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UNIVERSITY OF CALIFORNIA

Los Angeles

PET Imaging of Nucleoside Metabolism
for Individualized Therapy

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular and Medical Pharmacology

by

Jason Thanh Lee

2012

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ABSTRACT OF THE DISSERTATION

PET Imaging of Nucleoside Metabolism for Individualized Therapy

by

Jason Thanh Lee

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2012

Professor Caius G. Radu, Chair

Molecular imaging diagnostics such as Positron Emission Tomography (PET) have significantly improved cancer patient management. PET allows the non-invasive, whole body imaging of metabolic processes and has been extensively used to detect lesions and monitor patient response to therapeutic intervention. Despite the development of a large number of PET probes, only a limited few find utility for imaging cancer. Furthermore, complex probe metabolism may render image interpretation difficult. With these factors in mind, my work focuses on improving PET imaging of nucleoside metabolism for cancer detection and prediction of treatment response.

The thymidine analog, 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT), is extensively used as a measurement of tumor proliferation. However, its use is limited in tissues of murine origin, which do not avidly take up ^{18}F -FLT. Here, we describe a pharmaceutical intervention using dipyridamole (DPA) to extend the utility of ^{18}F -FLT PET for imaging mice tissues and tumors.

Administration of DPA ten minutes prior to injection of probe allowed visualization of thymus, bone marrow and spleen tissues in C57/BL6 mice and murine tumor models of melanoma, lung carcinoma, and leukemia.

The recent development of 1-(2'-deoxy-2'- ^{18}F -fluoro- β -D-arabinofuranosyl)cytosine (^{18}F -FAC), a PET probe for deoxycytidine kinase (dCK), provides a new tool to measure the other arm of salvage metabolism. We show that, in addition to phosphorylation by dCK, ^{18}F -FAC is highly susceptible to deamination and limits the utility of the probe for measuring dCK activity. We describe the development of a panel of L-analog probes that maintain affinity for dCK, low susceptibility to deamination, high uptake in dCK-expressing cell lines, and biodistribution in mice reflective of the tissue-expression pattern of dCK. Two lead compounds, 1-(2'-deoxy-2'- ^{18}F -fluoro- β -L-arabinofuranosyl)cytosine (L- ^{18}F -FAC) and 1-(2'-deoxy-2'- ^{18}F -fluoro- β -L-arabinofuranosyl)-5-methylcytosine (L- ^{18}F -FMAC), were validated in mice models of leukemia and autoimmunity.

We show that (i) ^{18}F -FAC tumor uptake is influenced by cytidine deaminase (CDA), a determinant of gemcitabine resistance; (ii) PET imaging with ^{18}F -FAC and L- ^{18}F -FMAC can stratify tumors for dCK and CDA activities; and (iii) application of this PET assay can predict differential tumor responses to dCK-dependent nucleoside analog prodrugs, gemcitabine and clofarabine, the latter being resistant to deamination. These results support PET in patient stratification and predictive medicine.

Findings here demonstrate the complexities of nucleoside analog probe metabolism and their effect on image interpretation. Understanding the biological relevance of these measurements, combined with complementary modalities such as magnetic resonance imaging (MRI), can provide new opportunities for personalized medicine.

The dissertation of Jason Thanh Lee is approved.

Johannes Czernin

Oliver Dorigo

Arion-Xenofon Hadjioannou

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Caius G. Radu, Committee Chair

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2012

DEDICATION

To my parents, Sam and Amanda Lee;
And my grandparents, Luc Luu, Thai Ly and Muoi Luu.

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ACKNOWLEDGMENTS

I would like to show my sincerest gratitude for the invaluable mentorship of Drs. Caius Radu and Johannes Czernin. You have shown me on a daily basis the importance of responsible science and constant vigilance of clinical translatability. Caius, your persistent oversight of my progress and encouraging me always to be better than I was yesterday has left a lasting impression that I take with me wherever I go. Johannes, you have taught me the value of dedication in the face of unrelenting challenges and I will never forget our determination to triumph through life's marathons.

I would like to thank Drs. Henry Huang, Arion-Xenofon Hadjioannou, Oliver Dorigo, and Antoni Ribas for their insight and guidance throughout my graduate studies and being an integral part of my doctoral committee.

I would like to thank Drs. Hsiang-I Liao, David Nathanson, Michelle XiaoXiao Li, and Chengyi Jenny Shu for their friendship, guidance and providing a place to thrive with our passion for science.

I would like to thank Dr. Michael Phelps, Dr. Samson Chow, Dr. Sherly Mossessian, and Sarah Starrett who made this wonderful department a home.

I would like to thank Dr. Owen Witte, Dr. Nagichettiar Satyamurthy, members of the cyclotron staff, Dr. David Stout and members of the Crump Preclinical Imaging Facility for providing me with one of the world's best places to study.

I would like to thank Dr. Liu Wei and Larry Pang with whom I have had the pleasure of working in creating one of the best imaging facilities and providing a familial place to work.

I would like to thank the members of the Radu/Czernin lab: Dr. Dean Campbell, Amanda Armijo, Dr. Arun Singh, Dr. Jennifer Murphy, Colette Martin, Elizabeth Dimitrova, Jordan Wengrod, Andrew Tran, Gerald Toy, Dr. Rachel Laing, Ryan Darvish, Emily Fitch, and many other colleagues who made graduate life exciting to say the least.

I would like to thank Yang Yang, Dr. Koon-Pong Wong, Dr. Christiaan Schiepers, Dr. Shahriar Yaghoubi, Dr. Elliot Landaw, and Freddie Daver for the most enjoyable of discussions in mathematics, kinetic modeling and physics of molecular imaging.

I would like to thank Michelle Tom, Evan Shih, Jeremy Work, and Lisa Ta for giving me a chance to teach science and through them become a better teacher for it.

I would like to thank Dr. Martin Allen-Auerbach, Dr. Ken Herrmann, Dr. Chintda Santiskulvong and Amber Luke for bridging my work into the clinic.

