Multimodality Molecular Imaging of [18F]-Fluorinated Carboplatin Derivative Encapsulated in [111In]-Labeled Liposome

Narottam Lamichhane
Virginia Commonwealth University

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Multimodality Molecular Imaging of $[^{18}\text{F}]$-Fluorinated Carboplatin Derivative Encapsulated in $[^{111}\text{In}]$-Labeled Liposomes

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Physics at Virginia Commonwealth University

by

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Bachelor of Science in Applied Physics and Chemistry

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March 21, 2014
Acknowledgements

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I dedicate my dissertation to humanity.
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List of Abbreviations

For the convenience of the reader, here are the lists of abbreviations.

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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$[^{18}\text{F}]$-FCP</td>
<td>cisdiaminmine ${2-(5-[^{18}\text{F}]-\text{fluoropentyl})-2\text{-methylmalonato-}\kappa^2, O, O'}$ platinum (II)</td>
</tr>
<tr>
<td>$^{19}\text{F}$-FCP</td>
<td>cisdiaminmine ${2-(5\text{-fluoropentyl})-2\text{-methylmalonato-}\kappa^2, O, O'}$ platinum (II)</td>
</tr>
<tr>
<td>$[^{18}\text{F}]$-FMISO</td>
<td>Fluoromisonidazole</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>FLT</td>
<td>3'\text{-deoxyfluorothymidine}</td>
</tr>
<tr>
<td>FMAU</td>
<td>$^{18}\text{F}$-1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) thymine</td>
</tr>
<tr>
<td>FPMA</td>
<td>2-(5-fluoro-pentyl)-2-methyl malonic acid</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photon Emission Tomography</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half Life</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Endothelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>3DCRT</td>
<td>Three-dimensional Conformal Radiation Therapy</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity Modulated Radiation Therapy</td>
</tr>
<tr>
<td>IGRT</td>
<td>Image Guided Radiation Therapy</td>
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<tr>
<td>SRS</td>
<td>Stereotactic Radiosurgery</td>
</tr>
<tr>
<td>SBRT</td>
<td>Stereotactic Body Radiation Therapy</td>
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<tr>
<td>mAbs</td>
<td>Monoclonal Antibodies</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>HER-2</td>
<td>Herceptin-2</td>
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<tr>
<td>CML</td>
<td>Chronic Myelogenous Leukemia</td>
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<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
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<tr>
<td>FDA</td>
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<tr>
<td>5-FU</td>
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<tr>
<td>HPMA</td>
<td>Hydroxypropylmethacrylamide</td>
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<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
</tr>
<tr>
<td>PgP</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>CTR1</td>
<td>Copper Transporter 1</td>
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</table>
GT  Glutathione Transferase
ATP  Adenosine Triphosphate
ABC  ATP-Binding Cassette
BCRP  Breast Cancer Resistance Protein
ERCC  Excision Repair Cross Complementation
MRP  Multidrug Resistance Protein
mRNA  Messenger RNA
LRP  Lung Resistance Protein
ICP  Induced Coupled Plasma
γ-rays  Gamma Rays
EC  Electronic Conversion
IT  Isomeric Transition
β  beta-minus
FOV  Field of View
NaI  Sodium Iodide
2D  Two Dimensional
3D  Three Dimensional
CdZnT  Cadmium Zinc Telluride
LOR  Line of Response
FBP  Filtered Backprojection
OSEM  Ordered Subsets Expectation Maximization
WB-DWI  Whole Body Diffusion Weighted Imaging
TK  Thymidine Kinase
AnxV  Annexin V
PLA  Polylactic Acid
PLGA  Poly-(lactic-glycolic)-Acid
PEG  Polyethylene Glycol
RGD  Arginylglycylaspartic acid
QMA  Quaternary Ammonium
NAH  Sodium Hydride
TBAF  Tetrabutyl Ammonium Fluoride
THF  Trihydro Furane
MeOH  Methanol
LiOH  Lithium Hydroxide
ACN  Acetonitrile
NaOH  Sodium Hydroxide
NMR  Nuclear Magnetic Resonance
RCY  Radiochemical Yield
RCP  Radiochemical Purity
TLC  Thin Layer Chromatography
HCl  Hydrochloric Acid
DCM  Dichloromethane
K18F/K222  Kryptofix
CO₂  Carbon dioxide
PBS  Phosphate Buffered Saline
FBS  Fetal Bovine Serum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>COLO205</td>
<td>Colorectal Carcinoma</td>
</tr>
<tr>
<td>KB</td>
<td>Human Cervical Carcinoma</td>
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<tr>
<td>SCLC</td>
<td>Small Cell Lung Carcinoma</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Carcinoma</td>
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<tr>
<td>A549</td>
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<td>Ovarian Carcinoma</td>
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<td>Benign Prostate</td>
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<td>Head and Neck Carcinoma</td>
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<tr>
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<tr>
<td>DSPC</td>
<td>1, 2-distearoyl-sn-glycero-3-phosphocholine</td>
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<td>Hydrogenated soy phosphatidylcholine</td>
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<td>Dipalmitoyl phosphatidylglycerol</td>
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<td>Cholesterol</td>
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<tr>
<td>DTPA-DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid</td>
</tr>
</tbody>
</table>
Abstract

Multimodality Molecular Imaging of $^{[18}\text{F}]$-Fluorinated Carboplatin Derivative Encapsulated in $^{[111}\text{In}]$-Labeled Liposomes

By Narottam Lamichhane, BS
A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Physics at Virginia Commonwealth University

Virginia Commonwealth University, 2014

Director: Jamal Zweit, PhD, DSc
Professor, Department of Radiology

Platinum based chemotherapy is amongst the mainstream DNA-damaging agents used in clinical cancer therapy today. Agents such as cisplatin, carboplatin are clinically prescribed for the treatment of solid tumors either as single agents, in combination, or as part of multi-modality treatment strategy. Despite the potent anti-tumor activity of these drugs, overall effectiveness is still hampered by inadequate delivery and retention of drug in tumor and unwanted normal tissue toxicity, induced by non-selective accumulation of drug in normal cells and tissues. Utilizing molecular imaging and nanoparticle technologies, this thesis aims to contribute to better understanding of how to improve the profile of platinum based therapy. By developing a novel fluorinated derivative of carboplatin, incorporating a Flourine-18 ($^{18}\text{F}$) moiety as an inherent part of the molecule, quantitative measures of drug concentration in tumors and normal tissues can be directly determined in vivo and within the intact individual environment. A potential impact of this knowledge will be helpful in predicting the overall response of individual patients to the treatment. Specifically, the aim of this project, therefore, is the development of a fluorinated carboplatin drug derivative with an inherent positron emission tomography (PET) imaging capability, so that the accumulation of the drug in the tumor and normal organs can be studied during the course of therapy. A secondary objective of this research is to develop a proof of concept for simultaneous imaging of a PET radiolabeled drug with a SPECT radiolabeled liposomal formulation, enabling thereby bi-modal imaging of drug and delivery vehicle in vivo. The approach is challenging because it involves development in PET radiochemistry, PET and SPECT imaging, drug liposomal encapsulation, and a dual-modal imaging of radiolabeled drug and radiolabeled vehicle. The principal development is the synthesis of fluorinated carboplatin $^{19}\text{F}$-FCP using 2-(5-fluoro-pentyl)-2-methyl malonic acid as the labeling agent to coordinate with the cisplatin aqua complex. It was then used to treat various cell lines and compared with cisplatin and carboplatin at different concentrations ranging from 0.001 µM to 100 µM for 72 hrs and 96 hrs. IC$_{50}$ values calculated from cell viability indicated that $^{19}\text{F}$-FCP is a more potent drug than Carboplatin. Manual radiosynthesis and characterization of $^{[18}\text{F}]$-FCP was performed using $^{[18}\text{F}]$-2-(5-fluoro-pentyl)-2-methyl malonic acid with coordination with cisplatin aqua complex. Automated radiosynthesis of $^{[18}\text{F}]$-FCP was
optimized using the manual synthetic procedures and using them as macros for the radiosynthesizer. $[^{18}\text{F}]-\text{FCP}$ was evaluated \textit{in vivo} with detailed biodistribution studies and PET imaging in normal and KB 3-1 and KB 8-5 tumor xenograft bearing nude mice. The biodistribution studies and PET imaging of $[^{18}\text{F}]-\text{FCP}$ showed major uptake in kidneys which attributes to the renal clearance of radiotracer. \textit{In vivo} plasma and urine stability demonstrated intact $[^{18}\text{F}]-\text{FCP}$. $[^{111}\text{In}]-\text{Labeled Liposomes}$ was synthesized and physiochemical properties were assessed with DLS. $[^{111}\text{In}]-\text{Labeled Liposome}$ was evaluated \textit{in vivo} with detailed pharmacokinetic studies and SPECT imaging. The biodistribution and ROI analysis from SPECT imaging showed the spleen and liver uptake of $[^{111}\text{In}]-\text{Labeled Liposome}$ and subsequent clearance of activity with time. $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ was developed and physiochemical properties were characterized with DLS. $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ was used for \textit{in vivo} dual tracer PET and SPECT imaging from the same nanoconstruct in KB 3-1 (sensitive) and COLO 205 (resistant) tumor xenograft bearing nude mice. PET imaging of $[^{18}\text{F}]-\text{FCP}$ in KB 3-1 (sensitive) and COLO 205 (resistant) tumor xenograft bearing nude mice was performed. Naked $[^{18}\text{F}]-\text{FCP}$ and $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ showed different pharmacokinetic profiles. PET imaging of $[^{18}\text{F}]-\text{FCP}$ showed major uptake in kidneys and bladder. However, $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ showed major uptake in RES in both PET and SPECT images. ROI analysis of SPECT image enabled by $[^{111}\text{In}$ corresponded with PET image enabled by $^{18}\text{F}$ demonstrating the feasibility of dual tracer imaging from the single nanoconstruct. Future work involves the intensive \textit{in vitro} characterization of $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ and detailed \textit{in vivo} evaluation of $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$ in various tumor models.
1.1 **Rationale**

Platinum based anti-cancer therapy is a major treatment modality for various solid tumors in clinic. For many patients however, treatment efficacy is still hampered by normal tissue toxicity and drug resistance, which limits adequate dose in tumors. In order to better understand the extent of therapeutic efficacy, in relation to drug concentration, *in vivo* live imaging of tissue based drug pharmacokinetics is needed. Therefore, to reduce the adverse effect caused by platinum drugs, to increase the delivery, and to optimize the response of tumor, an approach of encapsulating the platinum drug inside the liposome and combining it with radioisotope would provide theranostic and synergistic effect.

1.2 **Objective**

The overall objective of this project is to develop a multimodal nanocarrier drug delivery system to investigate the delivery, retention and efficacy of anticancer therapy through molecular imaging. The aim of this thesis project is to develop a novel multimodal therapy/imaging paradigm, where a novel Fluorine-18 labeled Carboplatin derivative, *cis*di*ammine*(2-(5-[18F]-fluoropentyl)-2-methylmalonato-κ²,Ο,Ο’*)platinum(II)*,([18F]-FCP) is encapsulated inside Indium-111 labeled liposome ([111In]-Labeled Liposome). The direction of this project is to combine the radiochemistry strengths that involve the ability to radiolabel organic molecules with [18F] and other radioisotopes to perform Positron Emission Tomography (PET/CT) and Single Photon Emission Tomography (SPECT/CT) imaging in small animal with high spatial resolution, to study the biodistribution and pharmacokinetics of the pharmaceutical load of the nanocarrier. This proposal where the radiolabeled drug is incorporated inside the radiolabeled vehicle is quite novel which would be useful in evaluating the fate of drug by tracking the PET
signal from $^{18}$F, whether it stays with the nanocarrier by tracking the SPECT signal from $^{111}$In or is separated from the nanocarrier and is accumulated in tissues by looking at the dual tracer profile. The ultimate objective of this project is to provide a quantitative theranostic agent that is capable of relating the tumor drug availability to efficacy of drug delivery and treatment. These dual advantages from a single nanocarrier construct will eventually be translatable to the clinic for the diagnosis of solid tumor and metastasis and to effectively kill tumors.

1.3 Statement of Aims
The specific aims of the project are as follows:

- **To synthesize and characterize $^{111}$In-Labeled Liposome nanocarrier to enable SPECT imaging.** Labeling a nanocarrier allows to study the *in vivo* kinetic profile of the vehicle. Liposome, as a nanocarrier stays in the blood circulation for a longer period of time to deliver an effective dose to tumor via enhanced permeability and retention effect (EPR). Optimal SPECT agents with medium half life are required for extended imaging of the nano-vehicle. Therefore, for this project, the liposome was labeled with $^{111}$In ($t_{1/2} = 2.8$ days, 171 keV, 90%, and 245 keV, 94%) to determine the pharmacokinetic profile of the nanocarrier.

- **To synthesize and characterize $^{18}$F-FCP as a theranostic drug for imaging and therapy.** Our goal is to test the efficacy of drug in tumors. This requires monitoring the drug’s therapeutic effectiveness in targeted site. Labeling the drug with $^{18}$F ($t_{1/2} = 110$ mins, 97% $\beta^+$) allows a pathway to determine the drug
metabolism, route of metabolism, clearance and tissue distribution \textit{in vivo}. It provides a non-invasive imaging flexibility to assess the drug accumulation and retention in normal tissues and tumors using PET.

- To encapsulate $^{[18}F\text{-FCP}$ in $^{[111}\text{In}\text{-Labeled Liposome nanocarrier to enable two distinct SPECT and PET signals from one construct.}$ Naked drugs once injected intravenously cause systemic toxicity due to non specific uptake. Encapsulation of drugs in nanocarrier reduces the exposure of drugs to normal tissue. This nanoconstruct (radiolabeled drug encapsulated in radiolabeled vehicle) provides \textit{in vivo} data on fate of the drug, vehicle and the construct itself using multi-modal imaging. \textit{In vivo} information on accumulation and clearing profiles facilitates in optimizing the nanoconstruct for future targeted imaging and delivery.

- To evaluate the doubly radiolabeled nanocarrier construct in tumor model using multimodal imaging. Determination of the behavior of the nanoconstruct in tumor is essential to determine the effectiveness of the delivery system. We evaluated our drug packaged liposomal carrier in tumor xenograft model. Dual tracer imaging using PET/SPECT was performed in nude mice inoculated with human epidermal cancer cell line KB 3-1 (Cervical Carcinoma) and COLO 205 (Colon Carcinoma) to assess the \textit{in vivo} pharmacokinetics of the nanoconstruct.

1.4 \textbf{Innovation}

This work is innovative in two aspects:
It proposes a novel dual modality guided imaging of a fluorinated drug encapsulated liposomal nanocarrier. Encapsulation of radiolabeled drug in radiolabeled vehicle will provide two distinct signals from one construct to study the overall pharmacokinetics of the drug and nanocarrier. This dual-modality imaging from one construct can offer synergistic advantages over a single modality.

Synthesis of $^{18}$F-FCP as a novel theranostic anticancer drug for imaging and therapy. This project is innovative because it proposes a new approach to individualized therapy by “in situ” measurement of drug concentration, using molecular imaging, during the course of therapy. This technological advance of an image-guided platinum drug therapy has been enabled by developing the $^{18}$F-FCP that retains very similar anti-tumor activity to the parent compound in various human cancer cell lines. This “drug biomarker”, could lead to how patient therapy can be individualized to affect the best possible response.

1.5 Impact
The impact of this work is tremendous in cancer imaging and therapy of platinum drugs. The proposed research will impact on how platinum drug therapy can be individually tailored to cancer patients, by directly monitoring drug treatment using PET imaging. As of our knowledge, this is the first time that a platinum drug is labeled with $^{18}$F, a non-invasive molecular imaging tracer for PET. The development of $^{18}$F-FCP allows tracking and measuring the drug treatment and its accumulation in tumor and normal tissues during the course of therapy using non-invasive multimodal imaging. This capability provides a tool to identify responders from non-responders and will also help
to understand susceptibility or resistance to treatment. Data from this project will provide direct measure of drug concentration in heterogeneous individual tumors in relation to response. Encapsulation of $[^{18}\text{F}]-\text{FCP}$ inside the liposome will also reduce the exposure of drug to normal tissue and hence reduce the normal tissue toxicity. In addition, encapsulation of drug inside the liposome will introduce favorable normal tissue pharmacokinetic profile compared to the naked drug and increases the tumor uptake of the drug by EPR effect and help in overcoming the tumor resistance.

### 1.6 Research Strategy/ Experimental Design

This research developed a liposome platform (Fig. 1) for the delivery of anticancer agents. Liposomes with lipid shell radiolabeled with $^{111}\text{In}$ were synthesized and were optimized for *in vivo* experiments. PEG modified phospholipids were used to provide a steric brush to minimize the lipid shell interactions from the blood components. Radiolabeled drug $[^{18}\text{F}]-\text{FCP}$ was encapsulated inside the $[^{111}\text{In}]-\text{Labeled Liposomes}$. $^{18}\text{F}$ is a positron emitter and has a potential for PET imaging to evaluate the *in vivo* kinetics of the drug. Carboplatin is water soluble that enables its encapsulation inside the liposome by using the hydration method or passive encapsulation method. $^{111}\text{In}$ is a gamma emitter which enables SPECT imaging. Radiolabeling the shell with $^{111}\text{In}$ helps to assess biodistribution and aim to follow the fate of the particle *in vivo* to obtain insights into the pharmacokinetics of the liposomes and to quantify the radioactive distribution in different tissues *ex vivo*. The liposome construct was evaluated in tumor xenograft model bearing nude mice. Biodistribution and imaging characteristics of encapsulated and free drug was determined in tumor bearing nude mice. Nude mice were inoculated with human.
epidermal cancer cell lines to establish a xenograft model with one sensitive and one drug resistant tumor to assess the drug resistance and retention.

Figure 1: Dual tracer labeled liposomal nanocarrier. $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]$-Labeled Liposome was synthesized and was evaluated in tumor xenograft bearing mouse model.
CHAPTER 2: BACKGROUND
2.1 Cancer
Cancer is a disease caused by uncontrollable division of abnormal cells in a part of the body that has the potential to invade other tissues. Normal cells stop dividing when they come into contact with like cells by a mechanism known as contact inhibition. However, in cancer cells, genetic mutation in DNA affects the normal cell growth and division. As a result, cancer cells do not have normal checks and balances that control and limit the cell division (Fig. 2). Cell division for both normal and cancer cells is through cell cycle that goes from resting phase, through active growing phase and then to mitosis. The inability of cells to divide and copy itself in division results in cell death. Various cancer cell phenotypes such as cells with unlimited proliferative potential, environmental independence for growth, evasion of apoptosis, angiogenesis, altered metabolism, invasion and metastasis to different parts of body are the hallmarks of cancer [1, 2].
Cancer is not one disease unlike many other local or organ specific diseases such as heart disease, kidney diseases etc. Cancer is a group of many diseases and is classified according to the fluid or tissue they initially originate from, or from the location in the body they are first developed. Pathologically, cancers are classified as below (Table 1).

**Table 1: Pathological classification of cancer**

<table>
<thead>
<tr>
<th>Types</th>
<th>Origin</th>
<th>Sub types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma</td>
<td>• Epithelial cells in the skin or tissues that line or cover the internal organs</td>
<td>• Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Squamous cell carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Basal cell carcinoma</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>• Bone, cartilages, blood vessels, fat, muscles, connective or supportive tissues</td>
<td>• Osteosarcoma (bone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Chondrosarcoma (cartilage)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>Originates in the cells of lymphatic system that develops in mature immune cells, mainly lymphocyte, spleen, thymus</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
<td>Non-Hodgkin’s lymphoma</td>
<td></td>
</tr>
<tr>
<td>Leukemia</td>
<td>Originates in hematopoietic cells residing in the bone marrow and blood</td>
<td></td>
</tr>
<tr>
<td>Acute myelogenous</td>
<td>Chronic myelogenous</td>
<td></td>
</tr>
<tr>
<td>Acute lymphocytic</td>
<td>Chronic lymphocytic</td>
<td></td>
</tr>
</tbody>
</table>

Cancer refers to a group of conditions that manifests the malignant behavior and uncontrolled cell growth leading to invasion. Malignant cells have tendency to move from their site of origin and disseminate to distant organs and form metastases. This ability of malignant cells is aided by large number of characteristics they possess. Migrations, ability to invade stroma and endothelial linings, dissemination through circulation, survival are some of the underlying physiological and biological properties of solid tumors that allow them to grow in secondary sites. Epithelial solid tumors have proved to be a major challenge in cancer treatment. Treatment of solid tumors is based on the type, location and stage of the tumor. Complexity and non-homogeneity of solid tumors pose an obstacle in the delivery of an efficient amount of drugs to the cancer cells in a safe and effective manner. Streamlines of techniques have been proposed for the delivery of cancer drugs to solid tumors in search of more effective therapies. Local only therapies such as ablation, radiation have garnered positive responses. However, local therapy is always not feasible to treat solid tumors, especially the metastases.

Several innovative approaches of delivering drugs into tumors using nanoparticles, liposomes, and micelles have been developed that can target tumor cells and help provide the information on the underlying biology of solid tumors.
2.2 Solid Tumors and Cancer Biology

Tumors comprise of heterogeneous population of cells that, over time, develop means to sustain their growth and survival despite ongoing immune responses or therapies [3-5]. Malignant solid tumors arise after various phases of genetic changes, which endow them with resistant, proliferative, and invasive capabilities. Such phases include anaplasia (loss of structural differentiation), neoplasia (abnormal proliferation), dysplasia (change in tissue phenotype) and hyperplasia where increased proliferative rate of cells leads to excess of normal appearing cells. When cells further accumulate genetic changes leading to abnormal and disorganized tissue growth, the phase is referred as dysplasia. This phase is followed by carcinoma in situ where cells accumulate further mutations, continue anaplastic growth, but still be limited within basal lamina; hence preventing from metastases. Further genetic insults result in malignant tumors, or often referred as cancer, cells increasingly become resistant to immune system or therapies and invade surrounding tissues or metastasize to distant organs [6, 7]. Metastases are often what cause death due to multiple organ failures in cancer patients.

