHL negative spheroids and more than 10-fold increase in sorafenib IC$_{50}$ was observed in 786-O VHL positive spheroids compared to monolayers (Figs. 19, 28). Sorafenib had a greater IC$_{50}$ value in 786-O VHL positive spheroids as compared to 786-O VHL negative spheroids, indicating VHL negative spheroids to be more sensitive to sorafenib (Fig. 28).

![Cytotoxicity of sorafenib in 786-O renal carcinoma cell spheroids](image)

**Figure 28.** Cytotoxicity of sorafenib in 786-O renal carcinoma cell spheroids. Spheroids were treated with sorafenib for 48 hours followed by their dissociation with 0.09 % Triton® X-100 and measurement of cell viability using CellTiter-Glo® Luminescent Cell Viability assay according to manufacturer’s instructions. The data represent mean ± standard deviation where n = 3.
5.18. DOSE-RESPONSE RELATIONSHIP AND COMBINATION INDEX OF CYTOTOXICITY OF SORAFENIB AND PAL IN COMBINATION IN SPHEROIDS OF RENAL CELL CARCINOMA

Similar to cell viability studies of sorafenib and PAL in combination in monolayers, a constant-ratio method proposed by Chou and Talalay was used to establish their dose-response relationship in spheroids of RCC. Combination index (CI) values for the combination treatment were calculated using CompuSyn software based on the classic isobologram equation of Chou and Talalay for combination of two or more drugs [154, 155, 169]. CI indicates whether drug combination may be synergistic (CI<1), additive (CI=1) or antagonistic (CI>1).

Treatment with PAL followed by sorafenib (data not shown) exhibited cytotoxicity similar to co-treatment with PAL and sorafenib in monolayers (Fig. 20). Hence co-treatment of PAL and sorafenib was the treatment sequence of choice in spheroids. Spheroids were treated with serial dilutions of sorafenib and PAL (data not shown) alone or in combination at the given constant molar ratios (1:5, 1:10 and 1:15 for 786-O VHL negative spheroids and 1:4, 1:8 and 1:12 for 786-O VHL positive spheroids).

Dose-response curves and CI values for combination treatments in 786-O spheroids are shown in Fig. 29. Combination data are presented in terms of sorafenib concentration. Similar to monolayers, sorafenib and PAL in combination at all concentration molar ratios, in both 786-O VHL negative and positive spheroids, showed a shift in the dose-response curves towards left compared to the curve of sorafenib treatment alone, indicating combination of sorafenib and PAL to be more potent than sorafenib monotherapy. The shift towards left of the dose-response curves for combination treatments became greater as the molar ratio of sorafenib to PAL increased in both 786-O VHL negative and positive spheroids. In both 786-O VHL negative and positive spheroids, a synergistic effect (CI at IC\textsubscript{50} < 1) was observed for combination of sorafenib and PAL at the IC\textsubscript{50} molar ratio (1:10 for VHL negative and 1:8 for VHL positive...
spheroids) and at lower than the IC$_{50}$ molar ratio (1:5 for VHL negative and 1:4 for VHL positive spheroids) (Fig. 29). Collectively, combination of PAL with sorafenib was shown to improve the cytotoxic effect of sorafenib in spheroids of RCC.

![Dose-response curves for cytotoxicity of sorafenib and PAL in combination in 786-O renal carcinoma spheroids.](image)

Figure 29. Dose-response curves for cytotoxicity of sorafenib and PAL in combination in 786-O renal carcinoma spheroids. Cells were co-treated with serial dilutions of sorafenib and PAL alone or in combination at molar ratios, as indicated, for 48 hours. Cell viability was then measured using CellTiter-Glo® Luminescent Cell Viability assay according to manufacturer’s instructions. For sorafenib and PAL combination treatments, concentration is plotted in terms of sorafenib dose. CI less than 1 indicates synergistic effect. The data represent mean ± standard deviation where n = 3.
RESULTS AND DISCUSSION OF SPECIFIC AIM 6.

To evaluate tumor growth inhibition to determine the effectiveness of palmitoyl ascorbate liposomes, sorafenib and their combination therapy in mice bearing tumors of renal cell carcinoma.