I would like to thank my family: Sharon Lee Yeh, Karen Lee Hitchens, Jonathan Lee, Nelson Lee, Steve Yeh, John Hitchens, Kim Luu Pena, Rodrigo Pena, Asa Young, Isidro Pena, Audrey Pena, Benjawz, and Finney who provided me the support, encouragement and a place to call

home when I needed it most. You all kept me grounded and forever a child at heart. You are my inspiration.

I would like to thank Irene Wong for always having words of wisdom and being there for me whenever I needed you most, for showing me how to enjoy life, and, most importantly, your dear friendship. I came out of my hiding because you showed me how to know myself.

I would like to thank the entire UCLA Badminton Team with whom, several hours a week, I can find camaraderie, spirit, competition, and a place unlike any other in the world. I would like to thank my doubles partner Dr. Chih-Ping Wang who has never stopped coaching me and, by doing so, brought this greatest of sports to a level I could not have imagined.

I would like to thank Dr. Nicole Green who helped guide me through life's challenges.

I would like to acknowledge Dr. Chengyi Shu (co-author), Dr. Dean Campbell (co-author), Andrew Tran, Jordan Wengrod, Dr. Owen Witte, Dr. Michael Phelps, Dr. Nagichettiar Satyamurthy, Dr. Johannes Czernin, and Dr. Caius Radu for allowing me to reprint "Novel PET Probes Specific for Deoxycytidine Kinase", Journal of Nuclear Medicine 2010.

I would like to acknowledge Dr. Dean Campbell (co-author), Dr. Nagichettiar Satyamurthy, Dr. Johannes Czernin, and Dr. Caius Radu for allowing me to reprint "Stratification of Nucleoside Analog Chemotherapy Using 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-D-Arabinofuranosyl)Cytosine and 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-L-Arabinofuranosyl)-5-Methylcytosine PET", Journal of Nuclear Medicine 2012.

I would like to acknowledge the Journal of Nuclear Medicine for allowing me to reprint Chapter 3 ("Novel PET Probes Specific for Deoxycytidine Kinase") and Chapter 4 ("Stratification of Nucleoside Analog Chemotherapy Using 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-D-Arabinofuranosyl)Cytosine and 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-L-Arabinofuranosyl)-5-Methylcytosine PET").

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Lee JT, Nair-Gill ED, Rabinovich BA, Radu CG, Witte ON. Imaging in Immunology Research. In: Kiessling F, Pichler B, eds. Small Animal Imaging: Basics and Practical Guide. Berlin: Springer-Verlag, 2011:565-583.

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*Lee JT**, Campbell DO*, Satyamurthy N, Czernin J, Radu CG. Stratification of Nucleoside Analog Chemotherapy Using 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-D-Arabinofuranosyl)Cytosine and 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-L-Arabinofuranosyl)-5-Methylcytosine PET. J Nucl Med 2012 Feb;53(2):275-80. *contributed equally to this work.

Chapter 1:

Introduction

1.1. Background

Cancer remains the current leading cause of death in developed countries and the second in developing countries (1). In the United States, new cancer diagnoses, or incidences, among men declined 0.6% annually from 1998 to 2008 and among women, 0.5% from 1998 to 2006, without any change from 2006 to 2008 (2). Reasons for the decline can be attributed to three areas: risk factors, screening and treatment (3). As exemplified by colorectal cancer, treatment alone accounted for 12% of the reduction in mortality between 1975 and 2000 based on microsimulation screening analysis; the remaining 88% was due to a decrease in risk factors and increased screening (3). In connection with optimal treatment, timely diagnosis also affects overall survival, as evidenced by the higher cancer mortality rate in developing countries where the disease tends to be diagnosed at later stages (1). Development in diagnostic techniques with prognostic and predictive value will improve therapeutic efficacy, lessen toxicity, and expedite assessment of treatment response (4).

Cancer is a disease characterized by the uncontrolled proliferation of normal cells. The earliest documentations of cancer and treatment date back approximately four thousand years (5). Douglas Hanahan and Robert Weinberg described the disease as exhibiting six fundamental and two emerging hallmarks acquired by cancer cells to promote survival, proliferation and dissemination (6). Cancer, they state, exhibits sustained proliferative signaling, evades growth suppressors, enables replicative immortality, resists programmed cell death, induces angiogenesis, activates tissue invasion and metastasis, evades host immune destruction, and has reprogrammed its energy metabolism.

1.2. Nucleoside metabolism in cancer

Energy metabolism supplies nutrients to support the continuous growth and proliferation requirements of cancer cells. These include glycolysis, amino acid metabolism, lipid metabolism and nucleoside metabolism. Nucleoside metabolism provides the precursors for the synthesis of new strands of deoxyribonucleic acid (DNA) during replication, repair of damaged regions and recombination (7). Nucleosides are phosphorylated by their respective kinases to form nucleotides, which are subsequently incorporated into DNA strands by DNA polymerases. Proliferating mammalian cells require a steady supply of nucleotides for DNA replication and repair of the human genome, which consists of 3×10^9 base pairs (8). High fidelity in these processes is dependent on a balanced supply of all four nucleotides (9). Imbalance of pools due to endogenous perturbations, such as changes in DNA metabolic enzyme expression, or exogenous insults, such as drugs that inhibit these enzymes, may interfere with normal DNA synthesis. Nucleoside metabolism maintains nucleotide pools via two pathways: de novo synthesis and salvage metabolism. In de novo synthesis, carbon sources such as glucose are metabolized through a series of enzymatic reactions to form ribonucleotides, which are subsequently converted to deoxynucleotides via ribonucleotide reductase. Salvage metabolism consists of nucleoside kinases that sequentially phosphorylate nucleosides to their triphosphate derivatives. These kinases are required for the activation of many nucleoside analog (NA) prodrugs in cancer and are targets of molecular imaging diagnostics such as positron emission tomography (PET).

1.3. Nucleoside kinases

Genetic and phylogenetic studies suggest nucleoside kinases diverged from a common ancestor with broad substrate specificity for all four nucleosides (10). Mammalian nucleoside

kinases consist of two cytosolic enzymes, deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1), and two mitochondrial enzymes, deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2). Each maintains specificity for certain subsets of the nucleosides: dCK mainly for deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG); TK1 for thymidine (dT); dGK for dA and dG; and TK2 for dT and dC (11). While all four kinases are subjects of anticancer and antiviral drug development, most therapeutics is directed towards dCK and to a lesser degree TK1 (10). The following work focuses on the relevance of these two cytosolic enzymes in cancer, development of diagnostics to assay their functions in vivo and applications to cancer treatment.