Various theories have been proposed as to how the tumor progression occurs. Although it is widely accepted now that the solid tumors are comprised of heterogeneous populations of cells, how the progression of tumor occurs had been debatable. Some of the theories proposed include linear progression model, multi/oligo-clonal progression model, and cancer stem cells and punctuated equilibrium model [8, 9]. Although linear progression model goes through the steps described above, it does little to explain the heterogeneity of the cells in the tumor mass. Oligoclonal progression model suggests that during progression of tumors leading to metastases, various populations of cells arise and coexist [8, 9]. Cancer stem cells and punctuated equilibrium model has garnered momentum as
of late. In many cases, patient relapse despite the optimal debulking and chemo/radiation/immune-therapies leading to no residual diseases. This has often attributed to the cancer stem cells, which are often thought to be more resistant and capable of giving rise to tumor cells [4, 8, 10, 11]. Stem cells have been described in various types of cancers including but not limited to breast, ovarian, and colon cancers [11-14]. They have been recognized to have increased resistance to chemo and radiation therapies and capable of inducing relapse and metastasis of tumors due to their regenerative capabilities. Presence of such cells limits the efficacy of traditional therapies. Hence, current studies have now started to take into account the importance of targeting such cell in the therapy regimen. Another phenomenon that often occurs during tumor progression is epithelial to mesenchymal transition (EMT) [15]. Although the causalities of this phenomenon in vivo is attributed to various growth factors and proteins such as TGF beta and EGF, many other factors are still being studied [15]. As of now, it has been clearly shown that EMT allows tumor cells of epithelial origin to be migratory through acquisition of mesenchymal phenotype and hence aid in metastases and disease severity by acquiring phenotypes like invasiveness and resistance to apoptosis [15, 16].

Solid tumors are adept in resisting immunotherapies and chemotherapies by means such as abilities to survive in anoxic to hypoxic conditions, constant mutations and selection of antigen loss variants to evade immune system, and ability to resist the radiation and chemotherapies by certain cells such as cancer stem cells which constantly give rise to new cancer cells whilst helping progression and metastases [17-19]. Solid tumors pose many problems not only to ones’ immune system by a simple fact that most tumor antigens are self-antigens; hence immune system does not mount a robust response
against them. On top of that, solid tumors foster such an environment which poses
difficulties to the therapies that are intended towards eliminating them. Most notable of
such difficulties includes the delivery of the drugs. A precise dose and contained delivery
of therapeutic agents limits the toxicities that are often associated with the cancer
therapies. Recent advances in the field of nanoparticle delivery combined with radiation
therapy have shown promising results in controlled delivery of drugs that are efficient in
selectivity and cytotoxicity. Although these recent advances need to be validated via
clinical trials before wide range of patients can benefit from them, progresses in such
technologies are certainly promising.

2.3 Imaging in Cancer Care
Imaging plays an important role in the diagnosis of cancer. Biomedical imaging has many
advantages including real time monitoring, accessibility without tissue destruction,
minimal or non invasiveness and can function over wide ranges of time and size scales
involved in biological and pathological processes [20]. Various multimodal biomedical
imaging techniques are being used in all phases of cancer management. The goal of
cancer imaging is to detect /and or image the smallest possible number of tumor cells,
ideally before the angiogenesis switch [21, 22]. Major problems in cancer treatment are
late diagnosis of cancer, lack of tools to visualize the treatment efficacy of the
biological/chemotherapy treatment, absence of in vivo revelation of signaling pathways
after radiation, chemo/biological therapy. Imaging provides a biological tool for the early
detection of cancer, in vivo drug distribution, heterogeneity of tumor, and direct way of
visualization of various pathways in vivo. Different metabolic pathways, cell proliferation,
cell death and variety of disorders can be studied to a greater understanding using newly
emerged biomarkers and molecular imaging modalities which provides a better way of tailoring an effective treatment of the diseased sites. Molecular imaging modalities imaging the hall marks of cancer is further explained in Chapter 3. Medical imaging in cancer care is mainly aimed to determine how far a cancer has spread, guide delivery of specific treatments, and to predict the response of treatment. Imaging is widely employed in cancer care for various applications including staging, diagnosis, therapy response and image guided therapy/ surgery.

2.4 Cancer Treatment and Therapy
The treatment of cancer mainly depends on the type and stage of cancer, possible side effects of the treatment regimen, the patient’s preference and overall health of the patient itself. The multidisciplinary approach of treating cancer is very essential in determining the treatment option for the patient. The most common cancer treatments include surgery, radiation therapy, biological therapy, and chemotherapy. These therapies may be used alone, or in combination depending on the stage and sensitivity of cancer towards the treatment. Adjuvant and neoadjuvant therapies are also a major regime of cancer treatment. However, various other therapies are also looked upon for the treatment options such as targeted therapy, immunotherapy, hyperthermia, phototherapy, hormonal therapy, bone marrow transplantation etc. These therapies are not in the scope of this thesis and won’t be discussed in detail. Different types of cancer cells have unique properties that may not be readily treatable using the currently available cancer treatment procedure. Nanotechnology has emerged as a field with potentials to detect and treat cancer using nanoparticles. Motivation of using nanoparticles for cancer detection is
prompted from the ineffectiveness of currently available general treatment regimens, difficulty in early detection of tumors and numerous side effects.

2.4.1 Surgery
Surgery is the primary treatment for many cancers. Surgical resection can completely remove some cancers. However, it should be noted that surgery is a local treatment and it only treats the parts of the body it is operated on. So, if the cancer is on its early stage and is contained in one area, surgery may be the only treatment required to completely treat the tumor. However, if the cancer has spread to other parts of the body, surgery may not be the right treatment option. Surgery is used in clinic for various reasons. Diagnosing of cancer is done by a surgical procedure called biopsy, where a small piece of tissue is removed to test for the presence of cancer cells, type of cancer cells, and the rate of growth. Surgery also reduces or prevents the risk of cancer. If a person is at high risk of particular type of cancer based on his/her family history/genetics, surgery may be able to reduce the risk of cancer. Woman with high risk of breast cancer may choose to have their breast removed. Reconstruction surgery is another field of surgery in oncology which made it possible to recreate the part of the body that was resected using other body tissues. Breast reconstructions after mastectomy, bladder reconstruction after bladder removal are some of the usual reconstruction surgeries done in oncology. Surgery is performed on the patients to remove or bypass blockage, to control pain caused by cancer that is pressing on a part of the body. Further, surgery may be used as a part of other treatments such as insertion of tube for chemotherapy, or biological therapy.
2.4.2 Radiation Therapy

Radiation therapy is the use of high energy x-rays or other particles to kill cancer cells. Radiation therapy remains an important component of cancer treatment with approximately 50% of all cancer patients receiving radiation therapy during their course of illness; it contributes to 40% of curative treatment for cancer [23]. Radiation used in the treatment of cancer is ionizing radiation which forms electrically charged particles that deposit energy in the cells of the tissue it passes through. The deposition of energy results in the genetic changes (damages deoxyribonucleic acid, DNA) of cells resulting in cell death. DNA is the biological target of radiation. Radiation therapy kills cancer either by directly interacting with cellular DNA and causing death or by inducing free radicals from the excitation or ionization of the water component of the cells which causes indirect damage to DNA. Regardless of the mechanisms of cell kill, the major goal of radiation therapy is to deprive cancer cells of their proliferating potential and leading to cell death. Therapeutic efficacy of radiation is achieved by various cell death pathways (Fig. 3)
Radiation can be used not only for treatment purposes but also for palliative treatment purposes to relieve patients from symptoms caused by cancer. Delivering radiation treatment can be accomplished either by using external beam radiation or by using internal beam radiation. External beam radiation delivers radiation treatment to cancer by aiming high energy rays from outside the body to the location of tumor. Internal beam radiation also known as brachytherapy delivers radiation treatment inside the body by using sealed radioactive sources or by sealed sources in catheters directly inserted into the tumor sites. Alike all cancer therapy, radiation therapy also aims to deliver as much dose to the tumor while sparing the normal tissues. Various radiotherapy techniques have been emerged over the period of time that encompasses new imaging modalities, delivery systems with powerful computers and software in advancing the method of treatment and

Figure 3: Types of cell death induced by radiation. Radiation mainly kills the cells either by apoptosis or mitotic catastrophe (adapted and modified from reference 28)
improving the understanding of radiobiology and radiation therapy. Technological
advances such as 3D conformal radiotherapy (3DCRT), Intensity modulated radiation
therapy (IMRT), Image-guided radiation therapy (IGRT), Stereotactic radiosurgery (SRS),
Stereotactic body radiation therapy (SBRT), Special procedures, Particle radiations
(electron, proton, neutron beams) have improved the therapeutic ratio of radiation
treatment.

2.4.3 Biologically Targeted Therapy

Spectacular progress in the field of cancer research unveiled the fundamental molecular
basis of cancer progression. Differences in molecular biology, physiology, morphology,
and pathways between the normal and malignant cells were well exploited which
provided the insights on the behavior of cancer mechanisms and proliferation. Based on
this knowledge, new biologically targeted therapeutic agents have been developed which
specifically targets the gene expression and signaling pathways deregulated in the cancer
cells. Cancer targeted therapies that use small molecules, peptides and antibodies have
made therapy more specific to tumor sparing normal tissues [24].
Some targeted therapies block specific enzymes and growth factor receptors involved in cancer cell proliferation. Targeted agents are categorized based on the mechanism of their action and the cells they target. Most of the targeted agents are small molecule drugs or monoclonal antibodies (mAbs). These agents perform their action either by inhibiting enzymes (tyrosine kinase inhibitors, proteosome inhibitors, signal transduction inhibitors, growth factors inhibitors etc), by inducing apoptosis, or by inhibiting the angiogenesis pathways (Fig. 4). Presently, there are few hundred targeted cancer agents.
in market including hundreds of new small molecule drugs and mAbs. Some of the selected targeted therapies in advanced clinical development are shown in Table 2 below.

Table 2: FDA approved targeted therapies that interfere with variety of cellular processes.

<table>
<thead>
<tr>
<th>Biologically targeted agent</th>
<th>Target(s)</th>
<th>Indication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>VEGF</td>
<td>• Colorectal cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Glioblastoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• NSCLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Renal carcinoma</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>EGFR</td>
<td>• Squamous cell carcinoma of head and neck</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Colorectal cancer</td>
</tr>
<tr>
<td>Gefitinib (Iressa)</td>
<td>EGFR</td>
<td>• NSCLC</td>
</tr>
<tr>
<td>Imatinib (Gleevec)</td>
<td>Tyrosine Kinases</td>
<td>• GI stromal cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Systemic mastocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Dermatofibrosarcoma protuberans</td>
</tr>
<tr>
<td>Trastuzumab (Herceptin)</td>
<td>HER-2</td>
<td>• Breast adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Gastric adenocarcinoma</td>
</tr>
<tr>
<td>Sorafenib (Nexavar)</td>
<td>VEGF, Tyrosine Kinases</td>
<td>• Advanced renal cell cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Dasatinib (Sprycel)</td>
<td>Tyrosine Kinases (ABL)</td>
<td>• CML</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ALL</td>
</tr>
<tr>
<td>Lapatinib (Tykerb)</td>
<td>HER2, EGFR</td>
<td>• Breast carcinoma</td>
</tr>
</tbody>
</table>

2.4.4 Chemotherapy

Despite the success in the development of various therapeutic innovations, chemotherapy is still a preferred method of therapy for a wide range of cancer [23, 25, 26]. Chemotherapy uses chemical substances that act electively on cells that are going through
mitotic cycle. The objective of chemotherapy in cancer is the selection of cytotoxic drugs and dose levels which predominantly is used in the treatment of metastases and dissemination without causing severe host toxicity. For the majority of solid tumors, chemotherapy is used to reduce the tumor volume and palliate symptoms. Most of the cases, chemotherapy has been used as an adjuvant to surgical and radiotherapy which results in the eradication of the subclinical metastases and leads to the prolongation of survival reducing the risk of recurrence. Chemotherapy can either be palliative or curative depending on the primary location and the extension of the disease. Depending upon the therapeutic scheme, chemotherapy can use a single therapeutic agent or a combination of substances. Primary tumors are heterogenic in nature and consist of metastatic potential, growth rate, immunogenicity, antigenecity and intrinsic response to cytotoxic drugs [26]. During the evolution stage of heterogenic tumors, some of them have favorable response to the cytotoxic agents while others do not. Therefore, for successful chemotherapy the drugs selected should be active when they are used alone, have known mechanism of actions and should not have toxicity grade higher than admissible grade. The maximal therapeutic effect is obtained with moderate drug toxicity and monitoring of the effects of the drug in the patient. However, the effect of chemotherapy is not strictly focused on cells or neoplastic tissues but have toxic action on the majority of normal cells especially on regions where normal physiological replacement of non-tumor cells occurs at a rapid rate. This results in the non-specific toxicity of cytotoxic agents. Wide ranges of chemotherapeutic agents (Table 3) are used in cancer care depending on the type of cancer and the mode of action of the antineoplastic agents. Chemotherapeutic agents act on metabolism and the
pharmacological and toxicological effect of cytotoxic agent depends on the drug concentration in the specific site of action. Thus the main aim of local delivery of cytotoxic agent to the disease site is to increase the local concentration in the specific area and minimize the exposure to the surrounding normal healthy cells.

Table 3: Classification of chemotherapeutic agents used in cancer treatment

<table>
<thead>
<tr>
<th>Types of Agents</th>
<th>Target</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum Coordinates</td>
<td>• Binds with DNA</td>
<td>• Cisplatin, Carboplatin Oxaliplatin</td>
</tr>
<tr>
<td>Antimicrotubules Agents</td>
<td>• Binds with microtubules and stop mitosis</td>
<td>• Taxanes (Paclitaxel, Docetaxel)</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>• Interfere with DNA and RNA</td>
<td>• Vincaalkaloids (Vincristine, Vinblastine)</td>
</tr>
<tr>
<td>Antitumor Antibiotics</td>
<td>• DNA intercalation</td>
<td>• Methotrexate</td>
</tr>
<tr>
<td>Alkylating Agents</td>
<td>• Covalently bind to DNA</td>
<td>• Fluopyrimidines (5-Fluorouracil, Capecitabine)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Anthracyclines (Doxorubicin, Daunorubicin)</td>
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<tr>
<td></td>
<td></td>
<td>• Camptothecins (irinotecan, topotecan)</td>
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<tr>
<td></td>
<td></td>
<td>• Cyclophosphamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Nitrosoureas (Carmustine)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Alkane sulfonates (Busulfan)</td>
</tr>
</tbody>
</table>

2.4.4.1 Platinum Therapy
Platinum based compounds such as cisplatin, carboplatin and oxaliplatin are widely used in clinic for the treatment of various malignancies. Since the accidental discovery of the biological properties of Cisplatin (cis-diamminedichloroplatinum (II) (Fig. 5)) in 1965 by Rosenberg et al, the era of platinum based anticancer therapy was introduced in clinic. Currently, cisplatin is one of the most commonly used anticancer drugs as a single agent, or in combination for the treatment of wide spectrum of malignancies such as testicular,
ovarian, bladder, cervical, head and neck, small cell and non small cell lung cancers [27]. However, cisplatin lacks tumor selectivity and the cumulative toxicities of cisplatin such as nephrotoxicity, ototoxicity, neurotoxicity, peripheral neuropathy limit the adequate dose administration [27-29]. Long term or high dose therapy may lead to severe anemia. To circumvent these adverse effects of cisplatin, several second and third generations platinum based drugs have been synthesized over the past few decades. Several of these second and third generation drugs are currently in clinical trials, however only few of these drugs were approved for use in humans.

Carboplatin, (cis diammine-[1,1-cyclobutylidicarboxylato-(2)O,O’] platinum (II)) (Fig. 5), is a second generation analogue of cisplatin. Carboplatin differs from cisplatin by having a cyclobutylidicarboxylato, a didentate ligand as the leaving group in place of two chlorides in cisplatin. Carboplatin is less toxic than cisplatin and exhibits antitumor activities to same types of tumors as cisplatin. As a result, carboplatin has replaced cisplatin in various treatment regimes in clinic. Owing to its reduced side effects, carboplatin is well tolerated by patients and can be used at higher doses than cisplatin [29]. Hearing loss and peripheral neuropathy are less common with carboplatin treatment, and nephrotoxicity is less as compared to cisplatin. However, visual changes or loss of vision has been reported with high doses of carboplatin and myelosuppression, principally thrombocytopenia is a dose limit factor for carboplatin [29, 30].
The mode of action for cisplatin and carboplatin is similar. The principal biological target of platinum drugs is nuclear DNA. After the intravenous injection, cisplatin enters the cells via passive diffusion. In response to the lower chloride concentration inside the cytoplasm (20mM) as compared to the serum (100mM), cisplatin undergoes activation by the aquation of one of two of its chloride leaving groups to form cis-\([\text{Pt(NH}_3\text{)}_2\text{Cl(OH}_2\text{)}]^+\). This positively charged species is able to bind to the N7 position of purine bases, especially guanine to form either monofunctional or bifunctional adducts (Fig. 6) depending on the displacement of either one or two leaving groups \([29, 31, 32]\). Predominant binding occurs between two adjacent guanine based, forming 1.2 intrastnad cross links. These adduct cause distortions in the DNA double helix, and blocks replication and transcription of DNA helix. These distortions are eventually recognized by a number of cellular proteins that will ultimately trigger apoptotic cell death.

Aquated species of carboplatin and cisplatin are same. However, the rate of aquation of carboplatin in phosphate buffer with a pH of 7 and at 37°C is 100 folds slower than that for cisplatin \([33]\).
Figure 6: Cisplatin adduct formation and binding with DNA. The adducts formed by cisplatin and carboplatin are same.

Oxaliplatin, cis-[(1R,2R)-1,2-Cyclohexane1,2-diamine]-[ethanedioato-O,O'] platinum(II) (Fig. 5) is another FDA approved drug which is known to treat cisplatin resistant tumors. Structurally, oxaliplatin differs from cisplatin by replacing two ammine ligands with (1R,2R)-cyclohexane-1,2-diamine (R,R-dach). Due to this structural difference, the adducts formed by oxaliplatin differs from the DNA adducts formed by cisplatin and
carboplatin. The drug mainly forms GpG intrastand adducts with bulky hydrophobic dach ligand pointing into DNA major growe and preventing the binding of DNA [34].

Oxaliplatin is widely used in clinic for metastatic colon cancer as a single agent or as a combination therapy with 5-FU and folinic acid.

The two other platinum based drugs that have been approved were nedaplatin and loboplatin. Nedaplatin, Diamine[hydroxyacetato 92-]O-O’] platinum(II), is a second generation cisplatin analogue which is approved for the treatment of non-small cell lung cancer, esophageal cancer and head and neck cancers in Japan. Nedaplatin is more water soluble that cisplatin and possess similar anticancer properties as of cisplatin with lesser side effects [29].

Loboplatin, ([2-Hydroxypropanoato(2-)-O1,O2][1,2-cyclobutanedimethanamine-N-N’]-platinum(II), has been approved for the treatment of chronic myelogenous leukemia, inoperable metastatic breast cancer and small cell lung cancer in China. Thrombocytopenia is the most commonly observed dose limiting toxicity associated with loboplatin.

2.4.4.2 Development of Platinum Analogues
Motivation for the development of new platinum analogues prompted from the need to enhance the therapeutic index of the platinum drugs with reduced side effects and to increase their effectiveness against various platinum resistant tumors. Success of cisplatin triggered the intensive work on the discovery of new platinum based anticancer drugs. Cisplatin, Carboplatin, and Oxaliplatin are the first line platinum regimen for the treatment of various solid tumors. However, various other platinum analogues have been developed and tested in vivo for various solid tumors and platinum resistant tumors.
Orally administered JM216 (Satraplatin), sterically hindered ZD0473 (Picoplatin), ProLindac are three platinum analogues that are undergoing clinical trials in patients with refractory prostate cancer, small cell lung cancer respectively. Satraplatin is an orally active platinum drug that has shown antitumor activity against human lung, ovary, cervix, prostate and several platinum resistant tumors. Satraplatin is currently undergoing Phase I/II/III clinical trials in conjunction with various other anticancer drugs; docetaxel in the treatment of prostate cancer, paclitaxel in the treatment of NSCLC [35]. Picoplatin (cis-Amminedichlorido(2-methylpyridine)platinum(II)) is a sterically hindered platinum analogue that has a pyridine ring perpendicular to the plane of platinum atom. In vitro study demonstrated the antitumor activity of picoplatin in cisplatin and carboplatin resistant tumor. Currently, picoplatin is in Phase I/II studies for colorectal cancer in combination with 5-FU and leucovorin, in conjunction with docetaxel for prostate carcinoma [36]. ProLindac is a nanopolymeric formulation of platinum drug, where the active moiety of oxaliplatin is bound to the hydrophilic biocompatible polymer (hydroxypropylmethacrylamide,HPMA) to improve the uptake through EPR effect. Treatment with ProLindac showed superior tumor growth inhibition with reduced side effect was observed in mice with B16 melanomas and ovarian carcinomas compared to oxaliplatin [36]. ProLindac in combination study with paclitaxel is currently in Phase II clinical trail in advanced ovarian carcinomas. Several other platinum analogues had undergone clinical trails but discontinued because of the lack of antitumor activity and because of their toxicity. The development of new and better platinum drugs is still a demanding area of research. Nanotechnology opens a plethora of opportunities in developing drug delivery vehicles for the delivery of platinum drugs that will alter the
biodistribution and pharmacokinetics of the drug and reduce the side effects, and enhance the therapeutic efficacy. Several nanoparticle formulations of platinum drugs are currently in clinical trials. A key approach in cancer therapy research is to develop a drug delivery method that selectively delivers the drug to tumor site while reducing the toxicity to normal organs. Formulations of anticancer drugs with liposome have demonstrated significant reduction in side effect and altered pharmacokinetic profile that provides a unique opportunity to facilitate drug delivery to targeted sites. Therefore, this project utilizes liposomal drug delivery technology to engineer novel platinum drug formulation.