5.19. EFFECT OF COMBINATION OF PAL AND SORAFENIB ON TUMOR GROWTH IN VIVO

PAL treatment significantly delayed tumor growth and showed about 46% tumor growth inhibition compared to Control/PL and sorafenib showed slight delay in tumor growth with about 19% tumor growth inhibition compared to its vehicle (statistically insignificant) at the end of the treatment. PAL and sorafenib in combination at full dose of 15 mg/kg PA and 25 mg/kg sorafenib as well as at half dose of 7.5 mg/kg PA and 12.5 mg/kg sorafenib showed significantly higher tumor growth inhibition and tumor stabilization compared to PAL (15 mg/kg PA) or sorafenib (25 mg/kg) alone. Combination of PAL and sorafenib at half doses showed tumor growth inhibition of about 89% compared to PAL and 91% compared to sorafenib. Combination of PAL and sorafenib at full dose showed 71% inhibition of tumor growth compared to PAL and 77% compared to sorafenib treatment after 30 days from start of treatment. This indicated combination of lower doses of PAL and sorafenib to be more effective (Figs. 30, 31). The effect of combination treatment on tumor growth was affected by the dosing regimen. The split dose combination treatment, where PAL was administered first followed by sorafenib on the next day, did not inhibit tumor growth as effectively as the half dose and full dose groups. The split dose combination treatment showed significant tumor growth inhibition of about 58% compared to control groups, but showed only slight tumor growth delay and tumor growth inhibition (statistically insignificant) compared to PAL or sorafenib alone at the end of the study.
For the split dose group, tumors of 2 out of 4 mice disappeared after day 3 from the start of the treatment. So the data for this group does not indicate actual results due to small number of tumor-bearing animals for most part of the treatment (n=2), but suggest a trend followed by the treatment regimen.

Two mice were tumor-free at the end of the treatment period, one each in PAL treatment and combination of PAL and sorafenib at full dose treatment groups, indicating complete inhibition of tumor growth.

No significant change in body weight, which is an indicator of acute toxicity, was seen in any treatment group throughout the course of the treatment regimen (Fig. 32). However, 3 mice from the PAL and sorafenib combination at full dose treatment group died 2-3 days after the start of the treatment. It may be due to some inflammation or infection due to a possible error in oral administration.

% tumor growth inhibition was calculated as:

\[
\frac{(\text{TVveh Day } x - \text{TVveh Initial}) - (\text{TVt Day } x - \text{TVt Initial}) \times 100}{(\text{TVveh Day } x - \text{TVveh Initial})}
\]

Where, TVt Day x= tumor volume of treatment group on the final day after the start of the treatment (Day 30)

TVt Intial = tumor volume of treatment group at the start of the treatment (Day 0)

TVveh Day x= tumor volume of group with which the treatment group is compared on the final day of after the start of the treatment (Day 30)

TVveh Intial = tumor volume of group with which the treatment group is compared at the start of the treatment (Day 0)
Figure 30. Effect of administration of PAL, sorafenib and their combination on tumor growth in mice bearing 786-O VHL negative tumors. Mice were administered PAL i.v. via tail vein and sorafenib by oral gavage. Treatments were administered once every 4 days, with first dose on day 0. Doses: PAL: 15 mg/kg PA, SFB: 25 mg/kg sorafenib, PAL + SFB (full dose) and (split dose): 15 mg/kg PA and 25 mg/kg sorafenib, PAL + SFB (half dose): 7.5 mg/kg PA and 12.5 mg/kg sorafenib. The data represent mean ± standard deviation. *, ^ and # denote the groups are statistically very significant (p-value < 0.05) compared to Control/PL and Vehicle, SFB 25 mg/kg and PAL 15 mg/kg groups respectively.
Figure 31. Effect of administration of PAL, sorafenib and their combination on post-mortem volume and weight of mice-bearing 786-O VHL negative tumors. Mice were administered PAL i.v. via tail vein and sorafenib by oral gavage. Treatments were administered once every 4 days, with first dose on day 0. Doses: PAL: 15 mg/kg PA, SFB: 25 mg/kg sorafenib, PAL + SFB (full dose) and (split dose): 15 mg/kg PA and 25 mg/kg sorafenib, PAL + SFB (half dose): 7.5 mg/kg PA and 12.5 mg/kg sorafenib. The data represent mean ± standard deviation. *, ^ and # denote the groups are statistically very significant (p-value < 0.05) compared to Control/PL and Vehicle, SFB 25 mg/kg and PAL 15 mg/kg groups respectively.
Figure 32. Effect of administration of PAL, sorafenib and their combination on body weights of mice bearing 786-O VHL negative tumors. Mice were administered PAL i.v. via tail vein and sorafenib by oral gavage. Treatments were administered once every 4 days, with first dose on day 0. Doses: PAL: 15 mg/kg PA, SFB: 25 mg/kg sorafenib, PAL + SFB (full dose) and (split dose): 15 mg/kg PA and 25 mg/kg sorafenib, PAL + SFB (half dose): 7.5 mg/kg PA and 12.5 mg/kg sorafenib. The data represent mean ± standard deviation.
6. SUMMARY AND CONCLUSIONS