1.4. Nucleoside kinases: deoxycytidine kinase (dCK)

dCK directly and indirectly catalyzes the phosphorylation of all four nucleosides in DNA synthesis. dA, dC, and dG are initially phosphorylated by dCK to form dATP, dCTP and dGTP. Deamination, the removal of an amine group by cytidine deaminases (CDA), catalyzes the conversion of dC to deoxyuridine, an intermediary step in thymidine triphosphate (dTTP) synthesis. dCK has the rare ability to phosphorylate both natural and non-natural enantiomeric nucleoside and NAs (10). dCK exists in its active form as a homodimer. Reports are mixed on the expression level of dCK throughout the cell cycle: some suggest dCK is constitutively expressed throughout the cell cycle; others report dCK levels increase during S phase with post-translational activation reported to be via phosphorylation at residue Ser-74, and that ATM and/or ATR may be key kinases (7, 12-14). It is also negatively regulated via feedback inhibition by dCTP. dCK is highly expressed in hematopoietic tissues such as thymus, spleen, bone marrow and lymph nodes.

dCK is overexpressed in many cancer subtypes, particularly in leukemia and lymphoma. While the specific role of dCK in cancer is unknown, a possible hypothesis is that dCK plays a role in DNA damage response and mediating replication stress (13). This is supported by findings in our group in genetically modified mice lacking dCK. These mice are viable, but exhibit deficiencies in T and B lymphocyte development with certain bone marrow lineages such as erythroblasts exhibiting a high degree of phosphorylated histone H2AX indicative of DNA damage (15). dCK is also required to activate a panel of NA pro-drugs including, but not limited to, gemcitabine cytarabine and clofarabine (8). Resistance to many of these pro-drugs has been directly linked to decreased dCK activity (16).

1.5. Nucleoside kinases: thymidine kinase 1 (TK1)

Thymidine kinase 1 (TK1) is the first step in dTTP production. It primarily phosphorylates thymidine, and has a much lower specificity for deoxyuridine (10). TK1 is found in the cytoplasm and exists as a dimer or as a tetramer, the latter having higher affinity for thymidine. It is highly regulated throughout the cell cycle at the transcriptional, post-translational and enzymatic levels (10). Protein levels and activity are absent in non-dividing cells, reach maximum levels during S-phase when DNA synthesis occurs, and quickly decline in the G₂/M transition by ubiquitin-proteasome degradation mediated by a signaling sequence in the C-terminus (10). Additionally, TK1 occurs as a homodimer or homotetramer, the latter having a 30-fold greater catalytic efficiency for thymidine than the former. It is proposed that the homodimer dominates during the G₁/S border, but as TK1 concentration increases during S due to increased transcription, TK1 exists as tetramers. The presence of the two forms act to fine-tune TK1 activity throughout the cell cycle (17).

TK1 is used as a biomarker of proliferation in cancer, which is reflected in the clinical use of the TK1 substrate, 3'-deoxy-3'-¹⁸F-fluorothymidine (¹⁸F-FLT), in PET for assaying cancer proliferation in vivo (18). It is also reported that serum TK1 levels increase in some cancers and may be used as a prognostic marker (19). Major therapeutics activated by TK1 include NAs such as azidothymidine and ganciclovir. The Herpes simplex virus 1 TK is extensively studied as a possible suicide gene in chemotherapy to enhance the phosphorylation of pro-drugs at target sites such as tumors (20).

1.6. Nucleoside analogs (NA) prodrugs

NAs consist of pyrimidine and purine chemotherapeutic agents, with efficacy in both liquid and solid tumors (21). A unique quality of NAs is their similarities in structure, but diversity in clinical activities (22). These compounds act through several mechanisms: induction of DNA strand breaks and chain termination; inhibition of key enzymes such as in DNA repair; activation of polymerases; induction of apoptosis. dCK, which accepts both D- and L-analogs and purine and pyrimidines, is responsible for the initial step of NA activation, and is oftentimes the rate-limiting step (7).

Resistance to NA therapy is an active area of study. Amongst these are deficiencies in dCK and overactive metabolism by cytidine deaminase (CDA). Prolonged incubation of cancer cells with NAs generated resistant subclones deficient in dCK (23). Indeed, dCK deficiency is a reported mechanism of NA resistance in several clinical studies (16). Deamination inactivates some NAs and has been implicated in resistance to these drugs (16). Inhibition of CDA by tetrahydrouridine improves pharmacokinetics and pharmacodynamics of deamination-sensitive NA prodrugs (24, 25).

Other mechanisms of resistance include: insufficient intracellular concentrations of NA-triphosphates due to poor transport or increased catabolism; inability to achieve sufficient alterations in DNA strands or dNTP pools; modifications of cellular response to stress triggered by NAs: DNA break/repair and/or defective induction of apoptosis (16).

1.7. Diagnostics in cancer

Tumor biomarkers are used to detect, diagnose, and manage various types of cancer and rely on sampling of blood, urine, stool, tumor tissue or other tissues or bodily fluids of cancer patients (26, 27). For example, detection of prostate-specific antigen in serum is routinely used in the staging of prostate cancer because it is often secreted at higher levels by prostate cancer cells than normal cells. Alpha-fetoprotein levels in blood help to diagnose liver cancers and germ cell tumors. Such assays face limitations in sensitivity and specificity: not all patients with the same cancer type express the biomarker while normal tissues may express them above normal ranges; it is not possible to detect tumor heterogeneity. Treatment predictive biomarkers such as the epidermal growth factor receptor (EGFR) require biopsies, which also may not detect heterogeneity nor metastasis.

Advances in imaging technologies such as computed tomography (CT), magnetic resonance imaging (MRI) and PET have allowed assessment of biomarkers at the whole body level with minimal invasiveness. MRI and CT are used to determine changes in tumor size and define response to therapy for solid tumors using the Response Evaluation Criteria In Solid Tumors (RECIST) (28). More recently, new criteria were proposed for clinical assessment of therapy response based on PET (29).