2.4.4.3 Multidrug Resistance to Platinum
Resistance to chemotherapy is a problematic aspect in the molecular biology of tumors and one of the most significant barriers in improving the effectiveness of cancer treatment [37]. Multiple drug resistance (MDR), both intrinsic and acquired, is a serious problem in solid cancers. There are several mechanisms by which cancer cells may express intrinsic resistance to platinum, such as, (i) impaired drug delivery, (ii) extracellular factors, (iii) decreased drug uptake into tumor cells, (iv) increased drug efflux, (v) drug inactivation by detoxifying factors, (vi) decreased drug activation or binding to target, (vii) altered target, (viii) increased damage repair, (ix) tolerance of damage (x) decreased proapoptotic factors, (xi) increased antiapoptotic factors, (xii) altered cell cycling and (xiii) altered transcription factors [38]. Moreover, different tumors in the same individual or even different cells within the same tumor may exhibit chemoresistance heterogeneity. The tumor cell interactions with host factors in the
humans constitute unique tumor microenvironment, which may contribute to chemoresistance [38].

Resistance to platinum agents occur through various mechanisms including detoxification of platinum agents by glutathione or other anti-oxidants, decreased intracellular accumulation, increased DNA repair or DNA tolerance [38-41]. Platinum drugs enter the cells via passive diffusion down the concentration gradient. The facilitated transport of platinum drugs is enhanced by copper transporter-1 (CTR1) protein [39, 42-44]. Platinum resistant may be associated with the decreased CTR1 expression [45] in various cancers including lung cancer. Upon entering the cells, platinating drugs become aquated which interacts with DNA resulting cross-linking and inducing apoptotic cell death. Metallothiones and glutathione related metabolic enzymes such as glutathione transferase (GT) have a tendency to bind with the aquated platinum complexes and detoxify the platinum agents (Fig. 7).

![Diagram of the mechanism by which tumor resistance to cisplatin and carboplatin occurs.](image)

**Figure 7**: The mechanism by which tumor resistance to cisplatin and carboplatin occurs. Platinum drugs enter the cell through diffusion or by transporter, major being the copper transporters (CTR1). After the aquation of the platinum drugs, the activated aqua species preferentially reacts with species containing glutathiones of metallothioneins, mopping up the aquated species before DNA binding could occur (adapted and modified from reference³⁸).
Elevated level of these sulfur containing compounds has been associated with resistance in lung cancer [38, 39, 42, 45]. ATP-binding cassette (ABC) super families of transport family proteins are associated with resistance in various cancers. The higher expression of copper transporter protein ATP7B is implicated to the chemoresistance of cisplatin in lung cancer [38, 41]. Breast cancer resistance protein (BCRP), a transporter and a member of ABC) was expressed significantly higher in chemo resistant patient treated for advanced non-small cell lung cancer NSCLC [41]. Higher expression of excision repair cross complementation group 1 (ERCC1), a form of DNA repair enzyme, has been shown to have chemoresistance effect in NSCLC [40, 45]. Subfamily C of ABC transporters includes multidrug resistance protein (MRP1-3) [46]. Higher expression of MRP is attributed to platinum resistance in small cell lung cancer (SCLC), NSCLC and other solid tumors [41, 47, 48]. However, there are several studies that are contradictory to the above and have failed to identify clear markers for chemoresistance in lung cancer. In an in vitro study, mRNA and protein expression levels of P-glycoprotein (MDR1), multidrug resistance-associated protein 1 (MRP1), and lung resistance-related protein (LRP) were investigated and compared with the chemosensitivity and the intracellular/intranuclear cisplatin accumulation of patient derived NSCLC cell lines (Ma-10, Ma-31, and Ma-46). The results of that study indicate that MDR proteins (MDR1, MRP1, and LRP) may not play an important role in the chemoresistance and drug efflux of platinum drugs in tumor cells [49]. Despite the multifactorial nature of platinum resistance (Fig. 7), reduced intracellular drug accumulation is one of the most consistently identified features of cisplatin-resistant
cell lines [50, 51]. Furthermore, a recent clinical study established the relationship between tissue platinum concentration and response in NSCLC by analyzing tumor tissues excised from patients who received neoadjuvant platinum-based chemotherapy without radiation followed by curative surgical resection [52]. Earlier in 1988, platinum concentrations were determined in autopsy samples and found that the tumors of patients that responded to Cisplatin had higher mean tumor platinum concentrations than the tumors of patients that did not respond to Cisplatin [53]. These studies suggest that reduced platinum accumulation might be an important mechanism of platinum resistance and the tumor response to platinum based treatment appears to be directly related to drug concentrations in the tumor tissue in the clinical setting. However, there is no non-invasive means of determining the concentration of platinum in normal tissues and tumors in patients. Concentration of platinum in tumors in patients is usually determined by adducts level in tumor biopsies using ICP. This invasive method of determining the platinum concentration is not feasible in cancer patients, especially when the tumor has metastasized. Therefore, this project aims in developing $^{18}\text{F}$-FCP that will enable a quantitative non-invasive imaging using PET that will ultimately provide a feasible way of determining the concentration of platinum in tissue basis.
References

CHAPTER 3: MOLECULAR IMAGING IN CANCER THERAPY
3.1 Molecular Imaging in Cancer Therapy

Molecular imaging is an emerging medical discipline that can be defined as the fusion of visual representation, characterization, and quantification of biological processes at the cellular and sub-cellular levels within intact living organisms. This novel multidisciplinary field integrates cellular and molecular biology, medical physics, bioinformatics, biomathematics with diagnostic imaging techniques to produce a new imaging paradigm. Molecular imaging provides a platform that reflects cellular and molecular pathways and \textit{in vivo} mechanisms of disease present within the context of physiologically authentic environments through the images produced using latest technological advancements. In the era of molecular medicine, the information on the complex biology of disease progression, drug metabolism and working therapies is crucial. Molecular imaging plays a pivotal role in understanding disease, in terms of biomarker diagnostics, early detection of disease, individualized treatment and potential prevention within the intact living subject. Molecular imaging detects not only the presence of the disease process, but can potentially also quantify its extent and severity, as well follow the course of disease over time [1]. Molecular imaging allows clinicians to detect the tumor location as well as to visualize the expression and activity of specific molecules and processes that influences the tumor behavior [2]. Traditionally, changes in tumor volume a general way of monitoring treatment response. However, singular measure of treatment response is not sufficient to predict the overall survival of the patient. Critical molecular features of neoplasm and its underlying pathophysiological mechanisms such as angiogenesis, metabolism, proliferation, and apoptosis can be
studied in details using molecular imaging probes that target those specific pathways (Fig. 8)

Figure 8: Molecular imaging probes and their cellular targets (reproduced from reference 3)

Molecular imaging has the capacity to go beyond macroscopic imaging to noninvasively characterize biological process at the cellular and sub cellular level. Depending on the molecular tumor specificity, suitable molecular imaging modalities can be employed to study the biology. While several imaging modalities are employed to study physiologic and disease processes, response to therapy; radionuclide imaging stands out as the most commonly utilized imaging modality to exploit the molecular details.
3.2 Radiotracer Imaging

Imaging with radioisotopes allows obtaining the functional information unlike x-ray computed tomography (CT) which only provides the anatomical information. Radiotracer imaging employs radiotracers/radioligands that have the ability to interact with molecular targets involved in cancer. The development of imaging modalities such as PET and single photon emission tomography SPECT allows the in vivo visualization of biological processes at the molecular and cellular level. Both of these imaging modalities involve the use of exogenous radioactive nuclides injected intravenously to obtain an image of the distribution within the body after administration. This project focuses on SPECT and PET which are further detailed in the sections below.

3.2.1 Single Photon Emission Tomography

Single photon emission computed tomography, commonly known as SPECT is an imaging technique in nuclear medicine. It is very similar to the conventional planar imaging using a gamma camera. The fundamental basis for SPECT imaging is the detection of monoenergetically emitted γ-rays from intravenously injected exogenous SPECT radionuclides (Table 4).

Table 4: Commonly used single photon emitting radionuclides

<table>
<thead>
<tr>
<th>Nuclides</th>
<th>Half Life</th>
<th>Emission Energy</th>
<th>Mode of Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In</td>
<td>2.80 days</td>
<td>171, 245 keV</td>
<td>EC (100%)</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>6.02 h</td>
<td>142 keV</td>
<td>IT (100%)</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13.22h</td>
<td>159 keV</td>
<td>EC (100%)</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8.03 days</td>
<td>364 keV</td>
<td>$\beta^-$ (100%)</td>
</tr>
</tbody>
</table>

EC: Electron Conversion, IT: isomeric transition, $\beta^-$: beta-minus

Basically, when a SPECT radioisotope decays, one or more $\gamma$-rays having particular energies are emitted in random directions from within the subject after the radiotracer injection. These $\gamma$-rays pass through a multichannel lead collimator (parallel hole or pin hole in order to give access to give the best spatial resolution/sensitivity/FOV) which restricts the view of the detector to those events originating from the area to be imaged. Optimal window is selected based on the direction of the photons reducing the detection of scattered photons. The selected photons traveling along single line of response corresponding to the individual aperture in the collimators are detected by sodium iodide (NaI) crystal. The crystal after the detection of the photons emits light which is amplified by photomultiplier tubes and is recorded. The gamma detector in SPECT rotates around the subject to detect the emissions. 2D images would be acquired by rotating the gamma camera at different angles. The acquired 2D image set is converted to 3D image data using tomographic reconstruction algorithm (Fig. 9).

![Schematic representation of SPECT System](image)

Figure 9: Schematic representation of SPECT System

SPECT is a rapidly changing field and various areas of research in the development of new hardware and image processing algorithms is being carried out. The use of longer
lived half life radionuclide and lower cot of gamma cameras make SPECT imaging widely available for pre clinical and clinical use. SPECT benefits from longer half lives radioisotopes that enable imaging studies to be conducted over longer period of time. Recent advancements on nanotechnology further advanced the field of molecular imaging with radiolabeled nanoparticles. Extrinsically or intrinsically radiolabeled nanoparticles with targeting moieties can interrogate specific molecular and cellular events in living organisms. $^{111}$In-Quantum dots, $^{125}$I labeled single walled carbon nanotubes, $^{111}$In labeled monoclonal antibodies linked iron oxide nanoparticles are some of the examples of nanoparticles used for SPECT imaging in pre clinical animal models [4]. The major advantage of SPECT is superior spatial resolution and it can potentially allow for simultaneous imaging of multiple radionuclides. However, for a given size of detector and FOV, SPECT suffers from tradeoff between resolution and sensitivity [5, 6]. Recent advances on the development of cadmium zinc telluride (CdZnT) direct conversion detectors allow higher intrinsic resolution and effectively eliminates the dead space at the edges of detectors [5, 7, 8]. Recent advances in iterative reconstruction algorithms provide the flexibility and versatility to SPECT imaging.

3.2.2 Positron Emission Tomography
Positron Emission Tomography, commonly known as PET imaging is a widely used molecular imaging modality which provides 3D image of functional processes in the body. PET scanner consists of full detector ring with hundreds of coincidence coupled detectors. After the administration of positron emitting radionuclide (Table 5), there would be a tracer uptake by different organs.
Table 5: Commonly used positron emitting radionuclides

<table>
<thead>
<tr>
<th>Nuclides</th>
<th>Half Life</th>
<th>Max. Energy/MeV</th>
<th>Mode of Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}$F</td>
<td>110 min</td>
<td>0.64</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>20.3 min</td>
<td>0.97</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>$^{13}$N</td>
<td>10 min</td>
<td>1.20</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>$^{15}$O</td>
<td>2 min</td>
<td>1.74</td>
<td>$\beta^+$</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>12.7 h</td>
<td>0.66</td>
<td>$\beta^+/EC$</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>68.1 min</td>
<td>1.90</td>
<td>$\beta^+/EC$</td>
</tr>
<tr>
<td>$^{76}$Br</td>
<td>16.2 h</td>
<td>4.00</td>
<td>$\beta^+/EC$</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>4.18 days</td>
<td>2.14</td>
<td>$\beta^+/EC$</td>
</tr>
</tbody>
</table>

$\beta^+$: beta plus; EC: Electron Conversion

The tracer decay naturally producing a positron (positive electron) which travels a certain distance (depending on its energy) and interacts with a free or loosely bound negative electron. This annihilation of one positive and one negative electron with each other results in their masses being converted into two photons, consisting of 511 keV energy being given off at approximately 180º to each other (Fig 10). After the detection of one photon by a detector, a small amount of light is produced that is amplified as an electrical signal and is detected as a single count. After this detection, the system waits for another single count to occur in another detector. If these two single counts occur in the prescribed time interval, these photons are paired together and stored as a coincident event (true detection, Fig. 10). This detected coincident event signifies that annihilation occurred along the line connecting the two detectors referred to as line of response (LOR). The integrals of annihilations event is measured along the LOR and is stored in a 2D
matrix called sonogram. Tomographic reconstruction methods such as filtered back projection (FBP) and ordered subsets expectation maximization (OSEM) are employed to reconstruct images from sonogram data [9].

Figure 10: Principle of PET imaging: positron emitting radionuclide yields a positron that travels a short distance and annihilates with an electron and produce two collinear 511 keV photons. These photons exit the body and are detected by an array of scintillation crystals

However, there is a probability of scatter or attenuation of the annihilated gamma rays before exiting the body because of their interaction with the tissues. If one or both of the γ-rays undergoes compton scattering before reaching the detector, the direction of the photon is likely to change and the scanner could incorrectly position the resulting coincidence event to wrong LOR (Fig. 11). Scattered coincidence event adds statistical noise to the signal. Random coincidence events (Fig. 11) are caused by the incorrect pairing of the two photons arising from different annihilations within the coincidence window of the detectors. Likely caused by the attenuation of one of the photons, or lacking of detection of photon by the system, random coincidence adds noise to PET image [6].
Figure 11: Depiction of the non-colinearity of the annihilation photons; scattered coincidence occurs when one or both of the photons are scattered but are still detected in the same energy and coincidence window in the detector (A); random coincidence occurs when two photons arising from two different annihilation events are detected in the same coincidence window (B).

Owing to the high resolution, high sensitivity instrumentation for γ-ray detection and unlimited tissue penetration, interest in in vivo imaging using PET is growing. Positron emitting radiotracers are primarily used to tag small molecules that are recognized by enzymes or bind to receptors and other targets [10]. As a result, numerous PET radiotracers have been developed for a wide range of applications (Table 6).

Table 6: Commonly used PET radiopharmaceuticals (adapted from reference 18)

<table>
<thead>
<tr>
<th>PET Probe</th>
<th>Application</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>[F-18] fluorodeoxyglucose</td>
<td>Glucose Metabolism</td>
<td>Accumulation as sugar phosphate</td>
</tr>
<tr>
<td>[F-18] fluoride</td>
<td>Bone Scanning</td>
<td>Incorporation into bone</td>
</tr>
<tr>
<td>[F-18] fluoromisonidazole</td>
<td>Hypoxia</td>
<td>Redox indicator</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>[F-18] fluorothymidine</td>
<td>Proliferation</td>
<td>Incorporation into DNA</td>
</tr>
<tr>
<td>[C-11] acetate</td>
<td>Fatty acid oxidation</td>
<td>Uptake and clearance by fatty acid</td>
</tr>
<tr>
<td>[C-11] choline</td>
<td>Protein metabolism</td>
<td>Incorporation into phospholipids</td>
</tr>
</tbody>
</table>

### 3.3 Molecular Imaging in Image Guided Therapy

Morphological imaging with an assessment of the biological function and metabolism is an invaluable tool for assessing the overall success of the treatment. Image guided therapy uses imaging techniques to aid in providing localization and targeting of diseased tissue, visualizing the extent of malignancies and particularly helps in specificity and identifying cancer. Image guided surgeries and minimally invasive interventions emerged over the past few decades replaced the traditional invasive approaches and helped in monitoring and controlling treatments. In modern medicine, nuclear medicine imaging techniques such as PET/SPECT, advanced imaging technologies such as MRI, CT entered into interventional and surgery suites to complement on the already available imaging techniques such as X-ray and ultrasound. Molecular imaging guided surgeries; MRI guided focused ultrasound surgeries, recently developed intraoperative imaging techniques substantially increased the versatility of modern interventional system and accelerated the process of image guided therapy. Integrated PET-MR promised a new avenue in radiation oncology for dose planning and therapy owing to the properties like superior resolution, soft tissue contrast and multi-planar capabilities. An ongoing clinical trail is using [18F]-FMISO dynamic PET-CT and functional MRI for imaging tumor hypoxia in locally advanced unresectable non-small cell lung carcinoma [1]. Further
work on using CT-MR on verifying the delivery of planned dose to the clinical target volume in prostate cancer, assessing the dose to the organs at risk are some of the connections between MR diagnostics and dose planning system in radiotherapy which highlights the use of molecular imaging in image guided therapy [11].

3.3.1 Molecular Imaging in Staging and Tumor Expression

Molecular imaging can improve all aspects of clinical cancer care [12]. Application of PET-CT in oncology has substantially influenced the management of cancer patients. PET-CT has the ability to generate morphological and anatomical information in a single examination that will provide an efficient and accurate approach on tumor staging. FDG-PET imaging takes advantage of alterations in glucose metabolism, which is a hallmark of cancer. Prospective studies have shown the higher sensitivity and specificity of this imaging principle [12]. Therefore, FDG-PET has high accuracy for staging of various solid tumors and is intensively used in clinic widely. Several new PET tracers have emerged that can improve the staging of cancer using PET. $^{11}$C-choline PET has very high specificity and sensitivity for staging and restaging of prostate cancer. Many studies have evaluated whole body-diffusion weighted imaging (WB-DWI) as a potential alternative for the detection of metastatic lesion. Tumor metabolism study using conventional MRI/MRS may serve as a predictive marker for prostate cancer.

Molecular PET imaging plays a pivotal role in identification and quantification of specific targets and receptor expression in vivo. Various new imaging probes employed in nuclear imaging have proven to be useful in detecting the presence of various targets due to the consequence of drug target interaction. $^{11}$C-raclopride for dopamine D2 receptors and $^{125}$I-SD7015 for cannabinoid receptor are two of the many examples of imaging drug
probe used to obtain qualitative and quantitative data in the tumors of central nervous system[13]. $^{64}$Cu-VEGF in PET imaging is another approach of an imaging probe that directly interacts with the target. Vascular endothelial growth factor receptor (VEGFR) is associated with tumor angiogenesis. $^{64}$Cu-VEGF directly binds to VEGFR and PET imaging of $^{64}$Cu-VEGF helps in assessing the tumor target expression. However, synthesizing a new customized imaging probe needs individual characterization for specificity and sensitivity and is costly and time consuming. Reporter gene imaging provides an alternative to direct target imaging. Several approaches have been emerged to encode reporter gene with specific enzymes or for receptor capable of binding the radioactive ligand. Using molecular imaging techniques such as MRI/ MRS, optical or radionuclide imaging, reporter genes help in the evaluation of level of expression of specific genes, receptor activities and protein-protein interactions [14].

3.3.2 Tumor Metabolism Imaging
High specific and sensitive imaging of increased metabolic rates for glucose, amino acids, or lipids associated with malignant tissues have led to a remarkable success of tumor metabolism imaging in recent years. PET imaging with glucose analog, $[^{18}F]$-FDG, is the most commonly used imaging technique to study glucose metabolism of cancer cells in vivo. After intravenous injection, $[^{18}F]$-FDG is recognized and is transported across the cell membrane by sodium independent glucose transporters mainly Glut-1, Glut-3 which is phosphorylated by hexokinase to form $[^{18}F]$-FDG-6-phosphate. FDG-6-phosphate is not a substrate for glycolysis and cannot go further glycolytic pathway because of the fluorine atom at the C2 position prevents $[^{18}F]$-FDG-6-phosphate from further degradation. As a result, the metabolite gets trapped within the tumor cells which is
exploited for tumor metabolism imaging by PET imaging [15]. PET imaging using $^{18}$F-FDG has been used as an indicator of therapy response. PET imaging of FDG takes advantage of one of the hallmark of cancer, namely the Warburg effect; increased glucose metabolism and conversion of glucose carbon to lactate are the characteristics of cancer cells as compared to normal cells [12]. This characteristics of malignant cells is attributed to the overexpression and increased activity of glucose transporters in the cell membrane of malignant cells [16]. However, specificity of $^{18}$F-FDG still remains. After the therapeutic intervention, tumors usually show inflammatory responses leading to a transient increase in glucose metabolism. $^{18}$F-FDG also accumulates in non-cancer tissues (usually tissues with high metabolic activity such as inflammatory lesions, brain, heart) and the interpretation of the imaging signal may be confounded by metabolic flare phenomena [17].

Various other alterations such as changes in amino-acid transport, protein synthesis, including the overexpression of cholin kinase (ChoK) is observed in malignant tumors. Complementary information on tumor biology is further useful for the diagnosis and therapy monitoring of tumors. Non-selective uptake of $^{18}$F-FDG lead to efforts to establish amino acids based tracers for tumor metabolism imaging (Fig. 12). Tumor cells have increased amino acid transport. $^{11}$C-labeled and $^{18}$F labeled amino acids such as methionines, tyrosines, and methyltyrosines are widely used for accurate metabolic cancer imaging [17]. Tracers were mainly used in brain tumors in which they showed excellent contrast between normal brain and tumors and exhibited higher sensitivity in tumor detection. Therefore, amino acids have the probable advantage over FDG in the imaging of brain tumors since the background tracer accumulation is lower as compared
to FDG. Although amino acids have a potential in differentiating between inflammation and malignancy, various studies have suggested that uptake of amino acid might occur in benign regions such as ischemic brain, infarction, scar and in irradiated studies [15]. Therefore, monitoring of treatment response of tumor using amino acid must be validated with further studies.