We can conclude the following from our studies:

1. PAL showed good cell association in 786-O renal carcinoma cells and higher cytotoxic effects than AA in murine and human renal carcinoma cells, but not in non-cancerous cells *in vitro*.

2. Increased extracellular ROS production and apoptosis induced by PAL as compared to AA are the main mechanisms by which PAL exert their effects in renal carcinoma cells. Both 786-O VHL negative and positive renal carcinoma cells exhibit these mechanisms of action. PAL also induced changes in cell cycle distribution in 786-O VHL negative cells arresting the cells in S and G2/M phase of the cell cycle. GLUT receptors were also found to be involved to some extent in the association of PAL to renal carcinoma cells.

3. Spheroids of renal carcinoma cells were formed with consistent growth profiles for both 786-O VHL negative and positive renal carcinoma cells.

4. PAL showed strong cytotoxicity in both 786-O VHL negative and positive renal carcinoma cell spheroids compared to AA. PAL were well-distributed in spheroids located mainly towards the periphery.

5. Sorafenib exhibited strong cytotoxicity in both renal carcinoma cells and spheroids. A synergistic cytotoxic effect was observed for the combination of sorafenib and PAL, at their IC\textsubscript{50} molar ratio and at lower than their IC\textsubscript{50} molar ratio, compared to cytotoxicity of sorafenib or PAL alone in both renal carcinoma cells and spheroids. Sorafenib also showed increased ROS generation and sorafenib and PAL in combination resulted in higher ROS production compared to sorafenib or PAL treatment alone.

6. *In vivo*, PAL showed significant tumor growth inhibition and tumor growth delay in mice bearing tumors of RCC. Combination of PAL and sorafenib showed increased tumor growth inhibition and tumor stabilization compared to PAL or sorafenib treatment alone.
In summary, PAL can serve as an effective delivery system of ascorbate to RCC *in vitro* and *in vivo* and as a potential alternative to AA in cancer therapy with PA delivered by these liposomes showing strong anticancer activity *in vitro* in renal carcinoma cells and *in vivo* in mice bearing tumors of RCC.

Combination of PAL with the chemotherapeutic drug, sorafenib, can improve the cytotoxic effect of sorafenib to achieve enhanced efficacy in the treatment of RCC.
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8. APPENDIX

8.1. LIST OF PUBLICATIONS

The data in this thesis work is not published as yet. It is in the process (manuscript preparation) of publication.

Articles published:


8.2. LABORATORY SAFETY TRAINING

Following trainings were undertaken under Northeastern University Office of Environmental Health & Safety:

1. CHEMICAL HYGIENE TRAINING Part 1- Right To Know

2. CHEMICAL HYGIENE TRAINING Part 2- Laboratory Safety and Hazardous Waste Management

3. BIOSAFETY AND BLOODBORNE PATHOGENS TRAINING
8.3. OCCUPATIONAL HEALTH AND SAFETY TRAINING FOR WORKING WITH ANIMALS IN RESEARCH AND TEACHING

Following requirements were completed for working with Animals in Research and Teaching:

- Participated in the training program.
- Prepared research protocol for *in vivo* studies.
- Enrolled in the Occupational Health and Safety Program.
- Received Animal Facility Orientation from DLAM Staff.