1.8. PET imaging in cancer

PET is a molecular imaging technology that measures the function of biological processes at the whole body level (30). In oncology, PET is used for the detection, staging and monitoring of cancer and its response to therapy (31). Clinicians use PET for identification of tumor lesions, evaluation of tumor response to therapy and identification of emerging resistance of metastases to treatment. Advantages of integrating PET into treatment selection and planning “include eliminating unnecessary diagnostic procedures, prompt selection of optimum treatment, improved radiotherapy targeting, avoidance of futile aggressive treatment, and improved quality of life” (32).

A large repertoire of PET probes are available that cover the range of hallmarks of cancer defined by Hanahan and Weinberg (33). Radiolabeled thymidine and thymidine analogs such as ^{11}C -thymidine, ^{18}F -FLT and 1-(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-thymine (FMAU) are potential biomarkers of proliferation and cancer replicative immortality, either as substrates of TK1 or the mitochondrial TK2 (34). ^{18}F -FDG PET takes advantage of cancer's evasion of growth suppressors and reprogramming of energy metabolism, resulting in increased glycolytic activity (35). Imaging cancer's resistance to cell death include markers of apoptosis such as radiolabeled annexin V (36). The induction of angiogenesis by cancer have been imaged with radiolabeled anti-VEGF antibodies or ^{64}Cu -VEGF, and the presence of hypoxia by radiolabeled fluoromisonidazole and other nitroimidazoles (37, 38). These biomarkers may complement and provide assessment of tumor metastatic potential (39). Indirect PET imaging with a reporter probe/reporter gene system allows visualization of cancer's interaction with immune cells such as in adoptive immunotherapy (40). The reprogramming of cancer metabolism includes other

metabolic pathways such as lipid metabolism, amino acid metabolism and nucleoside metabolism, each of which have been studied using PET (41, 42).

1.9. ^{18}F -FDG PET

Amongst those in metabolic imaging, 2-deoxy-2- ^{18}F -fluorodeoxyglucose (^{18}F -FDG) is by far the most extensively used in the clinic for cancer diagnosis, staging and monitoring of treatment (43, 44). Its utility is based on the ‘Warburg effect’, which states that tumor cells have elevated rates of glycolysis and lactate production regardless of oxygen availability, thereby allowing great contrast with most adjacent normal tissues (45, 46). ^{18}F -FDG is an analog of glucose and, upon transport into cells, is phosphorylated and trapped by the initial and rate-limiting enzyme, hexokinase. The addition of the phosphate group introduces a negative charge that prevents diffusion back across the plasma membrane, effectively trapping the probe in tissues of interest and allowing detection by PET scanners. ^{18}F -FDG has been successful in monitoring tumor response to treatment in lymphoma and several solid tumors. A limitation is its retention in non-malignant cells that also exhibit significant hexokinase activity, namely the brain and myocardium, but also the digestive tract, thyroid gland, skeletal muscle, bone marrow, and genitourinary tract and benign pathologic tissues present in healing bone, lymph nodes, joints, and sites of infection or inflammation (47). These physiologic regions of ^{18}F -FDG uptake limit the ability to differentiate cancer cells from surrounding normal tissue. More broadly, there exists a need for PET biomarkers that extend beyond glycolysis to differentiate the metabolic heterogeneity of tumor masses and guide development of drugs that target such pathways (46).

1.10. ^{18}F -FLT PET

Radiolabeled thymidine and thymidine analogs have been studied as potential markers of nucleoside metabolism. Thymidine is a key molecule in DNA synthesis and cellular proliferation because it is the only nucleoside that is incorporated into DNA, but not RNA, and is metabolized by cell-cycle dependent TK1 (48). The thymidine analog, ^{18}F -FLT, is the most widely studied probe for proliferation (48). It is transported into cells via nucleoside transporters and phosphorylated by TK1 and nucleoside kinases to its triphosphate form. Unlike thymidine, it is not susceptible to thymidine phosphorylase and lacks the 3'-hydroxy necessary for DNA incorporation. Applications include predicting treatment response to mTOR inhibitors and chemotherapeutics, monitoring stem cell localization and bone marrow engraftment. While ^{18}F -FLT is used as a marker of tumor growth, studies have shown that ^{18}F -FLT uptake does not always reflect cellular proliferation: ^{18}F -FLT competes with circulating thymidine, which can be sufficiently high to reduce probe uptake (49); certain chemotherapeutics alter metabolic enzyme activity independent of proliferation (48, 50).

The National Oncology PET Registry, a collaborative effort to assess the impact of ^{18}F -FDG PET on cancer patient management, indicated that, on average, physicians changed intended treatments in 38% of patients based on findings from ^{18}F -FDG PET scans (51). ^{18}F -FDG PET correlated with disease progression and response to chemotherapy and radiotherapy (52). While ^{18}F -FLT is less sensitive than ^{18}F -FDG at detecting tumor lesions, it has marginally greater specificity in detecting malignancies (50). Because ^{18}F -FLT is used as a measure of tumor proliferation, it is potentially more specific at detecting responses to cytostatic therapies than ^{18}F -FDG (48).

1.11. ^{18}F -FAC PET

Our group has sought to develop new PET probes that complement the metabolic measurements of ^{18}F -FDG and ^{18}F -FLT for assessing functions of highly proliferative tissues such as cancer and those in T cell-mediated immunity. Whereas most tissues rely on *de novo* production of deoxyribonucleoside precursors for DNA synthesis, cancer and activated T cells also utilize the nucleoside salvage pathway, which recycles deoxyribonucleosides from the extracellular milieu. A differential screening strategy was employed to identify small molecule probes that would preferentially be transported, phosphorylated and retained in activated T cells via the nucleoside salvage pathway (42). 1-(2'-Deoxy-2'- ^{18}F -fluoro- β -D-arabinofuranosyl)cytosine (^{18}F -FAC), an analog of dC and the pro-drug gemcitabine, was identified and validated to be a substrate for dCK. ^{18}F -FAC PET detected differential uptake in spleen, thymus, and lymph nodes in C57/BL6 mice and localized immune activation in the MSV/MuLV model of anti-tumor immunity. Consistent with the pathology associated with systemic autoimmunity observed in *fas*^{null-lpr} mice, the accumulation of ^{18}F -FAC indicated enlargement of the spleen, thymus, and lymph nodes when injected into these mice. Adult human biodistribution studies show uptake in spleen, bone marrow and muscle (53). ^{18}F -FAC is currently undergoing clinical studies in lymphoma and ovarian and pancreatic cancer patients.