Phosphocholine is the most abundant phospholipid found in the cell membrane. Elevated level of phosphocholine have been detected by various spectroscopic studies in variety of tumors, including breast, prostate and brain tumors [18]. Imaging approach based on the increased need of cancer cells for the cell membrane component phosphatidylcholine, increased transport and phosphorylation of choline lead to synthesis and pre clinical and clinical evaluation of choline based tracers. Radiolabeled choline and choline analogs (\(^{11}\text{C}\)-choline, \(^{18}\text{F}\)-choline, \(^{18}\text{F}\)-fluoroethylcholine) have been used to study tumor lipid metabolism by PET and have shown rapid accumulation in variety of human cancers including prostate, gliomas, non small-cell lung cancer and esophageal cancer [19]. Recent studies have also shown the avid uptake of \(^{11}\text{C}\)-acetate in various malignancies. Studies on the uptake of \(^{11}\text{C}\)-acetate correlated the uptake with the fatty acid synthase expression levels in cancer cells and attributed the observation to the anabolic pathway which uses acetate for synthesis of fatty acids and lipids [19].
3.3.3 Tumor Proliferation Imaging

Abnormal cellular proliferation is a hallmark of cancer. Imaging and measuring proliferation is a potentially broad and specific target for the assessment of tumor progression and response to therapy. DNA synthesis is the most directly related metabolic process to cell division. Most of the non-invasive methods of imaging the cell proliferation have focused on the thymidine (TdR) salvage pathway of DNA synthesis because TdR contains the only pyrimidine or purine base that is unique to DNA [20]. $^{11}$C-thymidine, which was used for detecting tumors and for the assessment of therapy, was limited by the short half life and rapid catabolism of TdR upon injection. Several
analogs of TdR have been radiolabeled with isotopes for imaging tumor proliferation. However, the most studied TdR analogs till this point are $^{18}$F-labeled-3′-deoxyfluorothymidine ($[^{18}$F]-FLT) and $^{18}$F-1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) thymine ($[^{18}$F]-FMAU).

Insight on the pharmacokinetics of $[^{18}$F]-FLT indicates the prospective assessment of disease response and identification of rapidly proliferating tumor cells. $[^{18}$F]-FLT PET provides the data on the activity of thymidine kinase (TK) activity. After injection, $[^{18}$F]-FLT enters the cell and is phosphorylated by thymidine kinase 1(TK1) to $[^{18}$F]-FLT-monophosphate [21]. Additional kinases act on $[^{18}$F]-FLT-monophosphate and convert $[^{18}$F]-FLT-monophosphate to diphosphate and triphosphate (Fig. 13). Replacement of hydroxyl group at the 5′ position (Fig. 13) of deoxyribose prevents $[^{18}$F]-FLT-triphosphate from cleavage by thymidine phosphorylase and from incorporating into DNA therefore making it as a substrate for TK1. This phosphorylation makes $[^{18}$F]-FLT too polar to exit the cell via membrane and hence gets trapped in the cell. Since, TK1 (cytosol) is a key enzyme in the DNA synthesis through the salvage pathway in cell, the degree of accumulation of $[^{18}$F]-FLT behaves as a biomarker for proliferation.
Figure 13: Thymidine salvage and de novo synthesis pathway. $[^{18}\text{F}]-\text{FLT}$ is transported into the cells and phosphorylated to $[^{18}\text{F}]-\text{FLT}$ monophosphate ($[^{18}\text{F}]-\text{FLTMP}$) which can be further phosphorylated to diphosphate and triphosphate. However, due to the substitution of OH with $^{18}\text{F}$ in 5-position, $[^{18}\text{F}]-\text{FLT}$ is not incorporated into the DNA. (adapted and modified from reference 14)

$[^{18}\text{F}]-\text{FMAU}$ has been developed for proliferation imaging with labeling at 2'-fluoro position of the sugar with $^{18}\text{F}$ (Fig. 14). Preliminary clinical studies have shown that the accumulation of $[^{18}\text{F}]-\text{FMAU}$ in tumors comparable to that seen in human studies with $[^{18}\text{F}]-\text{FLT}$ [20]. $[^{18}\text{F}]-\text{FMAU}$, having a 3'-hydroxyl group acts as a substrate for thymidine kinase 2 (TK2) in mitochondria and gets incorporated into DNA. However, the primary limitation of $[^{18}\text{F}]-\text{FMAU}$ is that it appears to be the poor substrate for cytosolic TK1. Cytosolic TK1 is dependent on cell cycle with higher expression during S, G2, M phases in dividing cells. Mitochondrial TK2 expression is independent of cell cycle.
Therefore, the visualization of TK2 activity using $^{18}$F-FMAU with PET imaging may be less accurate index of cell proliferation [22]. $[^{18}\text{F}]-\text{FLT}$ PET provides data on TK1 activity and $[^{18}\text{F}]-\text{FMAU}$ provides data on TK2 activity. TK1 and TK2 are independent enzymes and the information received with changes in $[^{18}\text{F}]-\text{FLT}$ and $[^{18}\text{F}]-\text{FMAU}$ respectively provides different biological information on cell proliferation and mitochondrial mass in tissue. Therapeutic regimen using cytotoxic agent changed the TK activity. Hence, the biological effect of therapy on $[^{18}\text{F}]-\text{FLT}$ and $[^{18}\text{F}]-\text{FMAU}$ accumulation and its implication must be validated further with scientific discussion [22].

![Chemical Structure](image)

Figure 14: Chemical Structure of (A) $[^{18}\text{F}]-\text{FLT}$; (B) $[^{18}\text{F}]-\text{FMAU}$

### 3.3.4 Cell Death Imaging

Cell death imaging plays an important role in detecting the onset of cell death in tumor. Cell death is an essential biological and pathological process in an organism that is critical for the correct formation of organs and tissue development. Cell death is hallmark of various pathological conditions including cancer. Imaging that can locate and identify cell death will greatly increase the understanding of cell death and its implications on diseases and therapeutic responses. Currently, there is no probe that has
been approved for routine cell death imaging in clinic. Various cell death biomarkers and probes have been developed exploiting various cellular events (Fig. 15) and tested in vivo. Few of the imaging probes reached clinical trials but failed. The major challenges in developing a cell death imaging agent include the choice of a relevant molecular target, the accurate detection of the temporal and spatial occurrence of cell death, optimization of probe pharmacokinetics, and the minimization of non specific tissue binding and maximization of tissue contrast [23].

Figure 15: Cellular events that have been exploited for imaging cell death (adapted and modified from reference 16)

Annexin-V (AnxV) is the most widely used probe for phosphatidylserine-based detection of apoptosis that has reached phase III clinical trial. AnxV forms trimer-based lattices on
the surface of apoptic cells, inducing strong cooperative phosphatidylserine binding and driving cellular internalization. Despite its early promise for detecting apoptosis, AnxV showed suboptimal pharmacokinetic profile and non specific binding to tissues[24]. Synaptotagmin-I (SynI) is a synaptic-vesicle associated membrane protein that contains C2A and C2B domain and is responsible for Ca$^{2+}$ dependent binding to phosphatidylserine and phosphatidylinositol. Approaches on radiolabeling the C2A domain with $^{99m}$Tc or $^{18}$F for visualizing cell death in animal model resulted in heterogenous probe mixtures. Recent studies on fluorescently labeling that C2Am domain had better specificity for detection of apoptosis and necrosis [25]. $^{18}$F-ML-10, a PET based tracer was introduced as small molecule agent capable of detecting the plasma membrane depolarization and acidification of the apoptotic cell [24, 26]. Assessment of correlation of early changes after radiation therapy using 18F-ML-10 in various solid tumors is currently taking place [23]. Uptake of Caspase-3/-7specific PET radiotracer $^{18}$F-ICMT-11 ($^{18}$F-(S)-(1-(2-fluoroethyl)-1H-[1,2,3]-triazol-4-yl)methyl)-5-(2,4-difluorophenoxy)methyl)- pyrrolidine-1-sulfonyl)isatin) after drug treatment was almost double, predicting the early detection of drug induced tumor apoptosis in xenograft models of lymphoma [27]. Structurally well defined probe development and its clinical translation for simultaneous imaging of cell death will help clinicians diagnose onset of diseases and tailor individualized treatment options.


CHAPTER 4: NANOPARTICLE DELIVERY OF ANTI-CANCER DRUGS
4.1 Drug Delivery Systems

The effectiveness of anticancer therapy depends on adequate delivery of therapeutic agents and enhanced retention in solid tumors. Conventional chemotherapy drugs suffer from lack of selectivity hence they cause significant damage to the normal proliferating cells of tissues and organs. In addition, some mainstream drugs also suffer from limited aqueous solubility and require solvent formulations that contribute to toxicity (e.g. paclitaxel formulation in Cremophor oil). The poor efficacy is further exacerbated by increased drug efflux due to activation of transmembrane protein efflux pumps, such as Pgp, MRP, BCRP etc. and detoxification by glutathione; resulting in less drug retention, and ultimately leading to drug resistance. Tools to address some of these problems require rationale approaches to quantitatively measure tumor and normal tissue concentrations of systemically administered drugs. Advancements in the development of nanoscale drug delivery vehicles, with distinct physical and biochemical properties for delivery applications, have the potential to improve the therapeutic index of currently available drugs by increasing the drug efficacy. Examples of these nano-carriers include nanoparticles, such as polymers, inorganic molecules, microbubbles, micelles, dendrimers, and liposomes. Platinum drugs are a major class of anticancer drugs in clinic. However the treatment efficacy of platinum drugs is hampered by multidrug resistance and increased normal tissue toxicity. This thesis project exploits the field of nanotechnology and introduces radiolabeled liposome ([111In]-Labeled Liposomes) as a drug delivery vehicle for novel fluorinated carboplatin derivative to study the pharmacokinetics and efficacy of liposomal platinum.
4.2 Nanoparticles in Drug Delivery

Colloidal particles with size ranging between 1 to 100 nm are known as nanoparticles [1, 2]. Nanoparticles have emerged as a promising approach to overcome the delivery barriers in solid tumors and specificity limitations of conventional drugs. Various types of nanoparticles based on metals, lipids, polymers and biological nanoparticles have been investigated for drug delivery systems. Each of these nanoparticles has its own advantages and disadvantages depending on its application. A brief comparison of these nanoparticles on drug delivery application is shown in Table 7. Nanoparticles have the potential to reduce drug toxicity and achieve steady state therapeutic levels of drugs over an extended period of time. The small size of nanoparticles offers advantages like increased cellular uptake and accessibility to locations that are not available to larger particles [2]. Flexible surface chemistry of nanoparticles allows possible conjugation of ligands and targeting molecule forming potentially effective new chemical entities that ultimately improves drug solubility, circulation time, stability and biodistribution of particles in the body. The surface properties and size of nanoparticles plays an important role in the blood opsonization process and clearance kinetics. The small size of nanoparticles allows penetration of cell membrane, binding and stabilization of proteins and lysosomal escape after endocytosis [3]. Opsonization of hydrophobic particles occurs more quickly than that of hydrophilic particles. Increasing the hydrophilicity by modifying the nanoparticle surface with chains of hydrophilic and flexible polymers reduces surface adsorption of blood serum proteins and shield nanoparticles from opsonins reducing clearance by monocytes and macrophages. The ability to functionalize the nanoparticles with targeting moieties, imaging agents, and drug payloads enables the synthesis of multimodal complexes that may provide patients with improved treatment
specificity and highly sensitive imaging capabilities to monitor treatment progress and outcomes [4]. Conjugating targeting molecules to the surface of nanoparticles enables manipulation of biodistribution by exploiting difference in molecular interactions between normal and malignant cells [5]. Nanoparticle targeting can be done with active and passive mechanisms. Passive targeting mechanism, known as enhance permeability and retention (EPR), takes advantages of the leaky vasculature and poor lymphatic drainage of cancer tissues. Active targeting mechanism has targeting molecules conjugated to the surface of nanoparticles and takes advantage of molecular recognition interactions such as ligand-receptor and antibody-antigen [6, 7]. However, defining highly specific and nonimmunogenic targeting agents that are able to transport nanocarriers to target tissues still remains as one of the challenges in nanomedicine.

![Table 7: Nanoparticle systems for drug delivery applications](image)

<table>
<thead>
<tr>
<th>Types of nanoparticles</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Applications</th>
</tr>
</thead>
</table>
| Inorganic Nanoparticles | • Inert and biocompatible  
• Easy surface modification  
• Stable over broad range of temperature and pH | • Slow biodegradation  
• Repeated administration causes toxicity | • MR contrast agents  
• In vivo cancer detection  
• Thermal therapy  
• Targeted delivery |
| Eg silica based, cadmium based, metal based, ceramic based | | | |
| Polymeric Nanoparticles | • FDA approved  
• Biodegradable  
• Easy surface modification  
• Entrapment of polar and non polar drugs | • Polymer toxicity  
• Low entrapment load  
• Rapid release  
• Immunogenicity | • Targeted drug delivery  
• In vivo cancer diagnosis |
| Eg PLA, PLGA | | | |
| Microbubbles | • FDA approved  
• Controlled release  
• Targeted delivery  
• Ease of synthesis and modification | • Short blood half life  
• Gas core exhaled through lungs | • Targeted drug delivery  
• Contrast agents |
<table>
<thead>
<tr>
<th></th>
<th>Easy surface modification</th>
<th>In vitro toxicity</th>
<th>Targeted drug and gene delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrimers</td>
<td>Precise size control</td>
<td>Compatibility issues may cause hemolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High drug carriage due to branched structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelles</td>
<td>FDA approved</td>
<td>Low hydrophobic core</td>
<td>Targeted drug delivery</td>
</tr>
<tr>
<td></td>
<td>Entrapment of both polar and non polar drugs</td>
<td>Limited drug loading</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thermodynamically stable</td>
<td>Drug precipitation in physiological fluid</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>FDA approved</td>
<td>Short shelf half life</td>
<td>Gene delivery</td>
</tr>
<tr>
<td></td>
<td>Biodegradable, non toxic, non antigenic</td>
<td>Inflammation</td>
<td>Controlled drug delivery</td>
</tr>
<tr>
<td></td>
<td>Surface amenable for modification</td>
<td></td>
<td>Targeted drug delivery</td>
</tr>
<tr>
<td></td>
<td>Low systemic toxicity</td>
<td></td>
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<tr>
<td></td>
<td>Prolonged half life</td>
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<td></td>
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<tr>
<td></td>
<td>Entrapment of both hydrophilic and hydrophobic drugs</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Controlled drug release</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3 **Liposomes in Drug Delivery**

Liposomes are spherical self closed structures composed of phospholipids that form multilamellar, concentric bilayer vesicles, with lipid layers separated by layers of aqueous media [1, 8-10]. The chemical composition, structure and colloidal size of liposome can be controlled by preparation methods that will be useful in various applications. Controlling the reaction conditions, liposomes can be manufactured with a different diameter ranging from several nanometers to micrometers. They benefit from spherical membrane, surface characteristics which may be charged or uncharged based on the selection of phospholipids. Amphiphilic characteristics of liposomes allow them to
solubilize a wide range of compounds. Owing to its amphiphilic characters, liposomes offer several advantages as a drug delivery vehicle. Liposomes can be used to load both hydrophobic and hydrophilic drugs. Therefore, liposomal drug delivery system is widely used in delivering anti-cancer agents. Liposome are the first therapeutic nanomedicine to reach commercialization with the FDA approval of Doxil in 1995 [11]. The success of liposomes as a drug delivery vehicle can be attributed by its successful translation to clinic (Table 8).

Table 8: Currently used liposomal drugs in clinic

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil/Caelyx</td>
<td>Doxorubicin</td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Myocet</td>
<td>Doxorubicin</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>Daunoxome</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma, breast and Lung cancer</td>
</tr>
<tr>
<td>Amphotec</td>
<td>Amphotericin-B</td>
<td>Fungal infections, Leishmaniasis</td>
</tr>
<tr>
<td>Fungizone</td>
<td>Amphotericin-B</td>
<td>Fungal infections, Leishmaniasis</td>
</tr>
<tr>
<td>AmBisome</td>
<td>Amphotericin-B</td>
<td>Fungal infections</td>
</tr>
<tr>
<td>Abelcet</td>
<td>Amphotericin-B</td>
<td>Fungal infections</td>
</tr>
<tr>
<td>VENTUS</td>
<td>Prostaglandin-E1</td>
<td>Systemic inflammatory diseases</td>
</tr>
<tr>
<td>ALEC</td>
<td>Dry protein-free powder of DPPC-PG</td>
<td>Expanding lung diseases in babies</td>
</tr>
<tr>
<td>Topex-Br</td>
<td>Terbutaline sulfate</td>
<td>Asthma</td>
</tr>
<tr>
<td>Depocyt</td>
<td>Cytarabine</td>
<td>Cancer Therapy</td>
</tr>
<tr>
<td>Novasome</td>
<td>Smallpox vaccine</td>
<td>Smallpox</td>
</tr>
</tbody>
</table>
Liposomes have been, and continue to be the most intensively researched drug delivery systems over few decades. However, conventional liposomes are cleared rapidly from the circulation by the macrophages which are located mainly in the liver, spleen and bone marrow [8, 12, 13]. The use of modified flexible hydrophilic polymers such as polyethylene glycol (PEG) which provides a protective hydrophilic layer at the surface of the liposome reduced the clearance of liposome from reticuloendothelial system (RES) [9, 14, 15]. Such PEGylated liposome, also known as Stealth liposome, have prolonged circulation time [10, 16], an improved pharmacokinetic profile compared with that of a free drug [17].

Tumor cells grow indefinitely by forming new vessels to provide nutrients for growth by a process called angiogenesis. During the formation of these new vessels, solid tumors have the presence of intercellular gaps in the endothelium. One of the major characteristics of solid tumors is the lack of effective lymphatic drainage. The intracellular gaps along with this lack of lymphatic drainage provide a leeway for the extravasation of molecules with a large particle size into tumor cells. This passive accumulation phenomenon in solid tumors is known as enhanced permeability and retention effect (EPR). The EPR effect is considered a heterogeneous process because of the high variability between different types of tumors, from patient to patient, and within the tumor itself where there is a vast difference in vascular permeability [18-20].
Therefore, the EPR effect associated with liposome results in different pharmacokinetics of the liposomal formulation of drugs compared to the naked drug. The ability to modify the surface of the liposome enables selective targeting of the liposome and liposomal anticancer drugs via specific ligands against antigens expressed on malignant cells. Targeting strategies are exploited to optimize the drug bioavailability to obtain higher accumulation of liposome accumulation. However, it should be noted that improved therapeutic efficacy is not necessarily a consequence of increased liposomal delivery to the target but seem to be model dependent [21]. Several studies have described the potential methods of active targeting the liposome with specific antibodies (HER-2), targeting peptide (RGD), and ligands targeting proteins expressed on cancer cell membrane (folate). Although some studies showed a marginal improvement in survival with ligand targeted liposome, for many targets, ligand mediated targeting of liposomes will result in little or no therapeutic improvement over passive targeting [22]. Various strategies have emerged with liposomal drug delivery to obtain elevated tumor to normal drug ratios. Externally triggered light and thermo sensitive liposome, receptor targeted pH and endogenous enzymatically triggered liposomes are some of the advanced and prospective strategies explored on liposomal drug delivery.

Acid triggered release of the liposomal content has been one of the most widely investigated release mechanism of liposome. pH sensitive liposomes, after internalization witnesses the change in the acidic environment where the enzymatic activity releases the content of liposomes. Another approach of acid triggered liposomes is the use of fusogenic lipid to synthesize liposomes. Lipoplatin, currently in phase III clinical trial, is an example of fusogenic liposome that fuses with the lipid layer of the cell membrane,
internalizes and releases cisplatin on the cytoplasm. Light triggered mechanism of release is based upon the isomerization, fragmentation of polymerization of lipids upon photoexcitation. PEG- liposome formulation containing bis-SorbPC, a photosensitive lipid can polymerize and causes leakage in the lipid bilayer upon UV-excitation [23]. Plasmogen photooxidiation as a triggered release strategy proposed by Thompson and co-workers [24] relies on an increase in membrane permeability upon photooxidative cleavage of plasmenylcholine to single chain surfactants. Hyperthermia or heat triggered release has significant advantage over other triggering concepts in that liposome tumor accumulation was increased due to the increased blood flow and vascular permeability [21]. Several liposomal formulations using DPPC/DSPC lipids have been designed and studied in vitro to determine the rate of release when heated at 42°C for 30 min [25, 26]. Hyperthermia as a triggered release is limited to tumor site that is accessible to local hyperthermia. Enzymatic release via the upregulated enzymes in tumor tissue is another triggered release mechanism. This mechanism utilizes the cell associated proteases for the enzymatic drug release from liposomes [27]. Liposomal formulations of drug offer the advantage of increasing the efficacy of chemotherapeutic drugs while reducing the toxic side effects. This strategy of liposomal formulation of drugs has been used for various platinum drugs. SPI-077 is a PEGylated liposomal formulation of cisplatin which is composed of HSPC, CH and PEG-derivatized phosphatidylethanolamine (PEG-PE). Although SPI-077 exhibited longer circulation half life, higher anti-tumor efficacy and a decrease in toxicity compared to free drug, SPI-077 revealed only modest anti-tumor efficacy in phase I and II studies [28].
Lipoplatin, PEGylated liposomal formulation of Cisplatin, is composed of anionic phospholipid dipalmitoyl phophatidyglycerol (DPPG), HSPC, CH and PEG-derivatized phosphatidylethanolamine (PEG-PE) as depicted in Fig. 16. Lipoplatin, nanoparticle with 110 nm diameter, has the ability to target tumors and metastasis via EPR effect after intravenous administration. Lipoplatin has shown increased concentration in tumors and metastases at levels up to 200 fold higher compared to the adjacent normal tissue [29].

The increased accumulation of Lipoplatin is attributed to the anionic lipid DPPG which has the ability to survive the lysosome, endosome or cytoplasmic enzyme and to cross the cell membrane allowing the encapsulated cisplatin to enter the nuclei [18]. During Phase I and II trials, Lipoplatin has shown anti-tumor efficacy similar to that of a free drug in advanced squamous cell carcinoma of the head and neck with reduced renal and hematological toxicity. Therefore, Lipoplatin has progressed into Phase III clinical trial. Lipoxal, a liposomal formulation of oxaliplatin was developed using similar technology as Lipoplatin and is composed of HSPC, CH, DPPG, PEG-PE. The mechanism of cell
uptake and internalization is similar to Lipoplatin since both formulations use the same lipid components. Preclinical studies carried out on tumor bearing mice proved Lipoxal to be less toxic to normal tissues without losing its efficacy. However, in Phase I clinical trial, mild myelotoxicity, nausea and few cases of peripheral neuropathy were observed at doses of 300-350 mg/m² [30]. No serious side effects were observed below 300 mg/m². Further studies should be done to demonstrate the antitumor efficacy of Lipoxal.

Various other targeted liposomal formulations of platinum derivatives are reported in literature. Suzuki et al reported transferrin conjugated oxaliplatin encapsulated PEgylated liposome and its superior antitumor efficacy than non-targeted and free oxaliplatin in in vivo model [31]. Folate receptor targeted liposomal formulation of carboplatin reported by Chaudhury et al showed improved therapeutic efficacy in ovarian tumor xenograft model compared to free drug or non-targeted liposomes [32]. There are currently no targeted liposomal platinum derivatives approved for clinical use. Studies involving the targeted liposomes should be further investigated before clinical translation.

This project introduces liposomes as a drug delivery vehicle for a newly synthesized radiolabeled platinum drug, [^{18}F]-FCP. The liposome surface is radiolabeled with ^{111}\text{In} to enable distinct SPECT imaging in contrast to PET imaging from [^{18}F]-FCP. This enables real time visualization of the radiolabeled drug and nanoparticle using bi-modal non invasive imaging. Further details on the synthesis and evaluation of [^{18}F]-FCP and the liposomal formulation are discussed in Chapter 5 and 6 respectively.
References


CHAPTER 5: FLUORINATED CARBOPLATIN DERIVATIVE: A NOVEL THERANOSTIC ANTICANCER DRUG FOR THERAPY AND IMAGING
5.1 Introduction

This chapter deals with the development and evaluation of fluorinated carboplatin derivatives. This chapter presents the development of cold reference compound, F-19 labeled carboplatin, cis[2-(5-fluoropentyl)-2-methylmalonato-κ²,O, O']platinum(II) (19F-FCP), and its in vitro cytotoxicity evaluation in comparison with cisplatin and carboplatin. Further, this chapter also presents the development of F-18 labeled carboplatin derivative ([18F]-FCP) and its in vivo evaluation in normal and xenograft model.

Platinum based anti-cancer drugs play a pivotal role in the treatment of various solid tumors in clinic today. Since the accidental discovery of its biological properties by Rosenberg et al [1] many years ago, Cisplatin has made a major impact in the therapeutic treatment of testicular and ovarian cancers, and along with carboplatin, is still widely used today for the treatment of ovarian, cervical, head & neck and lung cancers including advanced non-small cell lung cancer (NSCLC). Like other cytotoxic agents, however, adequate dosage administration of platinum chemotherapy is still hampered by the toxic effects of the drug on normal tissues and functions. Both tumor regression and normal organ toxicity are directly related to amount of drug that reaches the tissues. Reduced retention of drug in the tumor is further compromised by drug efflux due to multi-drug resistance (MDR) and limited drug penetration. Currently, there is no direct means by which the amount of platinum drug in the tumor and normal tissues can be determined in individual patients, a major obstacle in knowing which patient tumors have how much drug and hence which patients are more likely to respond to treatment. Despite the multifactorial nature of platinum resistance, reduced intracellular drug accumulation is one of the most consistently identified features of Cisplatin-resistant cell lines [2-4],
including resistant NSCLC cell lines [5-10]. More recently, a recent clinical study established a relationship between tissue platinum concentration and response in NSCLC, which suggests that reduced platinum accumulation might be an important mechanism of platinum resistance in the clinical setting [11]. The use of radio labeled drug candidates and PET/SPECT imaging, in early drug development, provides unique information that bridges the gap between molecular biology, pharmacology, pathology and the design of new generation therapeutic strategies and drug systems. For example, by labeling a chemotherapeutic ([\(^{18}\)F]-Paclitaxel) agent with a positron emitting radionuclide and by monitoring the kinetics of the radioactivity in the tumor, \textit{in vivo} chemoresistance have been studied [12, 13]. Two approaches of radiolabeling platinum drugs; one using Pt isotopes to synthesize drug and the other using \(^{13}\)N to label cisplatin have been published. Baer et al [14] described the microscale synthesis of various platinum compounds labeled with \(^{191}\)Pt. Leal et al [15] established the production of radioactive cisplatin with and without a cadmium capsule by irradiating cisplatin with thermal and epithermal neutrons flux. This approach irradiates an intact drug to convert stable Pt to radioactive Pt. However, this approach could produce radiation induced damage to the chemical structure of the compound. Speigeleer et al [16] developed a microscale synthesis of labeling cisplatin with cyclotron produced \([^{13}\)N]. Areberg et al [17] synthesized cisplatin using \(^{191}\)Pt, \(^{193m}\)Pt, and \(^{195m}\)Pt and calculated the absorbed doses to various organs and tissues as well as effective doses from the radionuclides after administration to humans. Thus, significant research efforts so far, to improve the overall efficacy of platinum-based therapy in solid tumors, have all emphasized the need to know the most important factors related to response: accumulation and retention of drug in tumors in a heterogeneous
tumor, which is affected by a unique microenvironment such as tumor hypoxia. In light of this, this research project aims to better utilize and update on the present therapeutic norm of platinum-based anticancer therapy and this has led us to develop and evaluate a $[^{18}\text{F}]$-FCP, by introducing a molecular imaging entity $[^{18}\text{F}]$-labeled malonic acid coordinated to the drug which enables, through non-invasive PET imaging, quantitative measurement of drug concentration in multiple tumors and normal tissues within an individual patient. The rest of this chapter details the approach and methodologies developed for the radiosynthesis of $^{19}\text{F}$-FCP and $[^{18}\text{F}]$-FCP including in vitro, ex vivo and in vivo imaging studies.

5.2 Experimental Section

5.2.1 Materials
All reagents and solvents were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. All cold reactions were performed under argon atmosphere in oven-dried glassware. Flash chromatography was performed employing Sigma-Aldrich 230-400 mesh60 Å silica gel. TLC (Sigma-Aldrich, Saint Louis, MO) was performed using silica gel-coated aluminum plates with F-254 indicator (250 mm, 20 cm20 cm, Whatman). NMR (Piscataway, NJ) spectra (1H-NMR, 13C-NMR, and $^{19}\text{F}$-NMR, $^{195}\text{Pt}$-NMR) were obtained using Varian Mercury 300 MHz and Varian Inova 400 MHz (Sigma-Aldrich, Saint Louis, MO) using tetramethylsilane as an internal standard. Aqueous $[^{18}\text{F}]$-fluoride was produced by the $^{18}\text{O}$(p,n)$^{18}\text{F}$ reaction, in a PET tracer cyclotron (GEMedical Systems, Wausheka, WI, USA), by the irradiation of an isotopically enriched 98% $[^{18}\text{O}]$ water (Rotem Industries, Ber Sheva, Israel) target. For fluoride trapping, Sep-Pak Light quaternary methyl ammonium (QMA) cartridge was
used in manual synthesis from Waters Corp. (Milford, MA, USA). Radio-HPLC analysis was carried using a Waters HPLC pump (Waters, Model 1525) equipped with UV detector (Waters, Model 2489) and radiation detector (Bio-scan, Model B-FC-3300) connected in series. For reverse phase HPLC, a Waters® Nova-Pak 4µ C18 150 x 3.9 mm column was eluted isocratically with 25/75 ACN/Water containing 0.1% TFA at flow rate of 1 mL/min. Radio-TLC was performed on a radio-TLC scanner (BioScan, Model AR/2000). Radioactivity was measured with dose calibrator Captintec CRC-15 PET. Gamma counting was measured by gamma counter (Gamma counter (LKB Wallac 1282 compugamma CS universal gamma counter/Perkin Elmer). Animals, normal adult athymic female nude mice, were purchased from Harlan laboratories, USA.

5.2.2 Synthesis of Cold Reference (18F-FCP)

5.2.2.1 Diethyl 2-(5-bromopentyl)-2-methylmalonate (1)
A solution of diethyl methylmalonate (6.0 g, 34.5 mmol) in dry dimethylformamide (50 mL) was treated portion wise with sodium hydride (1.5 g of a 60% dispersion, w/w, in mineral oil, 38 mmol) with cooling. The reaction mixture was stirred for 30 min at ambient temperature and treated with 1,5-dibromopentane (11.9 g, 51.8 mmol). The reaction mixture was heated at 50 °C overnight and quenched by adding 30% ammonium hydroxide solution. Distilled water (50 mL) was added in to the reaction mixture and extracted with dichloromethane (3 x 50 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by flash chromatography using 5% ethyl acetate in hexane to give alkyl malonate 1 as colorless viscous oil (8.0 g, 72%) (Scheme 1.): 1H-NMR (300 MHz)
(CDCl$_3$) $\delta$ 1.20 -1.33 (m, 2H), 1.25 (t, J = 7.32 Hz, 6H), 1.40 (s, 3H), 1.42 — 1.51(m, 2H), 1.81 — 191 (m, 4H), 3.39 (t, J = 6.73 Hz, 2H), 4.17 (q, J = 7.32 Hz, 4H); $^{13}$C-NMR (75 MHz) (CDCl$_3$) $\delta$ 13.9, 19.7, 23.3, 28.2, 32.3, 33.4, 35.1,53.4, 60.9, 172.1; ESI-MS [M+Na]$^+$ m/z calcd for C$_{13}$H$_{23}$BrO$_4$+Na 345.07 found 345.08. (NMR Fig 1)

5.2.2.2 Diethyl 2-methyl-2-(5-(tosyloxy)pentyl)malonate (2)

To a solution of alkyl malonate 1 (3.2 g 10 mmol) in dry acetonitrile (30 mL), silver tosylate (3.48 g, 12.5 mmol) was added. After the reaction mixture was refluxed for 24 h under an argon atmosphere, the reaction mixture was filtered through celite to remove the silver salt and solvent was evaporated under reduced pressure. The residue obtained from the solvent evaporation was purified by flash chromatography using 10% ethyl acetate in hexane to give tosylate precursor 2 as a colorless viscous oil (3.9 g, 96%): $^1$H-NMR (300 MHz) (CDCl$_3$) $\delta$ 1.18 — 1.25 (m, 2H), 1.23 (t, J = 7.22 Hz, 6H), 1.26 — 1.35 (m, 2H), 1.36 (s, 3H), 1.61 – 1.68 (m, 2H), 1.77 — 1.81 (m, 2H), 2.45 (s, 3H), 4.00 (t, J = 6.44 Hz, 2H), 4.16 (q, J = 7.22 Hz, 4H), 7.35 (d, J = 8.19 Hz, 2H) 7.78 (d, J = 8.19 Hz, 2H); $^{13}$C-NMR (75 MHz) (CDCl$_3$) $\delta$ 13.7, 19.4, 21.1, 23.2, 25.2, 28.1, 34.8, 53.1, 60.7, 70.1, 127.4, 129.6, 132.8, 144.4, 171.7; ESI-MS [M+Na]$^+$ m/z calcd for C$_{13}$H$_{23}$BrO$_4$+Na 437.16 found 437.43. (NMR Fig 2)

5.2.2.3 Diethyl 2-(5-fluoropentyl)-2-methylmalonate (3)

To a solution of tosylate 2 (2.1 g, 5 mmol) in dry THF (20 mL), a solution tetrabutylammonium fluoride TBAF (1 M solution in THF, 1.58 g, 6 mL, 6 mmol) was added dropwise under argon atmosphere. The reaction mixture was stirred at 60 °C for 3 h. The reaction mixture was cooled to room temperature and solvent was evaporated under reduced pressure. The product obtained from the solvent evaporation was purified
by flash chromatography using 10% ethyl acetate in hexane to give fluoropentyl malonate 3 as a light yellow colored viscous oil (1.1 g, 84%): $^1$H-NMR (300 MHz) (CDCl$_3$) $\delta$ 1.24 (t, $J = 7.32$ Hz, 6H), 1.25 -1.34 (m, 2H), 1.40 (s, 3H), 1.36 — 1.48 (m, 2H), 1.61 — 1.78 (m, 2H), 1.83 — 1.89 (m, 2H), 4.17 (q, $J = 7.32$ Hz, 4H), 4.34 (t, $J = 6.15$ Hz, 1H), 4.50 (t, $J = 6.15$ Hz, 1H); $^{13}$C-NMR (75 MHz) (CDCl$_3$) $\delta$ 13.87, 19.66, 23.79, 25.33, 25.40, 29.89, 30.15, 35.25, 53.43, 60.92, 82.49, 84.68, 172.12; ESI-MS [M+Na]$^+$ m/z calcd for C$_{13}$H$_{23}$FO$_4$Na 285.15 was found 285.31. (NMR Fig 3, 4)

**5.2.2.4 2-(5-Fluoropentyl)-2-methylmalonic acid (4)**

To a stirred solution of fluoropentyl malonate 3 (310 mg, 1.5 mmol) in THF:MeOH:H$_2$O (6:3:1, 15 mL) was added lithium hydroxide monohydrate (631 mg, 15 mmol). The reaction mixture was stirred 24 hr at room temperature. After the reaction was complete, the organic solvents were removed under reduced pressure. The aqueous layer was acidified with oxalic acid solution (1 mol) to pH 2-3 and then extracted with diethyl ether (3 x 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and then concentrated under reduced pressure. The residue was dissolved in chloroform, undissolved residue was filtered and the filtrate was concentrated under reduced pressure to give reference compound ([$^{19}$F]-FPMA) 4 (208 mg, 86%) as a white solid: mp = 102-104 °C: $^1$H-NMR (300 MHz) (CDCl$_3$) $\delta$ 1.32-1.47 (m, 4H), 1.49 (s, 3H), 1.65-1.78 (m, 2H), 1.90-1.94 (m, 2H), 4.37 (t, $J = 6.05$ Hz, 1H), 4.49 (t, $J = 6.05$ Hz, 1H) 10.48 (s, 2H); $^{13}$C-NMR (75 MHz) (CDCl$_3$) $\delta$ 20.09, 24.22, 25.59, 25.66, 30.14, 30.40, 35.63, 54.03, 83.04, 85.22, 178.43; $^{19}$F-NMR (300 MHz) (CDCl$_3$) (coupled) $\delta$ -218.88 (m); ESI-MS [M+Na]$^+$ m/z calcd for C$_9$H$_{15}$FO$_4$Na 229.09 found 229.09. $^1$H-NMR, spectra is shown in NMR Fig 5,6.
5.2.2.5 Non-radioactive Reference Compound ¹⁹F-FCP
Cisplatin 50 mg and silver nitrate 50 mg was dissolved in nanopure water (2.5 mL) and reaction was stirred at room temperature for overnight in protected light. After filtration of the precipitated silver chloride, one equivalent 2-(5-Fluoropentyl)-2-methylmalonic acid (4) was added, and the mixture was stirred at room temperature for 2 days. Product was purified by Sep-Pak purification method using water as eluent. Finally, water fraction was lyophilized to give fluorinated carboplatin drug. Analysis of Carboplatin Derivative $^1$H-NMR (300 MHz) (D$_2$O) $\delta$ 1.32-1.47 (m, 4H), 1.49 (s, 3H), 1.65-1.78 (m, 2H), 1.90-1.94 (m, 2H), 4.50 (t, $J = 6.05$ Hz, 1H), 4.66 (t, $J = 6.05$ Hz, 1H); $^{19}$F-NMR (300 MHz) (D$_2$O) $\delta$ -218.88 (m); $^{195}$Pt-NMR (300 MHz) (D$_2$O) $\delta$ -1596 (broad singlet).(NMR Fig 7)

5.2.3 Manual Synthesis and Characterization of [¹⁸F]-FCP
Radiosynthesis of [¹⁸F]-FPMA was carried out using the tosylate precursor. [¹⁸F] Fluoride was produced by the $^{18}$O (p,n)$^{18}$F reaction in a PETtrace cyclotron by the irradiation of an isotopically enriched [$^{18}$O] water target using a 16 MeV proton beam. [¹⁸F] Fluoride was then passed through a QMA Sep-Pak that was preconditioned with (0.25 mol) potassium carbonate solution followed by sterile water. The trapped [¹⁸F] Fluoride was eluted with a solution having 0.08 mL of potassium carbonate (0.25 mol) and 0.42 mL of sterile water into 10 mL V vial containing a solution of Kryptofix (12 mg) in dry acetonitrile (0.8 mL). The reaction mixture was dried with the addition of dry acetonitrile at 80 °C. After drying 4 mg tosylate precursor in acetonitrile (0.8 mL) was added and the reaction heated at 110 °C for 10 min. The reaction mixture was cooled for 5 min and then solvent evaporated under vacuum and helium flow room temperature. Base hydrolysis was accomplished by the addition of methanolic NaOH solution in 2 mL of
(DCM:MeOH; 9:1) for 20 min at 45 °C. After solvent evaporation sodium citrate buffer (pH 3) and 3 mol HCl (1.5:0.5 mL) was added. The reaction mixture was passed through a preconditioned C18 column and eluted with 2 mL ethanol. The ethanol was evaporated at 45°C under vacuum and helium flow. The final [\(^{18}\text{F}\)]-FPMA was redissolved in water and aqua platinum complex was added to the activity vial at 75 °C for 30 min. In the reaction some of the unreacted starting compound, [\(^{18}\text{F}\)]-FPMA was observed by HPLC analysis. Final [\(^{18}\text{F}\)]-carboplatin derivative was purified by QMA Sep-Pak ion-exchange cartridge.

5.2.4 Automated Synthesis and Characterization of [\(^{18}\text{F}\)]-FCP
Encouraged by the feasibility of manual synthesis of [\(^{18}\text{F}\)]-FCP, we initiated the development of automated synthesis of [\(^{18}\text{F}\)]-FCP. Automation provides benefits of reduced duration of synthesis, reduced waste, reproducibility and scaled up reaction without personnel exposure.

Automated radiosynthesis of [\(^{18}\text{F}\)]-FCP was carried out on Allinone Synthesizer (Trasis, Belgium) (Fig. 17) via [\(^{18}\text{F}\)]-FPMA route as shown in the Scheme 2. Briefly, initial optimization of automated radiosynthesis [\(^{18}\text{F}\)]-FPMA and purification was carried out on Allinone Synthesizer based on earlier manual radiosynthesis. Radiochemical yield (RCY) and radiochemical purity (RCP) was confirmed by radio-HPLC. The radiosynthesis procedure for [\(^{18}\text{F}\)]-FCP was a three step reaction process. Each step was programmed as a sequence with appropriate macros in the automated synthesizer. Each sequence was optimized along the reaction progression to produce a final product, [\(^{18}\text{F}\)]-FCP, with high radiochemical yield.
Briefly, the manual synthetic procedure involves the synthesis of platinum aqua complex ahead of the reaction. The pre-made platinum aqua complex was placed in Allinone Synthesizer in a closed vial under nitrogen protection. After purification and evaporation of ethanol solvent from $[^{18}\text{F}]-\text{FPMA}$, it was incubated with platinum aqua complex at $82^\circ\text{C}$ for 30 mins to yield $[^{18}\text{F}]-\text{FCP}$. Final $[^{18}\text{F}]-\text{FCP}$ was characterized and radiochemical purity (RCP) was confirmed by radio-HPLC.

Figure 17: Automated synthesis of $[^{18}\text{F}]-\text{FCP}$ using AllInone Synthesizer. The cassette for the three step reaction was prepared using manifolds. Machine test was performed to verify the optimal vacuum, pressure and the movement of all components before placing the manifold in the synthesizer as shown above. After the placement of the cassette, the cassette test was performed to test the leak, vacuum in the reactor vials and the syringe positions. After all the tests were successful, reagent vials were placed as shown above and the pre programmed sequences for the synthesis was started for the complete synthesis procedure.
5.2.5 Cell Lines and Culture Conditions
All cell lines were maintained in humidified incubator at 37 °C and 5% CO₂. Human Cervical Adenocarcinoma (KB 3-1 and KB 8-5) were purchased from National Institute of Health (NIH). Lung Carcinoma (A549), Ovarian Carcinoma (SK-OV-3), Colorectal Carcinoma (COLO-205), Renal Carcinoma (A498) and Prostate Carcinoma (LNCap) was purchased from ATCC. Head and Neck Carcinoma (FaDu) cell line was a gift from Dr. Andrei Pugachev at Virginia Commonwealth Massey Cancer Center. Briefly, KB-3-1 and KB 8-5 cells were grown in DMEM/High glucose medium supplemented with 10% fetal bovine serum (FBS), 5mM L-Glutamine, Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (0.25 µg/ml). KB-8-5 cell lines were grown in similar media supplemented further with 10ng/ml colchicine. COLO-205, FaDu, A498, LNCap cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS Penicillin G (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (0.25 µg/ml). The cells were grown to 70-80% confluency before using for the experiments.

5.2.6 Tumor Xenograft/Animal Model
KB-3-1 and KB 8-5 cells were maintained in DMEM/High glucose medium supplemented with 10% fetal bovine serum (FBS), 5mM L-Glutamine, Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (0.25 µg/ml).
Female athymic nude mice, (4-6 weeks old, weight: 18-25 g) were purchased from Harlan laboratories, USA and provided with food and water ad libitum. Animal experiments were approved and performed according to the policies and guidelines of the Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. KB-3-1 and KB-8-5 cells were cultured as mentioned above and collected for implantation. Tumor cells 2.5x10⁶/100µL (KB 3-1), 2.5.0x10⁶/100µL (KB 8-5) in media without
serum) were injected subcutaneously into the dorsal region on the right shoulder (KB8-5) and left (KB 3-1) shoulder of athymic nude mice. Following subcutaneous implantation digital caliper measurement of tumor size was accessed, once the bulge caused by the tumor growth at the injection site was visible, (approximately 5 days). The tumor volume was calculated using the formula $0.523 \times (\text{length} \times \text{width} \times \text{thickness})$ and assessed thrice per week.