The success of present and future PET radiopharmaceuticals depends on knowledge of the mechanism of probe retention and their reflection on the underlying biology of interest. PET imaging, as a molecular-based technology, and the development of radiolabeled nucleoside analog probes provide valuable tools for the noninvasive, longitudinal study of nucleoside metabolism *in vivo*. In turn, information gained from these studies will expand our understanding of cancer, guide therapeutic development, and further the goal of personalized medicine.

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* Part of this chapter was adapted from: Lee JT, Nair-Gill ED, Rabinovich BA, Radu CG, Witte ON. Imaging in Immunology Research. In: Kiessling F, Pichler B, eds. *Small Animal Imaging: Basics and Practical Guide.* Berlin: Springer-Verlag, 2011:565-583.

Chapter 2:

Dipyridamole amplifies the sensitivity of
3'-deoxy-3'-¹⁸F-fluorothymidine PET in mice

Abstract

We describe a pharmacological approach that expands the utility of 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT) PET in mice by increasing the sensitivity of this tracer for imaging thymidine kinase 1 (TK1) activity in murine models of cancer. Immunocompetent (C57/BL6) mice were treated with various doses of the nucleoside inhibitor dipyridamole (DPA) and imaged by PET with ^{18}F -FLT at different time points. The specificity of the DPA ^{18}F -FLT assay to image TK1 activity by PET was assessed in TK1 wild type, heterozygous and knockout mice (TK1^{+/+}, TK1^{+/-}, TK1^{-/-}, respectively). The sensitivity of the DPA ^{18}F -FLT PET assay was investigated in several murine models of cancer. C57/BL6 mice treated with DPA 10 minutes prior to ^{18}F -FLT PET exhibited significantly increased percent injected dose per gram (%ID/g) in target tissues. Optimal signal to background was obtained with 3 hours ^{18}F -FLT uptake. TK1-deficient mice did not accumulate ^{18}F -FLT in proliferative tissues regardless of DPA treatment. Various highly proliferative murine tumors, otherwise undetectable by ^{18}F -FLT alone, were clearly visible when animals were treated with DPA. Treatment with DPA prior to ^{18}F -FLT imaging amplifies the sensitivity of ^{18}F -FLT in a TK1-dependent manner. This approach improves the utility of ^{18}F -FLT for imaging proliferation and for treatment monitoring in murine models of cancer.

Introduction

3'-deoxy-3'-¹⁸F-fluorothymidine (¹⁸F-FLT) Positron Emission Tomography (PET) is frequently used in preclinical and clinical settings to image tumor cell proliferation (1, 2). As an analog of thymidine, ¹⁸F-FLT metabolism partially reflects thymidine salvage: it is transported into cells by equilibrative and concentrative nucleoside transporters and phosphorylated by its rate-limiting enzyme, thymidine kinase 1 (TK1) (1, 3). ¹⁸F-FLT has clinical prognostic value in many cancers (4), but has limited utility for imaging proliferation in normal and malignant murine tissues (5, 6).

The success of an imaging probe such as ¹⁸F-FLT is dependent on adequate biodistribution; probe pharmacokinetics is fundamental in probe development (7, 8). The effective half-life of ¹⁸F-FLT, given as a combination of its radioactive and biological half-lives, determines target-to-background ratios and is reflective of target expression, drug-target affinity, metabolism, and mechanism of retention. Clearance rates will significantly impact target tissue uptake: tracer probes with fast clearance will have relatively low background, but will also exhibit reduced target tissue exposure.

Thymidine has much greater specificity for TK1 than does FLT (9). Serum thymidine levels vary greatly across species and are an order of magnitude greater in mice than in humans (10). Reproducibility of ¹⁸F-FLT PET may be improved by adjusting for serum thymidine levels (11). Current methods to increase sensitivity are based on reducing serum thymidine concentrations (1, 12, 13). Injections of thymidine phosphorylase, the enzyme that breaks down thymidine, or 5-fluoro-2'-deoxyuridine to inhibit thymidine synthesis improves ¹⁸F-FLT PET in rodents (1, 14).

A method that increases availability of ^{18}F -FLT to tissues with minimal effect on endogenous thymidine levels is desirable. In this study, we describe the use of dipyridamole (DPA), a nucleoside transport inhibitor, to amplify ^{18}F -FLT signals in highly proliferative murine tissues. Administration of DPA shortly before ^{18}F -FLT injection increases ^{18}F -FLT tissue accumulation in a TK1-specific manner. The results support the use of DPA for enhancing ^{18}F -FLT PET imaging of highly proliferative normal and malignant mouse tissues *in vivo*.

Materials and Methods

TK1 mice and murine cancer models

Animal studies were approved by the UCLA Animal Research Committee and were carried out according to the guidelines of the Division of Laboratory Animal Medicine (DLAM) at UCLA. TK1 heterozygous mice were purchased acquired from Mutant Mouse Regional Resource Centers. Whenever possible, littermate TK1^{+/+}, TK1^{+/-} and TK1^{-/-} mice were used in experiments.