5.2.7 In vitro Cytotoxicity of $^{19}\text{F}-\text{FCP}$

The cytotoxicity of $^{19}\text{F}-\text{FCP}$ was compared with cisplatin and carboplatin by incubating in various cell lines. The cell viability of COLO-205, SK-OV-3, FaDu, A549, A498, LNCaP, RWPE-1 and KB-3-1 cells treated with cis-platinum and carboplatin, and fluorinated carboplatin was evaluated using Cell Titer Glo assay kit (Promega, USA). Briefly, depending on the cell type, 3,000 to 4000 cells in 25 µl complete media were plated in opaque 384-well plate in triplicates for each cell type and cultured overnight in the incubator. The next day the cells were treated with 25 µl drug for final concentrations from 0.001 (µM) to 100 (µM) for 72 hrs and 96 hrs. At the end of incubation period 50 µl assay reagent was added to each well, and the cell viability was assessed by measuring the luminescence, detected by a plate reader (Beckman Coulter). The luminescence detected in untreated cells was used as control (100% Viability) to calculate percent viability in each treatment groups. Statistical analysis was done using Student’s t-test and the p value calculated based on two-tailed test using Microsoft Excel software. $P<0.05$ was considered statistically significant.
5.2.8 Biodistribution and in vivo Stability Analysis

Animal experiments were approved and performed according to the policies and guidelines of the Animal Care and Use Committee at Virginia Commonwealth University. The biodistribution of $[^{18}\text{F}]-\text{FCP}$ was measured in normal adult female nude mice (Harlan). Under 2% isoflurane, three group of three mice received an intravenous injection (tail vein) of $[^{18}\text{F}]-\text{FCP}$ (1.5-2MBq, in 200µL PBS, at the start of the experiment). Under anesthesia, mice were sacrificed by cervical dislocation at 5, 30, and 90 mins post injection. Whole blood was collected by cardiac puncture, other major organs were harvested and weighed, and the radioactivity in the organs/tissues was counted in a gamma counter (Gamma counter (LKB Wallac 1282 compugamma CS universal gamma counter/Perkin Elmer)). The blood activity was used to calculate the blood half life. Decay-corrected radiotracer uptake in each tissue at various time points was then calculated as the percent injected dose per gram of tissue (%ID/g).

In vivo stability of $[^{18}\text{F}]-\text{FCP}$ in plasma and urine was determined following radiotracer administration in normal female nude mice under 2% isoflurane anesthesia. Samples were collected at 5, 30, 60, and 90 minutes following intravenous injection of $[^{18}\text{F}]-\text{FCP}$. The collected samples of plasma were centrifuged for 10 mins at 10,000g. Supernatant from these samples was filtered through 0.2µ filter and 200µL plasma supernatant was injected into reversed-phase HPLC (column Waters Nova-Pak 4 µ C18 150× 3.9 mm, CH$_3$CN/H$_2$O 25:75, containing 0.1% TFA at flow rate 1mL/min). Urine samples were also filtered and analyzed in the same HPLC system.
5.2.9 PET Imaging Studies
Small animal PET/CT imaging studies were performed using a Siemens Inveon® Multimodality PET/CT system (Siemens Medical Solutions Inc., Knoxville, TN, USA). Ten minutes prior to imaging, the animals were anesthetized using 2% isoflurane at room temperature until stable vital signs were established. Once the animal was sedated, the animal was placed onto the imaging bed under mixtures of O₂ (1 mL/min) and 2% isoflurane for the duration of the imaging. [¹⁸F]-FCP (5.5-6 MBq/200 µL) was injected intravenously in a normal female nude mouse via the tail vein. Immediately following the injection, a 90 minute dynamic scan was performed.

Nude mice were innoculated with human cervical adenocarcinoma (KB) cell lines to establish a xenograft model with one P-glycoprotein (P-gp) non expressing (KB 3-1) and one Pgp expressing (KB 8-5) tumor per animal. [¹⁸F]-FCP formulation (5.5-6 MBq) was injected intravenously in xenograft nude mice via tail vein injection. Static summed up image data acquired 60 minutes post injection was acquired for 30 mins. The micro CT imaging was acquired following the PET imaging at 80kV and 500 µA with a focal spot of 58 µm.

PET images were reconstructed using Fourier Re-binning and Ordered Subsets Expectation Maximization (OSEM) 3D algorithm with dynamic framing every 60 seconds. Reconstructed images were fused and analyzed using Inveon® Research Workplace (IRW) software. For quantitation, regions of interest were placed in the areas expressing the highest radiotracer activity as determined by visual inspection. The resulting quantitative data were expressed in Percent Injected Dose per Gram (%ID/g).

5.2.10 Protein Extraction and Western Blot
The KB-3-1 and KB-8-5 tumors were excised from the tumor bearing mice, snap frozen in liquid nitrogen and stored in -80°C until further use. Approximately 80-100 mg of
tumor was excised and homogenized in 500 µL of lysis buffer (Pierce RIPA buffer) containing protease inhibitor (PI) (Halt protease inhibitor, Pierce) and EDTA (Pierce) in a hand held homogenizer on ice. The tumor lysate was collected and stored in -80°C. KB-3-1 and KB-8-5 cells were also collected and lysed in lysis buffer containing PI/DETA. The homogenate/cell lysate were spun down at 12,000 rpm for 15 min and the clear supernatant was collected for protein assay. The amount of protein in supernatant was estimated using BCA Protein assay kit (Pierce/Thermo scientific). Twenty µg of protein was resolved on 7.5% tris-glycine gel. The proteins were transferred onto PVDF membrane, at 100 V for 1 hr. To prevent non-specific binding of the antibody, the PVDF membrane was blocked with Odyssey blocking buffer (Cat No; 927-40000, LiCor) for 1 hr at room temperature (R.T). The blots were the incubated overnight at 4°C with primary antibodies, anti-P Glycoprotein antibody (F4) Cat No: ab80594 and rabbit β-actin mAb (Cell signaling, Cat No 4970) respectively. After washing the blot with PBS/0.1% Tween-20, for four times, the blot was incubated with secondary antibodies, IRDye 680LT Goat anti-mouse IgG and IRDye 800CW Goat anti-rabbit IgG (Li-Cor) respectively. The blot was rinsed in wash buffer and the bands for P-gp and β-actin on PVDF membrane was detected using Odyssey CLx. The image was analyzed and quantified using Image Studio 2.0 software.

5.3 Results

5.3.1 Synthesis of ¹⁹F-FCP
Scheme 1 describes the synthesis of the tosylated precursor and reference standard compound [¹⁹F] 2-(5-fluoro-pentyl)-2-methylmalonic acid (4). The reference non-radioactive compound 5 was synthesized in four steps starting with commercially
available diethyl methylmalonate. Diethyl methylmalonate was alkylated with 1,5-dibromopentane in presence of NaH to give alkylated compound 1 in 72% yield using the procedure of Astles et al. Excess 1,5-dibromopentane was used to avoid disubstitution. The bromide was displaced using commercially available silver tosylate to give a tosylated precursor 2 in 96% yield. The tosylate group was exchanged for the required fluoride by a nucleophilic displacement reaction using tetrabutylammonium fluoride (TBAF) to give diethyl 2-(5-fluoropentyl)-2-methyl malonate 3 in 84% yield. Fluoropentyl diethyl malonate ester 3 was hydrolyzed using lithium hydroxide under mild conditions to give 2-(5-fluoro-pentyl)-2-methyl malonic acid as a white solid with 50% overall yield. Finally, 2-(5-fluoro-pentyl)-2-methyl malonic acid 4 was incubated with platinum aqua complex for 2 days to give F-19 Carboplatin derivative 5. All the intermediate compounds and final compound were fully characterized by $^1$H-NMR, $^{13}$C-NMR, $^{19}$F-NMR, and ESI-MS analysis. Fluorination of compound 3 was confirmed by $^{19}$F-NMR, which showed the expected multiplet pattern and had a chemical shift in the expected region (218.88 for terminal (CH$_2$F)). The calculated molecular mass for purified 2- (5-fluoro-pentyl)-2-methyl malonic acid was in agreement with experimental value. Chemical purity was found to be greater than 98% as determined by analytical HPLC.
Scheme 1: Synthesis of cold reference compound $^{19}\text{F}$-FCP and tosylate precursor of $^{19}\text{F}$-FCP. Reagents and conditions: (a) 1,5-dibromopentane, NaH, DMF, 50 °C, 12 h, 72%; (b) silver tosylate, ACN, reflux, 12 h, 96%; (c) TBAF, THF, 60 °C, 3 h, 84%; (d) LiOH/H$_2$O THF/MeOH/H$_2$O (6:3:1), rt, 24 h, 86%; (e) platinum aqua complex, rt, 2 days.

5.3.2 *In vitro* Cytotoxicity of $^{19}\text{F}$-FCP
A key prerequisite for a drug analog is that it must not alter the anti-tumor activity of the parent drug. To directly compare the cytotoxicity of $^{19}\text{F}$-FCP with cisplatin and carboplatin, *in vitro* a panel of eight cell lines of different origin; COLO 205, SK-OV-3, FaDu, A549, A498, LNCaP, RWPE-1 and KB-3-1, was treated with $^{19}\text{F}$-FCP with cisplatin and carboplatin at concentrations from 0.001 (µM) to 100 (µM) for 72 hrs and 96 hrs. At the end of the incubation period, the percent viable cells were evaluated by measuring amount of ATP released from viable cells using Cell Titer-Glo reagent. The percent viability of each cell line at various concentrations of drugs was plotted and used to calculate the IC$_{50}$ for each drug. Table 9 reports IC$_{50}$ (50% growth inhibition concentration) values for each of the eight cell lines. Different cell lines exhibited different sensitivities to platinum compounds depending on their origins, morphologies, and tumorigenicities. Based on the cell viability data, the IC$_{50}$ values for the various
platinum compounds demonstrated that cisplatin reduced the cell viability to the greater extent than carboplatin, and \(^{19}\text{F}-\text{FCP}\). The cytotoxicity of \(^{19}\text{F}-\text{FCP}\) was slightly higher than carboplatin suggesting that \(^{19}\text{F}-\text{FCP}\) is more potent than carboplatin. Cytotoxic effect for all three compounds was time dependent. Increasing the incubation time from 72 hours to 96 hours reduced the IC\(_{50}\) values for all three compounds indicating that the cytotoxic activity of all three compounds was elevated with time.

Table 9: IC\(_{50}\) values of cisplatin, carboplatin and \(^{19}\text{F}-\text{FCP}\) in various cancer cell lines

<table>
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<tr>
<th>Cell line</th>
<th>Cisplatin ((\mu\text{M}))</th>
<th>Carboplatin ((\mu\text{M}))</th>
<th>Fluorinated ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 hrs</td>
<td>96 hrs</td>
<td>72 hrs</td>
</tr>
<tr>
<td>COLO 205</td>
<td>25.72±4.32</td>
<td>6.68±7.54</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>12.71±11.84</td>
<td>1.76±0.69</td>
<td>63.92±12.74</td>
</tr>
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<td>FaDu</td>
<td>4.38±2.28</td>
<td>1.22±0.59</td>
<td>45.78±15.65</td>
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<tr>
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<tr>
<td>LNCaP</td>
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<td>11.34±5.88</td>
<td>101.86±16.48</td>
</tr>
<tr>
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<td>3.08±1.48</td>
<td>56.16±5.18</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>9.11±4.02</td>
<td>12.83±0.10</td>
<td>88.94±33.59</td>
</tr>
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</table>

5.3.3 Radiosynthesis of \([^{18}\text{F}]\)-\(\text{FCP}\)

Scheme 2 represents manual and automated radiosynthesis of \([^{18}\text{F}]\) 2-(5-fluoropentyl)-2-methyl malonic acid (\([^{18}\text{F}]\)-FPMA) in two steps: (i) Kryptofix-mediated direct nucleophilic fluorination and (ii) NaOH base hydrolysis (iii) conjugation with platinum-aqua complex.

Radiofluorination of 2 was carried out in the presence of Kryptofix and K\(_2\)CO\(_3\) in acetonitrile, optimum conditions of 8-mg precursor, 110 °C, and 20 min. reaction time resulted in >90% RCY of non-hydrolyzed compound 6 as confirmed by radio-thin-layer chromatography (TLC) analysis. After radiolabeling, the acetonitrile was evaporated with
helium flow under vacuum, and the product was subjected to base hydrolysis. Complete hydrolysis was achieved by methanolic sodium hydroxide (3 M) in dichloromethane (DCM)/methanol (9:1) for 20 min at 45 °C. The final product was purified by solid phase extraction. After evaporation of the solvent, under vacuum and helium flow, the product was re-dissolved in water and adjusted to pH 2–3 using 3 M HCl. The reaction mixture was passed through a C18 Sep-Pak column, and the column was washed with 10 mL of water to remove free fluoride and byproducts of para-toluene sulphonic acid. The product was eluted with multiple 0.5 mL ethanol fractions. Greater than 90% of product radioactivity was eluted in fractions 3 and 4. The overall decay-corrected (yield of 7, 50 min after introduction of [\(^{18}\text{F}\)]-fluoride activity) isolated RCY of [\(^{18}\text{F}\)]-FPMA was 60%, and the radiochemical purity (RCP) was more than 98% in 50 min total synthesis time. RCP was confirmed by radio-HPLC. Co-injections with non-radioactive fluorinated compound gave the same retention time as [\(^{18}\text{F}\)]-FPMA = 4 min. Compound 7 was completely dried under helium flow under vacuum. Aqua platinum complex was added to the reaction vial 7 for further conjugation at 80 °C for 30 mins to yield the final compound. The final product 8 was purified by QMA anion exchange column. The product was eluted with multiple 0.5 mL water fractions. The decay corrected (yield of final product, 180 mins after the introduction of [\(^{18}\text{F}\)]-fluoride activity isolated RCY of [\(^{18}\text{F}\)]-FCP was 14.3±3.8% (n=3), and the radiochemical purity was more than 98% in 180 mins synthesis time. RCP was confirmed by radio-HPLC (Fig 17). Retention time of [\(^{18}\text{F}\)]-FCP was 1.43 mins (Fig. 18).
Scheme 2: Synthesis of $[^{18}\text{F}]-\text{FCP}$. Reagents and conditions: (a) K$^{18}$F/K$_{222}$, K$_2$CO$_3$, ACN, 110 °C, 10 min condition used for manual synthesis; (b) glass microfluidic device K$^{18}$F/K$_{222}$, K$_2$CO$_3$, ACN, 190 °C, condition used in automated microfluidic synthesis; (c) 3 mol NaOH, DCM/MeOH (9:1), 45°C, 20 min; (d) 3 M HCl, in manual and sodium citrate buffer (pH 2.79) in microfluidic synthesis, was used for pH adjustment (pH 2–3), (e) platinum aqua complex, 82 °C, 30 mins.

Figure 18: Sample was run on Waters® 4µ C18 150 x 3.9 mm column; ACN/Water (0.1% TFA) 25/75 with flow rate of 1mL/min; Fig A. RT = 3.38 min. of Radioactivity $[^{18}\text{F}]-\text{FPMA}$; Fig B: HPLC profile of Platinum coordination reaction; Fig. C: HPLC profile after purification RT=1.43 min of $[^{18}\text{F}]-\text{FCP}$. 
5.3.4 Biodistribution and *in vivo* Stability Analysis

Table 10 and Fig.19 show the biodistribution of [\(^{18}\text{F}\)]-FCP in female nude mice. The radiotracer demonstrated rapid blood clearance with less than 2\% of radioactivity remaining in the circulation after 1 h. The blood half life was calculated to be 10-12 mins. Most organs showed low accumulation of the radiotracer and radioactivity was predominantly cleared through the kidneys with greater than 95\% cleared 1 h after injection.

The *in vivo* stability of [\(^{18}\text{F}\)]-FCP in plasma and urine was assessed during the first 60 mins after intravenous administration in normal nude mice. The stability of [\(^{18}\text{F}\)]-FCP was analyzed by radio-HPLC at 5, 30, 60, and 90 mins post administration. The radio HPLC peak of the plasma samples injected appeared at the same retention time as the initial injected radiotracer (Fig. 20), demonstrating an intact [\(^{18}\text{F}\)]-FCP 60 min-post injection. No radioactivity was detected in 90 min samples which could be attributed to the fast physiological clearance of the radiotracer from the blood. The radio HPLC peak of the urine samples injected also appeared at the same retention time as the initial injected radiotracer demonstrating the intact [\(^{18}\text{F}\)]-FCP (data not shown).

Table 10: Biodistribution of intravenously injected [\(^{18}\text{F}\)]-FCP in nude mice (n=3 per time point). Data are presented as %ID/g (mean±sem) values determined through gamma counting

<table>
<thead>
<tr>
<th>Organs</th>
<th>5 Mins</th>
<th>30 Mins</th>
<th>90 Mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4.56±0.67</td>
<td>1.09±0.62</td>
<td>0.99±0.57</td>
</tr>
<tr>
<td>Heart</td>
<td>1.5±0.09</td>
<td>0.62±0.36</td>
<td>0.71±0.41</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.20±0.018</td>
<td>1.27±0.73</td>
<td>1.37±0.79</td>
</tr>
<tr>
<td>Liver</td>
<td>4.54±0.50</td>
<td>3.48±2.01</td>
<td>2.28±1.31</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.43±0.50</td>
<td>0.91±0.53</td>
<td>1.70±0.98</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.36±0.37</td>
<td>0.75±0.43</td>
<td>0.38±0.22</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.84±0.31</td>
<td>2.28±1.31</td>
<td>1.65±0.95</td>
</tr>
<tr>
<td>Kidneys</td>
<td>10.47±2.77</td>
<td>4.98±2.88</td>
<td>4.63±2.67</td>
</tr>
<tr>
<td>Skin</td>
<td>4.40±0.70</td>
<td>1.30±0.75</td>
<td>1.13±0.65</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.56±0.73</td>
<td>0.69±0.40</td>
<td>0.678±0.38</td>
</tr>
<tr>
<td>Brain</td>
<td>0.22±0.01</td>
<td>0.10±0.06</td>
<td>0.12±0.072</td>
</tr>
</tbody>
</table>
Figure 19: Biodistribution of [18F]-FCP in normal adult female nude mice (n=3) at 5, 30, 90 mins after injection. Radio tracer uptake in % ID/g was determined by gamma counting.

<table>
<thead>
<tr>
<th>Organ</th>
<th>5 mins</th>
<th>30 mins</th>
<th>90 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur</td>
<td>1.02±0.17</td>
<td>0.78±0.45</td>
<td>0.53±0.30</td>
</tr>
</tbody>
</table>

Figure 20: Plasma sample was run on Waters® 4u C18 150 x 3.9 mm column; ACN/Water (0.1% TFA) 25/75 with flow rate of 1mL/min at different time point; Fig. A: 05 min; Fig. B: 30 min; Fig. C: 60 min.
5.3.5 PET Imaging Studies

$[^{18}\text{F}]-\text{FCP}$ imaging in nude mice was performed to establish PET imaging feasibility and biodistribution. Dynamic images acquired for 90 mins illustrate the full body distribution of $[^{18}\text{F}]-\text{FCP}$ (Fig. 21). $[^{18}\text{F}]-\text{FCP}$ had the highest uptake in kidneys. Accumulation of radiotracer was also seen in lungs and liver in early time points. High uptake in the kidneys seems to reflect urinary excretion of the tracer which can be attributed to the radioactivity present in the urine during the scan. Images showed the subsequent clearance of radiotracer with time. Ex-vivo biodistribution data on the tissue distribution correlated to the imaging data.

Figure 21: A female nude mouse was injected (i.v) with 5.5-6 MBq of $[^{18}\text{F}]-\text{FCP}$ derivative and imaged using an Inveon PET/CT scanner (SIEMENS,USA). Dynamic image data acquired between 5, 30, 60 and 90 minutes post injection was reconstructed (FBP and OSEM/MAP) and analyzed.
Static summed up images acquired 90 mins post radiotracer administration illustrate the full body distribution of $^{18}$F-FCP in tumor bearing mice (Fig. 22). Major uptake was observed in liver and abdominal region. Quantification of the 90-min static summed up scans of xenograft tumors (KB3-1 and KB 8-5) with ROI analysis showed differential uptake of radiotracer in these tumors. Images showed a significant difference in uptake between Pgp (+ve) and Pgp (-ve) tumor.

Figure 22: Nude mice were innolated with human cervical adenocarcinoma (KB) cell lines to establish a xenograft model with one P-glycoprotein (P-gp) non expressing (KB 3-1) and one Pgp expressing (KB 8-5) tumor per animal. $^{18}$F-FCP (5.5-6 MBq) was injected intravenously in xenograft nude mice via tail vein injection. Imaging was carried out 90 mins post injection using Inveon PET/CT (Siemens, USA). Static summed up image acquired 90 mins post injection was reconstructed (FBP and OSEM3D/MAP) and analyzed. Image showed that P-gp non expressing (KB 3-1) tumor has slightly higher uptake of radiotracer than P-gp expressing KB 8-5 tumor.
5.3.6 Protein expression and Western Blotting
The PET image of the KB-3-1 and KB-8-5 tumor bearing mice showed subtle difference in the accumulation of $[^{18}\text{F}]-\text{FCP}$. To determine whether this differences in the accumulation was attributed to level of P-gp, tumors from the imaged mice were taken, homogenized and immunoblotted against P-gp. As a loading control β-actin level in the tumor were analyzed. The results after normalizing the signal of P-gp to β-actin showed that the level of expression of P-gp in KB-8-5 tumor was twice compared with KB-3-1 tumor.

5.4 Discussion
One of the major hindrances in platinum therapy is the assessment between the amount of drug administered, the amount of drug that reaches the targeted therapeutic site and overall therapeutic efficacy of the treatment. Non-invasive studies of the tissue concentrations are rare using radiolabeled platinum. Areberg et al [18] studied the antitumor effect of radioactive cisplatin ($^{191}\text{Pt}$) on nude mice and demonstrated that $^{191}\text{Pt}$-cisplatin is a more effective drug than cisplatin in retarding tumor growth on tumor growing nude mice. Patient studies by Areberg et al [17, 19] demonstrated the use of radiolabeled with $^{191}\text{Pt}$, $^{193m}\text{Pt}$, $^{195m}\text{Pt}$ to visualize the uptake of platinum in tumors and tissues non-invasively after cisplatin treatment. Dowell et al [20] used $^{195m}\text{Pt}$ labeled cisplatin and carboplatin to estimate the amount of platinated drug and its metabolites at the tumor site and at selected organs using noninvasive imaging. However, the major problems with the known platinum radionuclides ($^{191}\text{Pt}$, $^{193m}\text{Pt}$, $^{195m}\text{Pt}$, $^{197}\text{Pt}$) is the limited supply of the highly enriched platinum isotope needed for the reactor production, and that the cross sections needed for the production of platinum radionuclides from cyclotron is not very favorable [20].
This study demonstrated the feasibility of utilizing our previously published work on \([^{18}\text{F}]-\text{FPMA}\) [21] in the synthesis of \([^{18}\text{F}]-\text{FCP}\). Ease of production and well suited for routine use of \(^{18}\text{F}\) in nuclear medicine application, \(^{18}\text{F}\) remains the radionuclide of choice for PET/CT. In this project, we have presented the results of the radiosynthesis of \([^{18}\text{F}]-\text{FCP}\), a theranostic anticancer drug for imaging and therapy, and its feasibility of in vivo imaging using PET in animal model. This project addressed a major need to enhance the overall efficacy of platinum-based chemotherapy in solid tumors, by introducing F-18 as a molecular PET imaging entity as part of the drug to enable the measurement of drug concentration in tumors and normal tissues. An important finding of the study was the very high stability of the drug in plasma and urine at 60 mins after administration (Fig 20). Such stability ensures that the drug is intact in vivo and the radioactivity measured throughout the study (in vivo imaging and ex vivo analysis) originates from the intact drug instead of the potential metabolites.

\([^{18}\text{F}]-\text{FCP}\) exhibited uniform pattern of tissue uptake in tissues and tracer clearance followed monoexponential blood kinetics. The blood half life is calculated to be 10-12 mins which is similar to the value presented in the literature for native carboplatin [22]. Following intravenous injection of \([^{18}\text{F}]-\text{FCP}\) in normal nude mice, quantification of the dynamic images of the 90 mins, the highest activity was recorded in kidneys, bladder and urine. Accumulation of \([^{18}\text{F}]-\text{FCP}\) was also recorded in liver and lungs in early time points with subsequent clearance with time which is consistent with the findings by Ginos et al [23]. Ex-vivo biodistribution data corresponded to the in-vivo findings with major accumulation of radiotracer in kidneys and liver. In vitro cytotoxicity assay on various cells lines demonstrated that \(^{19}\text{F}-\text{FCP}\) kills cancer cells with greater efficacy than carboplatin.
5.5 Conclusion

An automated radiosynthesis of $^{18}$F-FCP was developed, using our previously developed $^{18}$F-FPMA radiotracer. *In vivo* data showed a rapid blood clearance and accelerated renal elimination of intact radiotracer. Feasibility of non-invasive imaging and quantitation of $[^{18}\text{F}]$-FCP was demonstrated in normal and tumor bearing nude mice.
NMR Fig 1: $^1$H-NMR and $^{13}$C-NMR of Diethyl 2-(5-bromopentyl)-2-methylmalonate
NMR Fig 2: $^1$H-NMR and $^{13}$C-NMR of Diethyl 2-methyl-2-((tosyloxy)pentyl)malonate
NMR Fig 3: $^1$H-NMR and $^{13}$C-NMR of Diethyl 2-(5-fluoropentyl)-2-methylmalonate
NMR Fig4: $^{19}$F-NMR of Diethyl 2-(5-fluoropentyl)-2-methylmalonate
NMR Fig 5: $^1$H-NMR and $^{13}$C-NMR 2-(5-Fluoropentyl)-2-methylmalonic acid
NMR Fig 6: $^{19}$F-NMR of 2-(5-Fluoropentyl)-2-methylmalonic acid
NMR Fig 7: $^1$H-NMR, $^{19}$F-NMR and $^{195}$Pt-NMR [F]-FCP
References


CHAPTER 6: LIPOSOMAL CARBOPLATIN: A NOVEL DOUBLE RADIOLABELED NANOCONSTRUCT FOR DUAL TRACER IMAGING AND THERAPY
6.1 Introduction

This chapter deals with the development of liposomal carboplatin formulation. This chapter presents the development of $^{111}$In-labeled Liposomes and its in vivo evaluation in normal nude mice. Further, $^{18}$F-FCP encapsulation of $^{111}$In-labeled Liposomes to develop a dual tracer labeled nanocarrier and its in vivo evaluation in two different tumor xenograft bearing nude mice is also presented in this chapter.

Liposomes are self assembling vesicles composed of lipid bilayers and an enclosed fraction of the surrounding aqueous medium [1]. Pharmacokinetics and distribution of liposomes can be manipulated by modifying their size, charge, composition and surface modification [2, 3]. Amenable surface modification, amphiphilic nature and biodegradable properties of liposome make them an appealing vehicle for drug delivery. Several attempts of radiolabeling liposomes using various isotopes have been tried for a variety of reasons. The major aim of all of these studies using radiolabeled liposomes is to follow the fate of liposome in vivo and to measure the distribution of the nanoparticles quantitatively ex vivo. Information on the in vivo behavior of liposomes helps in optimizing the liposome for novel imaging and therapeutic approaches.

Varieties of techniques have been developed to label liposomes with radioisotopes. Radionuclides such as $^{99m}$Tc, $^{186}$Re, $^{67}$Ga, $^{111}$In were attached to the liposome using chelators or by encapsulating them in aqueous cavity [4]. This project focuses on labeling liposomes with $^{111}$In. Diethylenetriaminepentaacetic acid (DTPA)-derivatized liposomes have been widely used to radiolabel with $^{111}$In [5]. Harrington et al [6] reported detailed biodistribution and pharmacokinetics of $^{111}$In-DTPA-labelled pegylated liposome in nude mice bearing a human cancer xenograft. Umeda et al [7] demonstrated the imaging ability

This project focuses on designing $^{111}$In-Labeled Liposome as a drug delivery vehicle for $^{18}$F-FCP. To our knowledge, there is no study on the synthesis and development of carboplatin that combines $^{18}$F as a molecular imaging entity. Furthermore, there is no study that encapsulates $^{18}$F-FCP inside $^{111}$In-Labeled Liposome. Therefore, this project is quite novel in determining the fate of both the drug and the vehicle in vivo using non invasive multimodal imaging. This project develops a novel dual tracer labeled liposomal nanocarrier as a multimodal imaging/therapy paradigm.
6.2 Experimental

6.2.1 Chemicals

All chemicals were used as received without further purification. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (ammonium salt) (DPPE-DTPA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1, 2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[methoxy(polyethyleneglycol)-2000] ammonium salt (DSPE-PEG\textsubscript{2000}) was bought from Laysan Bio, Inc. (Arab, AL, USA). Cholesterol (Chol), cis-diamminedichloridoplatinum (II) (Cisplatin), cis-Diammine(1,1-cyclobutanedicarboxylato)platinum(II) (Carboplatin) were purchased from Sigma-Aldrich (Steinheim, Germany). Sephadex-G25 and all other materials were purchased from VWR.

Flash chromatography was performed employing Sigma-Aldrich 230-400 mesh silica gel. TLC (Sigma-Aldrich, Saint Louis, MO) was performed using silica gel-coated aluminum plates with F-254 indicator (250 mm, 20 cm20 cm, Whatman). NMR (Piscataway, NJ) spectra (1H-NMR, 13C-NMR, and 19F-NMR, 195Pt-NMR) were obtained using Varian Mercury 300 MHz and Varian Inova 400 MHz (Sigma-Aldrich, Saint Louis, MO) using tetramethylsilane as an internal standard. Aqueous \textsuperscript{18}F-fluoride was produced by the \textsuperscript{18}O (p,n)\textsuperscript{18}F reaction, in a PET tracer cyclotron (GemMedical Systems, Wausheka, WI, USA), by the irradiation of an isotopically enriched \textsuperscript{18}O water (Rotem Industries, Ber Sheva, Israel) target. For fluoride trapping, Sep-Pak Light quaternary methyl ammonium (QMA) cartridge was used in manual synthesis from Waters Corp. (Milford, MA, USA). Radio-HPLC stability analysis was carried using a Waters HPLC pump (Waters, Model 1525) equipped with UV detector (Waters, Model
and radiation detector (Bio-scan, Model B-FC-3300) connected in series. For reverse phase HPLC, a Waters® Nova-Pak 4µ C18 150 x 3.9 mm column was eluted isocratically with 25/75 ACN/Water containing 0.1%TFA at flow rate of 1 mL/min. Radio-TLC was performed on a radio-TLC scanner (BioScan, Model AR/2000). Radioactivity was measured with dose calibrator Captintec CRC-15 PET. Animals, normal adult athymic female nude mice, were purchased from Harlan laboratories, USA. 

\(^{111}\)In-indium chloride in 0.05M HCl (specific activity: 15.70mCi/mL) was purchased from Perkin-Elmer (Wellesley, MA) and Triad Isotopes (Richmond, VA).

### 6.2.2 \[^{111}\text{In}\]-Labeled Liposome Preparation

PEGylated liposome was synthesized using the method described by Mougin-degraef et al [10]. Briefly DPPC, Chol, DSPE-PEG\(_{2000}\), DPPE-DTPA (60:30:5:5 mole ratio) were mixed in a 10 ml vial and dissolved with Chloroform: Methanol (CHCl\(_3\):MeOH, 9:1). The solvent was evaporated under rotary evaporator to form a lipid film followed by high vacuum to remove the residual organic solvent. Dulbecco’s phosphate buffered saline (PBS; 1ml) was added and the suspension was sonicated at 50-60ºC for 10 mins. At the end of the incubation, the liposome solution was immediately chilled in ice. The resulting vesicles were extruded through a series of polycarbonate membrane at 50-60ºC. The extruded liposomes were purified using gel chromatography on Sephadex G-25 with PBS as an eluent.

### 6.2.3 Labeling Procedure

PEGylated liposome was labeled with \(^{111}\text{In}\) as previously described by Chow et al.[13]. Briefly, 500 µL of preformed liposome was incubated with 20 µL of \(^{111}\text{InCl}_3\) (Indium Chloride in 0.05 M HCl; 3.7–74 MBq) (Perkin Elmer/Triad Isotopes) in 20 µL of 3M
sodium acetate buffer (pH 5.2) and then incubated at 40ºC for 30 min. Unincorporated $^{111}$In was purified using gel chromatography (Sephadex G-25). The labeling efficiency was determined by measuring the activity in dose calibrator (CRC-15R, Capintec; Bioscan) for the liposome, filter and supernatant.

6.2.4 Biodistribution of $[^{111}\text{In}]}$-Labeled Liposome:
Animal experiments were approved and performed according to the policies and guidelines of the Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. Adult female nude mice were injected with $^{111}$InCl$_3$, $^{111}$In-DTPA, $^{111}$In-DTPA-DPPE, and $^{111}$In-Liposome through tail vein. The amount of radiotracer injected was (1.07± 0.03 MBq) in 200 µL PBS. At different time points post injection (1hr, 6hrs, 48hrs), mice were euthanized and key tissues were harvested. Tissues were counted in gamma counter and percent injected dose per gram (%ID/g) of the tissue was determined.

6.2.5 In vivo SPECT/CT Imaging of $[^{111}\text{In}]}$-Labeled Liposome
Micro-SPECT was performed using a multimodal (DPET/SPECT/CT) preclinical imaging system (Siemens, USA) having dual-head camera mounted with 2 multipinhole collimators (five 1.0-mm pinholes in each collimator, 51-mm trasaxial FOV, 40-mm radius of rotation and maximum resolution of 1.5 mm). Images were acquired over 360º in a total of 40 projections, resulting in a total imaging time of 30 min. Nude mice were intravenously injected with $[^{111}\text{In}]}$-Labeled Liposomes (8MBq, 200 µL) and 2, 48, 144 hrs later, were anesthetized with mixture of 2% isoflurane in oxygen and whole body micro-SPECT imaging was carried out in prone position as described above. Micro-CT was also performed with 75 kV and 500 µA at a resolution of 96 µm. The whole body scan time was 10 min. The SPECT images were reconstructed using an iterative
reconstruction algorithm (ordered-subset expectation maximization or OSEM3D) modified for the 5-pinhole geometry with a 20% energy window around the 171 keV photo peak of $^{111}$In. These images were then registered with CT images based on a transformation matrix previously generated using four $^{57}$Co landmarkers. Images were viewed and quantified using ASIpro, the image data analysis software. Regions of interest (ROIs), covering the entire organs, were drawn and the average counts were measured and the data was correlated to ex vivo biodistribution result from gamma counting.

6.2.6 Synthesis of $[^{18}\text{F}]$-FCP encapsulated $[^{111}\text{In}]$-Labeled Liposomes

6.2.6.1 Synthesis of $[^{18}\text{F}]$-FCP
$[^{18}\text{F}]$-FCP was synthesized using an automated synthesis method using Allinone Synthesizer (Trasis, Belgium) as described previously in section 5.2.4.

6.2.6.2 Labeled Liposome Preparation and Drug Loading
PEGylated liposome was synthesized using the method described by Mougin-degraef et al.[10]. Briefly, DPPC, Chol, DSPE-PEG$_{2000}$, DPPE-DTPA (60:30:5:5 mole ratio) were mixed in 10 ml vial and dissolved with Chloroform:Methanol ($\text{CHCl}_3$:MeOH, 9:1). The solvent was evaporated under rotary evaporator to form a lipid film followed by high vacuum to remove the residual organic solvent. Freshly synthesized $[^{18}\text{F}]$-FCP in PBS was added to the lipid film and was sonicated for 10 min at 60°C. At the end of sonication, the liposome solution was immediately chilled in ice. For the membrane radiolabeling with $^{111}$In, sodium acetate buffer (3M, pH 5.2) was added to liposome solution. $^{111}$InCl$_3$ was buffered with sodium acetate buffer (3M, pH 5.2) for 2 mins before incubating with buffered liposome solution at 40°C for 30 mins. The resultant dual labeled liposome nanoconstruct was purified using gel chromatography (Sephadex G-
The purified liposome nanoconstruct was further used for characterization and *in vivo* studies.

### 6.2.6.3 Characterization
Zeta Sizer Nano Series ZEN3600 was used to measure the hydrodynamic size and $\zeta$ potential of liposomes in PBS (pH~7.2). The radioactive reaction yield was calculated from gamma counting. The labeling and encapsulation efficiency were determined by counting the liposome suspension before and after chromatography on a PD-10 (Sephadex G-25) with a dose calibrator (CRC-15R, Capintec; Bioscan).

### 6.2.6.4 In Vivo Dual Tracer Imaging with Trimodal PET/SPECT/CT
Female nude hairless mice (Harlan Laboratories, USA), aged four-six weeks, inoculated with KB 3-1 and COLO 205 on dorsal regions on left shoulder and right flank respectively were employed for the *in vivo* imaging. $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]$-Labeled Liposome was administered via tail vein injection. Each mouse was injected with total activity of 15MBq of $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]$-Labeled Liposome. One hour post administration, mice were anesthetized by Isoflurane (2.0% flow rate) and kept under nose cone set up for imaging. Static summed up PET image data 60 minutes post injection was acquired for 30 mins. At completion of the PET imaging, without moving the specimen, the mouse bed was moved to the SPECT/CT imaging planes. A SPECT scan was performed using dual-head camera mounted with 2 multipinhole collimators (five 1.0-mm pinholes in each collimator, 51-mm trasaxial FOV, 40-mm radius of rotation and maximum resolution of 1.5 mm). Images were acquired over $360^\circ$ in a total of 40 projections, resulting in a total imaging time of 60 mins. Micro-CT was also performed.
with 75 kV and 500 μA at a resolution of 96 μm. The whole body scan time was 10 min (Fig. 23).

After the scan, the mice were returned to the cage. The same mice were SPECT scanned 24, 48 and 72 hours post administration.

![Figure 23: Sequential trimodal in vivo imaging with PET and SPECT probes was achieved through the above workflow. A. Injection of $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposomes via tail vein injection; B. PET imaging, C. SPECT imaging; D. X-ray CT imaging (modified from reference 18).](image)

### 6.2.7 Cell lines and Culture Conditions

Human cervical carcinoma (KB-3-1) and colorectal adenocarcinoma (COLO-205) cell lines were maintained in humidified incubator at 37 °C and 5% CO₂. Briefly, KB-3-1 cells were grown in DMEM/High glucose medium supplemented with 10% fetal bovine serum (FBS), 5mM L-Glutamine, Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (0.25 µg/ml). COLO-205 cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS Penicillin G (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (0.25 µg/ml). The cells were grown to 70-80% confluency before using for the experiments.
6.2.8 Xenograft Animal Model

Female athymic nude mice, (4-6 weeks old, weight: 18-25 g) were purchased from Harlan Laboratories, USA and provided with food and water ad libitum. Animal experiments were approved and performed according to the policies and guidelines of the Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. KB-3-1 and COLO 205 cells were cultured as mentioned above and collected for implantation. Tumor cells (2.5×10^6/100µL (KB 3-1), 5.0×10^6/100µL (COLO 205) in media without serum) were injected subcutaneously into the dorsal region on the right flank (COLO 205) and left shoulder (KB 3-1) of athymic nude mice. Following subcutaneous implantation digital caliper measurement of tumor size was accessed, once the bulge caused by the tumor growth at the injection site was visible, (approximately 5 days). The tumor volume was calculated using the formula 0.523 × (length × width × thickness) and assessed thrice per week.

6.3 Results

6.3.1 [\(^{111}\text{In}\)]-Labeled Liposome Characterization

The mean diameter of PEGylated empty liposome was 168 nm, \(\zeta\) potential was -2.3mV. Labeling efficiency of \([^{111}\text{In}]\)-Labeled Liposome was greater than 90%.

6.3.2 Biodistribution Studies

6.3.2.1 Biodistribution of \(^{111}\text{InCl}_3\)

The biodistribution study performed with \(^{111}\text{InCl}_3\) showed major accumulation of \(^{111}\text{InCl}_3\) in liver (33.10±2.24 %ID/g) and spleen (12.35±1.82 % ID/g) at 6 hours post injection. The activity was subsequently cleared to (16.24±1.49 %ID/g) and (6.92±0.37% ID/g) at 48 hours in liver and spleen respectively. However, the kidney uptake was increased with
time (6.86±0.60, 6 hours to 14.54±1.48%ID/g, 48 hrs) suggesting renal clearance of $^{111}$In in later time point. Data representing the biodistribution profile is demonstrated in Table 11 and Fig. 24.

Table 11: Biodistribution of tail-vein injected $^{111}$InCl$_3$ (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g

<table>
<thead>
<tr>
<th>Organs</th>
<th>1hr</th>
<th>6hrs</th>
<th>48hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.03±0.08</td>
<td>2.12±0.21</td>
<td>0.63±0.13</td>
</tr>
<tr>
<td>Heart</td>
<td>1.63±0.11</td>
<td>1.06±0.17</td>
<td>1.48±0.08</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.93±0.12</td>
<td>1.93±0.27</td>
<td>2.21±0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>17.09±0.21</td>
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<td>16.24±1.49</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.73±0.52</td>
<td>12.35±1.82</td>
<td>6.92±0.37</td>
</tr>
<tr>
<td>Stomach</td>
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<td>0.92±0.36</td>
</tr>
<tr>
<td>Intestine</td>
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</tr>
<tr>
<td>Kidneys</td>
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<td>6.86±0.60</td>
<td>14.54±1.48</td>
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<tr>
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<td>Muscle</td>
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<td>0.86±0.15</td>
</tr>
<tr>
<td>Skull</td>
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<td>1.82±0.25</td>
<td>4.43±2.02</td>
</tr>
<tr>
<td>Brain</td>
<td>0.11±0.01</td>
<td>0.11±0.01</td>
<td>0.14±0.00</td>
</tr>
<tr>
<td>Femur</td>
<td>0.75±0.12</td>
<td>1.50±0.42</td>
<td>2.04±1.26</td>
</tr>
</tbody>
</table>
Figure 24: Biodistribution of tail-vein injected $^{111}$InCl$_3$ (1.07±0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate %ID/g.

### 6.3.2.2 Biodistribution of [$^{111}$In]-DTPA

Table 12 and Fig. 25 show the detailed biodistribution performed in nude mice. The biodistribution study performed with [$^{111}$In]-DTPA showed rapid accumulation of [$^{111}$In]-DTPA in kidneys (2.51±1.38 %ID/g) at 1 hour post injection. Rapid clearance from blood and minimal uptake was observed in other major organs. Major concentration of activity cleared out within an hour of administration.

Table 12: Biodistribution of tail-vein injected [$^{111}$In]-DTPA (1.07±0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g.

<table>
<thead>
<tr>
<th>Organs</th>
<th>1hr</th>
<th>6hrs</th>
<th>48hrs</th>
</tr>
</thead>
<tbody>
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<td>Blood</td>
<td>0.06±0.02</td>
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<td>0.00±0.00</td>
</tr>
<tr>
<td>Tissues</td>
<td>1 Hour</td>
<td>6 Hours</td>
<td>48 Hours</td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Heart</td>
<td>0.04±0.01</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.12±0.02</td>
<td>0.03±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>0.14±0.06</td>
<td>0.08±0.01</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.05±0.01</td>
<td>0.03±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.04±0.01</td>
<td>0.02±0.01</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.08±0.03</td>
<td>0.03±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Kidneys</td>
<td>2.51±1.38</td>
<td>0.83±0.17</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>Skin</td>
<td>0.24±0.10</td>
<td>0.08±0.01</td>
<td>0.03±0.00</td>
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<tr>
<td>Muscle</td>
<td>0.10±0.06</td>
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<td>0.00±0.00</td>
</tr>
<tr>
<td>Skull</td>
<td>0.14±0.10</td>
<td>0.03±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Brain</td>
<td>0.03±0.01</td>
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<td>0.00±0.00</td>
</tr>
<tr>
<td>Femur</td>
<td>0.14±0.06</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Figure 25: Biodistribution of tail-vein injected [111In]-DTPA (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g.
6.3.2.3 Biodistribution of $[^{111}\text{In}]-$DTPA-DPPE

In Table 13 and Fig. 26, the biodistribution study performed with $[^{111}\text{In}]-$DTPA-DPPE lipid showed major accumulation of $[^{111}\text{In}]-$DTPA-DPPE in liver (34.11±13.39%ID/g) and spleen (2.43±0.98% ID/g) at 6 hours post injection. The activity in liver declined to 7.71±0.44%ID/g in 48 hours. However, the spleen uptake increased in later time point to (45.11±3.01%ID/g) 48 hours post injection.