In subcutaneous tumor models, mice were injected subcutaneously in the right flank as follows: 0.25×10^6 B16 or 1×10^6 LLC cells resuspended in 100% phosphate-buffered saline (PBS) in C57/BL6 mice; 1×10^6 L1210 cells resuspended in 50% PBS and 50% MatrigelTM (354234, BD Biosciences) in SCID mice. Mice were imaged one week later when tumors reached approximately 0.5 cm in size. In the T cell leukemia model, bone marrow from C57/BL6 mice was harvested after 4 days of conditioning with 5-fluorouracil (150 mg/kg i.p.). Bone marrow was cultured for 48 hours in IL-3 (10 ng/mL), IL-6 (10 ng/mL) and mSCF (100 ng/mL) prior to undergoing two rounds of spinfection (24 hours apart) with frozen MSCV-p185-YFP or MSCV-YFP empty vector retrovirus stocks in the presence of polybrene. 1×10^6 nucleated p185

bone marrow cells in 200 mL phosphate buffered saline were injected via tail vein into recipient mice that had undergone lethal irradiation with 950 centigray (cGy) 6 hours prior. Mice were prophylaxed with trimethoprim-sulfamethoxazole for one month.

DPA treatment and mPET imaging

Dipyridamole (NDC 0641-2569-41, Baxter) was purchased from the UCLA Pharmacy. The vehicle solvent was prepared as 50 mg/ml PEG 600 and 2 mg/ml tartaric acid in water, pH 2.2-3.2. DPA was administered via intraperitoneal (i.p.) injection as indicated. Mice were kept under heating pads at all times at approximately 37°C.

¹⁸F-FLT was synthesized as described elsewhere (15). The radiochemical purity of the probe was >99% and the specific activity was >1000 Ci/mmol. Static microPET images were acquired for 600 sec, followed by 3D histogramming and reconstruction with a zoom factor of 2.1 using 3D-OSEM with 2 iterations followed by MAP with 18 iterations (beta=0.1). Whole-body MicroCAT images were acquired with the X-ray source based at 70 kVp and 500μA and exposure time of 480 sec. A Feldkamp reconstruction algorithm was applied. Images were analyzed using OsiriX Imaging Software version 3.8.

Statistical analysis

Data are presented as mean ± SD. All *p* values were determined with unpaired, two-tailed T tests and *p* values less than 0.005 were considered to be statistically significant. GraphPad Prism 5 software was used for statistical analysis.

Results

DPA amplifies the sensitivity of ¹⁸F-FLT PET in mice

DPA has been shown to potentiate anticancer chemotherapeutics targeting nucleotide synthesis by preventing cancer cells from maintaining their nucleotide pools via salvage metabolism (16, 17). L1210 cancer cells and *ex vivo* CD3-activated T cells exposed to DPA in culture exhibited decreased ^3H -thymidine retention (Supplemental Fig. 1). We reasoned that PET imaging with the thymidine analog ^{18}F -FLT might allow stratification of tumors that respond to DPA treatment. To test this hypothesis in normal tissues, we injected mice with various doses of DPA (50, 100 and 200 mg/kg) at different time points prior to ^{18}F -FLT injection and imaged them by PET between one and five hours. None of the conditions assayed demonstrated a reduction in ^{18}F -FLT signals compared to vehicle control. Unexpectedly, DPA treatment prior to injection of ^{18}F -FLT increased ^{18}F -FLT uptake in proliferative tissues.

Optimization of the assay was performed in C57/BL6 mice. The condition that induced greatest signal increase in target tissues was a single DPA intraperitoneal injection at 200 mg/kg 10 min prior to ^{18}F -FLT i.v. injection with a three hour probe uptake time. Amplification of ^{18}F -FLT signals relative to vehicle control occurred in highly proliferative bone marrow ($p < 3\text{e-}7$) and thymus tissues ($p < 8\text{e-}7$) (Fig. 1). Spleen amplification was variable. ^{18}F -FLT uptake was lowest in heart, liver and muscle. Notably, bladder signal was relatively low ($p < 0.008$). Similar changes in ^{18}F -FLT biodistribution following DPA treatment were observed in SCID mice (Fig. 3C).

DPA amplification of ^{18}F -FLT in mice is TK1-dependent

Cellular retention of ^{18}F -FLT requires TK1. To determine whether DPA amplification of ^{18}F -FLT signals retains TK1 dependence, TK1 wild type, heterozygous and knockout mice ($\text{TK1}^{+/+}$, $\text{TK1}^{+/-}$ and $\text{TK1}^{-/-}$, respectively) underwent ^{18}F -FLT PET on day 1 after vehicle and day 2 after DPA treatment. The overall ^{18}F -FLT profile of $\text{TK1}^{+/+}$ mouse was comparable to

C57/BL6 mice (Fig. 3A). Bone marrow ^{18}F -FLT uptake was greatest in TK1^{+/+} mice, marginally lower in TK1^{+/-} mice (not statistically significant) and at background levels in TK1^{-/-} mice (Fig. 3B).

DPA enhances the ability of ^{18}F -FLT PET to image murine models of cancer

We applied the DPA approach to murine tumors, which are undetectable by ^{18}F -FLT PET alone. All three subcutaneous tumor models of murine melanoma (B16), lung carcinoma (LLC) and leukemia/lymphoma (L1210) were detectable only when host mice were treated with DPA prior to ^{18}F -FLT (Figs. 3A, B and C). Uptake with DPA was highest in B16 (9.4 ± 2.1 %ID/g). We also applied the DPA approach to an endogenously growing malignancy. 5-fluorouracil-activated host bone marrow retrovirally-transduced with p210^{Bcr-Abl} was transplanted into sublethally irradiated recipient mice, which results in development of Bcr-Abl-driven myeloid leukemia (18). ^{18}F -FLT PET alone was unable to detect the leukemic expansion in bone marrow and extramedullary compartments such as spleen and thymus. Administration of DPA shortly before ^{18}F -FLT injection revealed bone marrow, thymus and an enlarged spleen, the splenomegaly characteristic of this tumor model (Fig. 3D).

Discussion

Cancer is characterized by the uncontrolled proliferation of host cells. ^{18}F -FLT PET has clinical value for assaying proliferation in various types of cancer and in treatment response (1, 19). ^{18}F -FLT PET has utility in imaging human xenografts in mice (Fig. 4). In contrast, the utility of ^{18}F -FLT PET for visualizing tumors of murine origin is limited (1).