Table 13: Biodistribution of tail-vein injected $[^{111}\text{In}]-$DTPA-DPPE (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as % ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g

<table>
<thead>
<tr>
<th>Organs</th>
<th>1hr</th>
<th>6hrs</th>
<th>48hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>17.59±4.53</td>
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<td>Heart</td>
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<td>Liver</td>
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<td>7.71±0.44</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.51±0.31</td>
<td>2.43±0.98</td>
<td>45.11±3.01</td>
</tr>
<tr>
<td>Stomach</td>
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</tr>
<tr>
<td>Intestine</td>
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</tr>
<tr>
<td>Kidneys</td>
<td>3.95±0.33</td>
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</tr>
<tr>
<td>Skin</td>
<td>0.97±0.27</td>
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</tr>
<tr>
<td>Muscle</td>
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<td>0.71±0.23</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>Skull</td>
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</tr>
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<td>Brain</td>
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<td>0.01±0.00</td>
</tr>
<tr>
<td>Femur</td>
<td>1.08±0.20</td>
<td>0.68±0.37</td>
<td>0.63±0.10</td>
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</tbody>
</table>
Figure 26: Biodistribution of tail-vein injected $[^{111}\text{In}]-\text{DTPA-DPPE}$ (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g.

6.3.2.4 Biodistribution of $[^{111}\text{In}]-\text{Labeled Liposome}$

The biodistribution study performed with $[^{111}\text{In}]-\text{Labeled Liposome}$ showed significant RES uptake. Prominent uptake of $[^{111}\text{In}]-\text{Labeled Liposome}$ in liver and spleen was observed, reaching maximum levels at 6 hours of 22.86±3.86, 73.33±12.13%ID/g respectively which decline gradually to (7.71±0.44%ID/g) and (45.01±2.94% ID/g) in 48 hours. Minimal uptake of $[^{111}\text{In}]-\text{Labeled Liposome}$ was observed in other major organs. The detailed biodistribution of $[^{111}\text{In}]-\text{Labeled Liposome}$ in normal nude mice is presented in Table 14 and Fig. 27.
Table 14: Biodistribution of tail-vein injected $^{111}$In-Labeled Liposome (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as % ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g

<table>
<thead>
<tr>
<th>Organs</th>
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<th>48 hrs</th>
</tr>
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<td>26.72±8.80</td>
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</tr>
<tr>
<td>Heart</td>
<td>5.83±0.73</td>
<td>1.11±0.05</td>
<td>0.21±0.00</td>
</tr>
<tr>
<td>Lungs</td>
<td>7.63±1.01</td>
<td>1.97±0.20</td>
<td>0.34±0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>15.05±1.07</td>
<td>22.86±3.86</td>
<td>7.71±0.44</td>
</tr>
<tr>
<td>Spleen</td>
<td>65.92±4.65</td>
<td>73.33±12.13</td>
<td>45.01±2.94</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.70±0.22</td>
<td>0.35±0.07</td>
<td>0.20±0.05</td>
</tr>
<tr>
<td>Intestine</td>
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<td>1.24±0.13</td>
<td>0.66±0.06</td>
</tr>
<tr>
<td>Kidneys</td>
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<td>Skin</td>
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<td>0.89±0.18</td>
<td>0.62±0.08</td>
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<tr>
<td>Muscle</td>
<td>0.73±0.21</td>
<td>0.39±0.09</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>Skull</td>
<td>3.35±0.48</td>
<td>0.94±0.21</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td>Brain</td>
<td>0.60±0.01</td>
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<td>0.01±0.00</td>
</tr>
<tr>
<td>Femur</td>
<td>1.28±0.10</td>
<td>0.99±0.18</td>
<td>0.6±0.10</td>
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</tbody>
</table>
Figure 27: Biodistribution of tail-vein injected \[^{111}\text{In}]\)-Labeled Liposome (1.07± 0.03) in nude mice (n=3 per time point. Data are presented as %ID/g (mean±sem) values determined through gamma counting. Tissues were collected, weighed and gamma emission was measured in a gamma counter to calculate % ID/g.

6.3.2.5 *In vivo* SPECT/CT Imaging of \[^{111}\text{In}]\)-Labeled Liposome

Radiolabeled liposome (8MBq/200 µL in PBS) was injected intravenously in a female nude mouse (28.55gms) via tail vein injection. Imaging was carried out using 171 keV energy gammas 2 hours, 48 hrs and 144 hrs post injection. The resulting images are depicted below in Fig. 28. The images show that prominent uptake of radiotracer was in liver and spleen in 2 hours which corresponding to the *ex vivo* biodistribution data. Later time points show the subsequent clearance of radioactivity from spleen and liver (Fig. 29).
Figure 28: In vivo SPECT imaging of normal nude mice injected with $[^{111}\text{In}]$-Labeled Liposome through tail vein 2, 48 and 144 hrs post injection. The images showed initial uptake of activity in RES and subsequent clearance with time.
Figure 29: The clearance profile of $^{111}$In-Labeled Liposome in specific tissues as shown in SPECT images measured by ROI analysis.

6.3.3 $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposomes

6.3.3.1 Size Measurement and ζ Potential.
Range of particle sizes was between 40 nm to 1μm with an average size of 221 nm.
Surface charge of liposome formulation was -2.11mV.

6.3.3.2 Entrapment and Labeling Efficiency:
Entrapment efficiency was calculated as the percentage of liposomal encapsulated $^{18}$F - FCP. Encapsulation efficiency of $^{18}$F-FCP was 38±2 % (n=3) of the liposomal formulation. The surface labeling efficiency of liposome with $^{111}$In was greater than 90% after 30 mins at 40º C.

6.3.3.3 Dual Tracer Imaging
In vivo dual tracer imaging was carried out in KB 3-1 (sensitive) and COLO 205 (resistant) tumor xenograft bearing nude mice following a sequential protocol as outlined in Fig 16 in experimental section. Sensitivity and resistance of tumor was established by in vitro cytotoxicity assay which was discussed in section 5.3.2. Mice received bolus injection of after an intravenous injection of $^{18}$F-FCP encapsulated $^{111}$In-Labeled
Liposomes. There was minimal animal manipulation because of the capability of automatic moving of bed between different imaging planes in trimodal system which conserves the full trimodal fusion. Since the dosing comes from the single nanoconstruct, that conserves the animal position, hence the spatial position was fixed for PET mode and SPECT mode. PET images acquired one hour post injection using the sequential protocol was automatically fused to CT. PET/CT image showed the uptake of radioactivity in RES, mainly liver and spleen. No bleed through of SPECT signal from $^{111}$In was visually noted in the PET images. SPECT images acquired using 171 keV $\gamma$’s from $^{111}$In one hour post injection was automatically fused to CT. SPECT/CT image demonstrated the accumulation of radioactivity in RES, mainly liver and spleen. This demonstrated the feasibility of dual tracer PET and SPECT imaging with $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]$-Labeled Liposomes using sequential protocol.

Comparative study performed using $[^{18}\text{F}]-\text{FCP}$ alone showed the accumulation of activity in kidneys and bladder (Fig. 30). Activity was also seen in bone marrow, COLO 205 tumor and KB 3-1 tumor. This implies that liposomal encapsulation of $[^{18}\text{F}]-\text{FCP}$ changed the pharmacokinetics of the $[^{18}\text{F}]-\text{FCP}$ by protecting it from systemic clearance through kidneys.
Figure 30: *In vivo* CT and PET/CT image of KB 3-1 (sensitive) and COLO 205 (resistant) tumor xenograft bearing nude mouse injected with 10 MBq of $[^{18}\text{F}]$-FCP through tail vein injection 1 hour post administration. Image shows the uptake of $[^{18}\text{F}]$-FCP in kidneys, bladder and marrow. KB 3-1(sensitive) has higher uptake than COLO205 (resistant). (A) Coronal Images (B) Volume Rendered Images
**Figure 31:** *In vivo* CT, PET/CT and SPECT/CT images of KB 3-1 (sensitive) and COLO 205 (resistant) tumor xenograft bearing nude mouse injected with 14 MBq of \[^{18}F\]-FCP encapsulated \[^{111}In\]-Labeled Liposome through tail vein injection 1 hour post administration. PET/CT Image shows the uptake of \[^{18}F\]-FCP encapsulated in \[^{111}In\]-Labeled Liposome RES. SPECT/CT image shows the uptake of \[^{18}F\]-FCP encapsulated in \[^{111}In\]-Labeled Liposome in RES. Both image corresponded to each other in the uptake profile demonstrating the feasibility of dual tracer imaging from a single nanoconstruct. (A) Coronal Images (B) Volume Rendered Images

**6.4 Discussion:**

Pegylated liposomal nanoparticles have been used as a vehicle for therapeutic agents. As a therapeutic agent, liposome should carry adequate load of drug.
Carboplatin, a cisplatin analog, is an anticancer drug used in clinic for the treatment of variety of solid tumors. Although carboplatin shows comparable efficacy to cisplatin in ovarian, bladder and esophageal carcinomas, it has inferior efficacy in germ cell tumors, head and neck cancer, and lung carcinomas [14]. Carboplatin comprises as a reasonable alternative to cisplatin in clinic for several treatment regimes. Afforded benefit of carboplatin over cisplatin is due to its toxicity profile. Carboplatin shows less nephrotoxicity, neurotoxicity and ototoxicity than cisplatin. However, myelosuppression is a dose limiting factor for cisplatin.

Resistance to platinum drug remains a huge obstacle in platinum therapy. Carboplatin resistance arises from reduced concentration of carboplatin reaching the tumor, increased efflux, intracellular detoxification by glutathione, increased DNA repair, decreased mismatch repair, defective apoptosis, and impairment in various signaling pathways [15]. Development of new platinum derivatives is prompted with an aim to deliver higher amount of platinum drugs to tumor and modulate the interaction with the DNA to overcome the resistance.

We addressed some of those limitations of platinum therapy by synthesizing a novel carboplatin derivative with non invasive imaging capability. We further extended the work by developing a radiolabeled liposomal formulation of the novel drug and investigated its in vivo pharmacokinetics using multimodal imaging. This study demonstrates the feasibility of optimal radiolabeling the DTPA-derivatized pegylated liposomes with $^{111}$In. Detailed in vivo biodistribution of $[^{111}$In]-Labeled Liposome contrast directly with the behavior of the components of liposome. $[^{111}$In]-DTPA cleared very rapidly from the circulation with most activity observed in kidneys suggesting renal
clearance which agrees to the data published by Harrington et al [6]. Pharmacokinetic profile of $^{111}\text{In}$-Labeled Liposome showed major uptake in RES. Major uptake was observed in spleen and liver. Gradual accumulation of radioactivity was observed in spleen with highest uptake (73.33±12.13 % ID/g) in six hours. Minimal accumulation of radioactivity was observed in other major organs. Subsequent clearance of radioactivity with time was observed in all organs. Feasibility of SPECT imaging with $^{111}\text{In}$-Labeled Liposome was demonstrated (Fig. 28). Images showed the initial accumulation of activity in liver and spleen which corresponds to the ex vivo biodistribution data. Extended imaging with $^{111}\text{In}$-Labeled Liposome was enabled because of the medium half life of $^{111}\text{In}$ ($t_{1/2}=2.8$ days). Images during later time points (48, 144 hours) showed the gradual clearance of activity from liver and spleen. In vivo biodistribution studies performed with various components of liposomes enabled comparative interpretation of $^{111}\text{In}$-Labeled Liposome with other components.

$^{18}\text{F}$-FCP was encapsulated into $^{111}\text{In}$-Labeled Liposome via passive encapsulation method. Encapsulation efficacy was 38% which was slightly higher than the values reported in the literature [16, 17]. Surface labeling with $^{111}\text{In}$ was greater than 90% which agrees to the value reported in literature [13].

Multimodal imaging has been very useful for researchers for a range of in vivo study. Single modality imaging such as SPECT, PET has been intensively used to gather in vivo functional data. Complexity of disease state and the unique pathways they express may not be easily visible for a single modality. Therefore, dual modality imaging further strengthens the capacity to gather robust data and can image different pathways simultaneously/sequentially which is otherwise not possible with a single modality.
Various researchers have presented dual modality imaging with combination of reporters and tracers. However, there are very few studies that combine two PET and SPECT radiotracers in a single nanoparticle. Chapman et al [18] demonstrated the dual tracer imaging feasibility of SPECT and PET probes in living mice using a sequential protocol. As part of the dosing protocol in the study Chapman et al injected SPECT tracer at first and acquired the SPECT images and injected the PET tracer after the SPECT scan is performed. Mastunari et al [19] studied the detection of viable myocardium using dual isotope simultaneous acquisition SPECT using $^{18}$F-FDG and 99mTc-sestamibi. To our knowledge, there is no study that performs the dual tracer PET/SPECT imaging from a radiolabeled drug encapsulated nanoparticle. In this study, we have demonstrated the feasibility of dual tracer sequential imaging with PET and SPECT using $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposome (Fig. 31). $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposome showed major uptake in RES in both PET and SPECT images. ROI analysis of SPECT image enabled by $^{111}$In corresponded with PET image enabled by $^{18}$F demonstrating the feasibility of dual tracer imaging from the single nanoconstruct. From our previous study with $^{111}$In-Labeled Liposome we observed the uptake of liposome in RES which is propagated in PET and SPECT images acquired from $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposome in an hour after administration. This suggests that the $^{18}$F-FCP is intact with $^{111}$In-Labeled Liposome. Comparison of PET images acquired with $^{18}$F-FCP alone (Fig. 30) and PET and SPECT images acquired with $^{18}$F-FCP is intact with $^{111}$In-Labeled Liposome showed different pharmacokinetic profiles. One important finding of this study was $^{18}$F-FCP uptake in KB 3-1 (sensitive) tumor was higher than COLO205 (resistant) tumor within an hour post intravenous
administration. The uptake of $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposome was not visible in tumors within an hour post intravenous injection. This can be attributed to the duration needed for the passive accumulation of liposome through EPR effect in tumors. Liposomal formulation of $^{18}$F-FCP changed the pharmacokinetics of carboplatin derivative. However, detailed in vivo evaluation using different mouse models and biodistribution studies is required to confirm the behavior of $^{18}$F-FCP encapsulated $^{111}$In-Labeled Liposome.

### 6.5 Conclusion

DTPA-derivatized pegylated liposome was labeled with $^{111}$In. $^{111}$In-Labeled Liposome was evaluated in vivo with biodistribution and SPECT imaging. $^{18}$F-FCP was encapsulated in $^{111}$In-Labeled Liposome. Dual tracer feasibility of PET/SPECT imaging with $^{18}$F-FCP was encapsulated in $^{111}$In-Labeled Liposome was demonstrated. Different pharmacokinetic profile of $^{18}$F-FCP alone and its liposomal encapsulation in $^{111}$In-Labeled Liposome was shown in tumor xenograft bearing nude mice.
References


CHAPTER 7: CONCLUSION AND FUTURE WORK
7.1 Summary of Conclusion
Radiolabeled carboplatin derivative $[^{18}\text{F}]-\text{FCP}$ has been synthesized with $^{18}\text{F}$ as a molecular imaging entity for the first time using malonic acid derivative. Manual synthesis of $[^{18}\text{F}]-\text{FCP}$ was optimized using low scale of activity. Purification of $[^{18}\text{F}]-\text{FCP}$ was optimized using anion exchange method. Automated synthesis of $[^{18}\text{F}]-\text{FCP}$ using radio synthesizer was optimized using the manual synthetic procedure and inserting them as macros for sequences. The radiochemical purity was characterized using radio-HPLC. Feasibility of in-vivo PET imaging of $[^{18}\text{F}]-\text{FCP}$ was demonstrated in normal and KB3-1 and KB8-5 tumor xenograft bearing nude mice. In vivo plasma and urine stability test confirmed the intact $[^{18}\text{F}]-\text{FCP}$ after intravenous administration. Detailed biodistribution in various tissues of $[^{18}\text{F}]-\text{FCP}$ was performed in normal nude mice.

Radiolabeled Pegylated $[^{111}\text{In}]-\text{Labeled Liposome}$ has been synthesized as a nanodrug delivery vehicle. Labeling efficiency was determined. The physiochemical characteristics, hydrodynamic size and zeta potential, were assessed using DLS. Feasibility of in vivo SPECT imaging with $[^{111}\text{In}]-\text{Labeled Liposome}$ was demonstrated. Detailed biodistribution of $[^{111}\text{In}]-\text{labeled Liposome}$ in various tissues was performed in normal nude mice. Comparative evaluation of tissue distribution of $[^{111}\text{In}]-\text{labeled Liposome}$ with $^{111}\text{InCl}_3$, $[^{111}\text{In}]-\text{DTPA-DPPE}$, and $[^{111}\text{In}]-\text{DTPA}$ was performed to determine the pharmacokinetic profiles of different components of liposome.

Finally, $[^{18}\text{F}]-\text{FCP}$ encapsulated $[^{111}\text{In}]-\text{Labeled Liposome}$; a dual labeled nanoconstruct, capable of providing two distinct PET and SPECT signals from a single nanoconstruct was developed. The physiochemical characteristics including size and zeta potential of
[\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome were assessed with DLS. Entrapment efficiency of [\textsuperscript{18}F]-FCP, labeling efficiency of [\textsuperscript{111}In]Cl\textsubscript{3} were determined. \textit{In vivo} PET imaging using [\textsuperscript{18}F]-FCP alone was performed in KB 3-1 and COLO 205 tumor xenograft bearing nude mice. Feasibility of dual tracer PET/SPECT imaging from a single nanoconstruct was demonstrated in KB 3-1 and COLO 205 tumor xenograft bearing nude mice. Comparison of pharmacokinetic profile of naked [\textsuperscript{18}F]-FCP with [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome demonstrated different routes of elimination of radiotracers.

\textbf{7.2 Future Work}

This is an ongoing work. The demonstration of dual tracer imaging feasibility of [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome establishes the proof of concept of liposomal formulation of a novel platinum derivative. However, this study did not address the intensive \textit{in vitro} characterization of [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome. \textit{In vitro} characterization such as plasma binding with naked [\textsuperscript{18}F]-FCP, [\textsuperscript{111}In]-Labeled Liposome and [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome needs to be established to get a detail understanding of the \textit{in vivo} properties of [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome. Detail biodistribution studies of [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome in normal and tumor xenograft bearing mice remains to be accomplished. Biodistribution study will demonstrate the pharmacokinetic behavior of [\textsuperscript{18}F]-FCP encapsulated [\textsuperscript{111}In]-Labeled Liposome. The information gathered from this will help interpret the behavior of the nanoconstruct which can further be compared with [\textsuperscript{111}In]-Labeled Liposome alone and [\textsuperscript{18}F]-FCP alone. Several factors must be considered when using a dual tracer probe for an optimal \textit{in vivo} imaging. Relative properties of each
radionuclide and its contribution to each modality must be configured before imaging. Although it is likely that PET detection of $[^{18}\text{F}]$-FCP excludes $[^{111}\text{In}]$-Labeled Liposome’s 171 keV energy, potential cross talk between $[^{18}\text{F}]$-FCP into the SPECT acquisition energy window and $[^{111}\text{In}]$-Labeled Liposome into the PET acquisition must be validated by phantom experiments.

The development of fluorinated carboplatin derivative lends a feasible platform for developing radiolabeled platinum derivatives with other isotopes. $[^{18}\text{F}]$-FCP enables PET imaging, a non invasive imaging technology that can be exploited to study the \textit{in vivo} pharmacokinetics of drug. Information such as drug distribution, drug ability for blood brain barrier penetration, bioavailability and tissue concentration and clearance of drug can be achieved by radiolabeled drug and non invasive imaging. $^{18}\text{F}$ is a positron emitter with a half life of 110 mins. Due to the short half life of $^{18}\text{F}$, $[^{18}\text{F}]$-FCP enables \textit{in vivo} pharmacokinetic studies for a short duration of time. Iodine-124 ($^{124}\text{I}$) is an isotope of iodine with a half life of 4.18 days. Its modes of decay are: 74.4\% EC and 25.6\% positron emission ($\beta^+\$). Feasibility of production of $^{124}\text{I}$ using a cyclotron offers an advantage because of the availability of cyclotron on site at VCU. Developing an $[^{124}\text{I}]$- labeled carboplatin derivative using malonic acid derivative will enable \textit{in vivo} PET imaging and prolonged \textit{in vivo} pharmacokinetic study which is otherwise difficult with $[^{18}\text{F}]$-FCP. Furthermore, encapsulation of $[^{124}\text{I}]$-labeled carboplatin derivative inside $[^{111}\text{In}]$-Labeled Liposome will enable dual tracer imaging using PET/SPECT. Liposomal formulation will change the pharmacokinetic profile of the drug and reduces the exposure of naked drug, hence less normal tissue toxicity and increased bioavailability. The medium half lives of
both isotopes (2.8 days of $^{111}$In, 4.8 days of $^{124}$I), enable prolonged in vivo study of the liposomal formulation using non invasive imaging.

In vivo therapeutic merit of $^{19}$F-FCP in comparison of parent compound needs to be demonstrated. Evaluation of the nephrotoxicity of $^{19}$F-FCP in comparison with its encapsulated formulation and the parent compound needs to be accomplished. Modulation of nephrotoxicity by nanoparticle free radical scavengers such as nanoceria should be investigated.

Developing $[^{18}$F]-FCP, $[^{124}$I]-labeled carboplatin derivative using an isotope of platinum, $^{195m}$Pt (half life 4.0 days, energy released from Auger electrons 23keV/decay) would enable dual therapy, cytotoxic and radiotherapy from the same platinum compound. This approach of dual therapy from Pt isotope with a molecular imaging entity attached to the radiopharmaceutical would provide theranostic and synergistic effect in cancer therapy and provide a platform in optimizing the response of therapy to treatment.
APPENDIX

Publications


Conferences and Presentations


Lamichhane N, Dewkar GK, Gobalakrishnan G, Thadigiri C, Zweit J. “Improved Radiosynthesis and In vivo Evaluation of $^{18}$F]-pentyl-2-methyl malonic acid for Apoptosis Imaging” Medical Physics Department BS session, Virginia Commonwealth University, Richmond, Virginia, December 2011.


VITA

Narottam Lamichhane was born in Mulpani, a small village in the suburbs of Kathmandu, Nepal. He graduated from Kathmandu Institute of Science and Technology in 2003. He received his Bachelor of Science in Applied Physics and Chemistry from Arcadia University, Glenside, PA in 2009. He moved to Richmond, VA in 2009 to start his graduate school in Medical Physics at Virginia Commonwealth University.