Here, we describe the development of a pharmacological intervention to amplify ^{18}F -FLT uptake in mice tissues using DPA. Intraperitoneal injection of DPA 10 min prior to intravenous

injection of ^{18}F -FLT in C57/BL6 mice significantly increased ^{18}F -FLT signals in highly proliferative tissues such as bone marrow, thymus and spleen. In contrast, TK1^{-/-} mice had uniformly low %ID/g across all tissues. Murine tumors, previously undetectable by ^{18}F -FLT PET, were clearly visible after administration of DPA.

While we have not determined the specific mechanism(s) by which DPA mediates ^{18}F -FLT tissue uptake, it is likely through an effect on probe clearance. All mice treated with DPA exhibited transient lethargy, hypothermia and significantly reduced renal clearance at the highest doses studied, but all recovered within hours of treatment. These symptoms are consistent with previous reports (20, 21). DPA increases extracellular adenosine levels by inhibiting adenosine reuptake and adenosine deaminase activity (20). Local adenosine concentrations mediate glomerular filtration by affecting vascular and tubular functions (reviewed in (21)). Our data supports this mechanism: tissue uptake of ^{18}F -FLT in mice treated with DPA negatively correlated with probe clearance as measured by ^{18}F -FLT concentration in bladder and bladder size (Fig. 1B, data not shown). Transient inhibition of renal function such as with adenosine reuptake or adenosine deaminase inhibitors may help validate this hypothesis.

The utility of probes such as ^{18}F -FLT depends on target affinity and clearance from non-specific tissues to enhance target-to-background ratios (7, 8, 22). We hypothesize that DPA inhibition of clearance accounts for the amplification of ^{18}F -FLT by increasing tissue exposure to the probe. Indeed, mice treated with DPA had increased %ID/g in the left ventricle, representative of the blood pool, in a panel of PET probes: ^{18}F -FLT, ^{18}F -FBU, ^{18}F -FCU, ^{18}F -FHBG and L- ^{18}F -FMAC.

Human xenografts were detectable by ^{18}F -FLT without DPA (Fig. 4). This is consistent with clinical data in which ^{18}F -FLT works well for imaging several types of cancers (19).

Previous reports attributed limited use of ^{18}F -FLT in rodent models to probe competition with circulating thymidine, which is 9-15 times higher in mouse than human plasma (10). Here, we controlled for circulating thymidine levels by engrafting human and mice tumors in SCID mice. Studies in our group and others show that thymidine is at micromolar levels in mice lymphoid organs and tumors, levels that can outcompete the picomolar levels of ^{18}F -FLT (data not shown, (13)). Species differences in tumor thymidine metabolism may contribute to the disparity: differences in transport; expression of active TK1; expression of thymidine phosphorylase, the enzyme that metabolizes thymidine to thymine (23, 24). Further complicating the problem is the lower affinity ^{18}F -FLT has for TK1 than does thymidine (9). Thus, differences in local thymidine levels mediated by distinct thymidine metabolism may lead to differential ^{18}F -FLT uptake. The extent of this problem in the clinic remains to be determined (25).

The improvement of ^{18}F -FLT for imaging mice tissues expands its utility in preclinical cancer research. This may be useful in studying disease progression in the many transgenic mice models of cancer (26). ^{18}F -FLT imaging of proliferation in models of immune disease and cancer immunotherapy would complement information from other probes such as ^{18}F -FDG and ^{18}F -FAC. Additionally, this new technique may allow monitoring of tumor response to treatments such as those targeting thymidylate synthase (27).

It is also important to note that DPA, as an inhibitor of nucleoside transporters, may modify the biology of interest. More detailed understanding of the effects of DPA is also warranted.

Conclusion

Administration of DPA improves the utility of ^{18}F -FLT PET for imaging proliferative normal and tumor tissues of murine origin in a TK1-dependent manner. A thorough understanding of DPA's mechanism of action in improving ^{18}F -FLT will broaden the use of this assay for imaging proliferation in vivo in preclinical research.

Figure 2.1

DPA treatment enables visualization by FLT PET/CT of highly proliferative tissues in mice. (A) WT C57/BL6 mice scanned on consecutive days with FLT without (-) and with (+) DPA (200 mg/kg administered i.p. 10 min prior to FLT injection). PET was performed 180 min after FLT injection. BM – bone marrow, Thy – thymus, Sp – spleen, Bl – bladder, GI – gastrointestinal tract. (B) Data quantification. $n \geq 5$ mice.

FIGURE 2.1

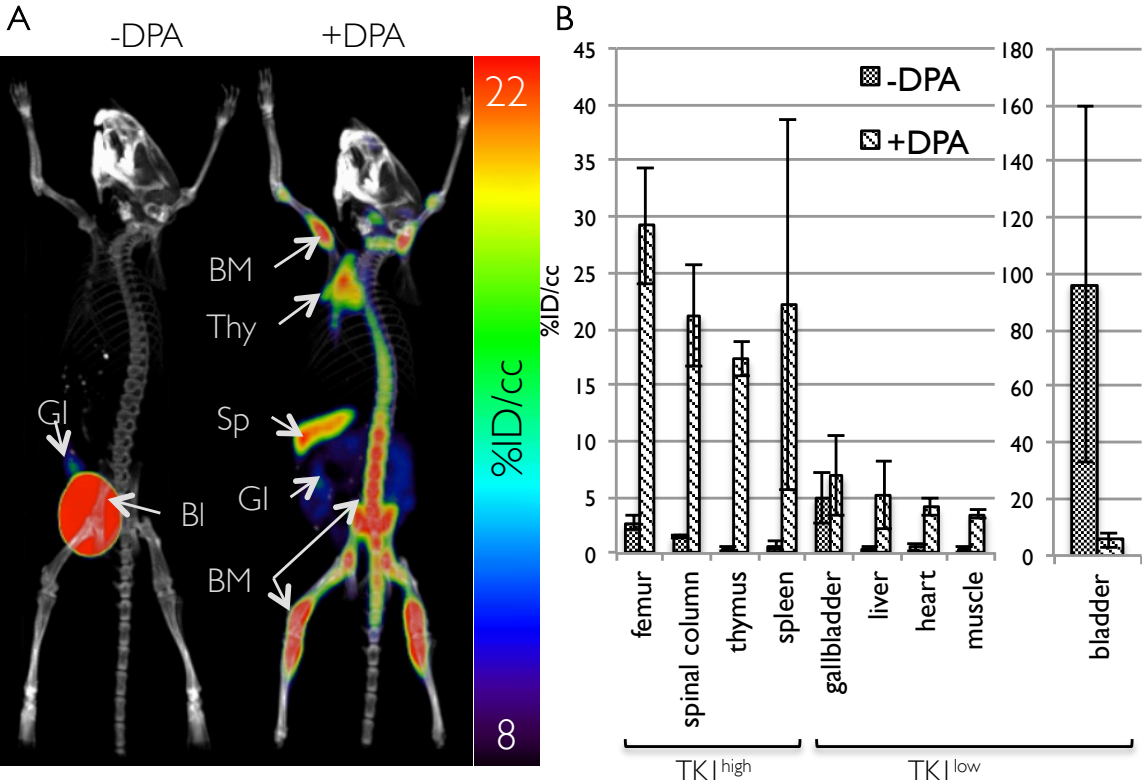


Figure 2.2

DPA amplification of ^{18}F -FLT PET signal in mice is TK1-dependent. (A) TK1^{+/+}, TK1^{+/-} and TK1^{-/-} mice underwent ^{18}F -FLT microPET/CT imaging on consecutive days without (not shown) or with DPA (200 mg/kg administered i.p. 10 min prior to ^{18}F -FLT injection). PET was performed 180 min after ^{18}F -FLT injection. BM – bone marrow, Thy – thymus, Sp – spleen, GB – gallbladder, GI – gastrointestinal tract, K – kidney, Bl – bladder. (B) Data quantification $n \geq 4$.

FIGURE 2.2

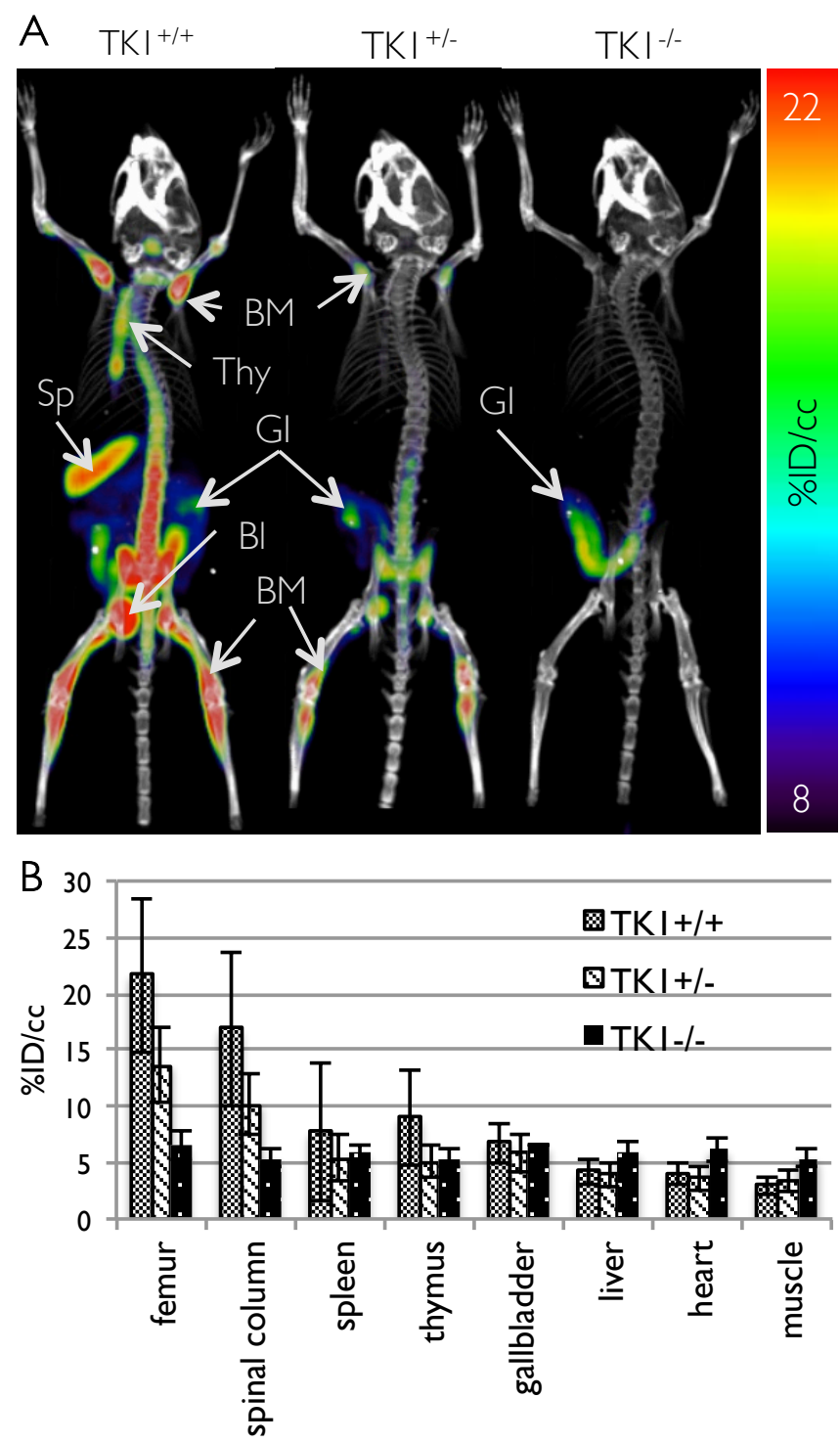


Figure 2.3

DPA treatment enables visualization of tumors in mice. Mice were scanned on consecutive days with FLT without (-) or with (+) DPA (200mg/kg administered i.p. 10 min prior to FLT injection). (A) B16- and (B) LLC-bearing C57/BL6 mice. (C) L1210-bearing SCID mice. (D) Bcr-Abl p185 leukemia in C57/BL6 mice. PET was performed 180 min after the FLT injection. Broken circle (---) encompasses tumor. n = 5 mice.

FIGURE 2.3

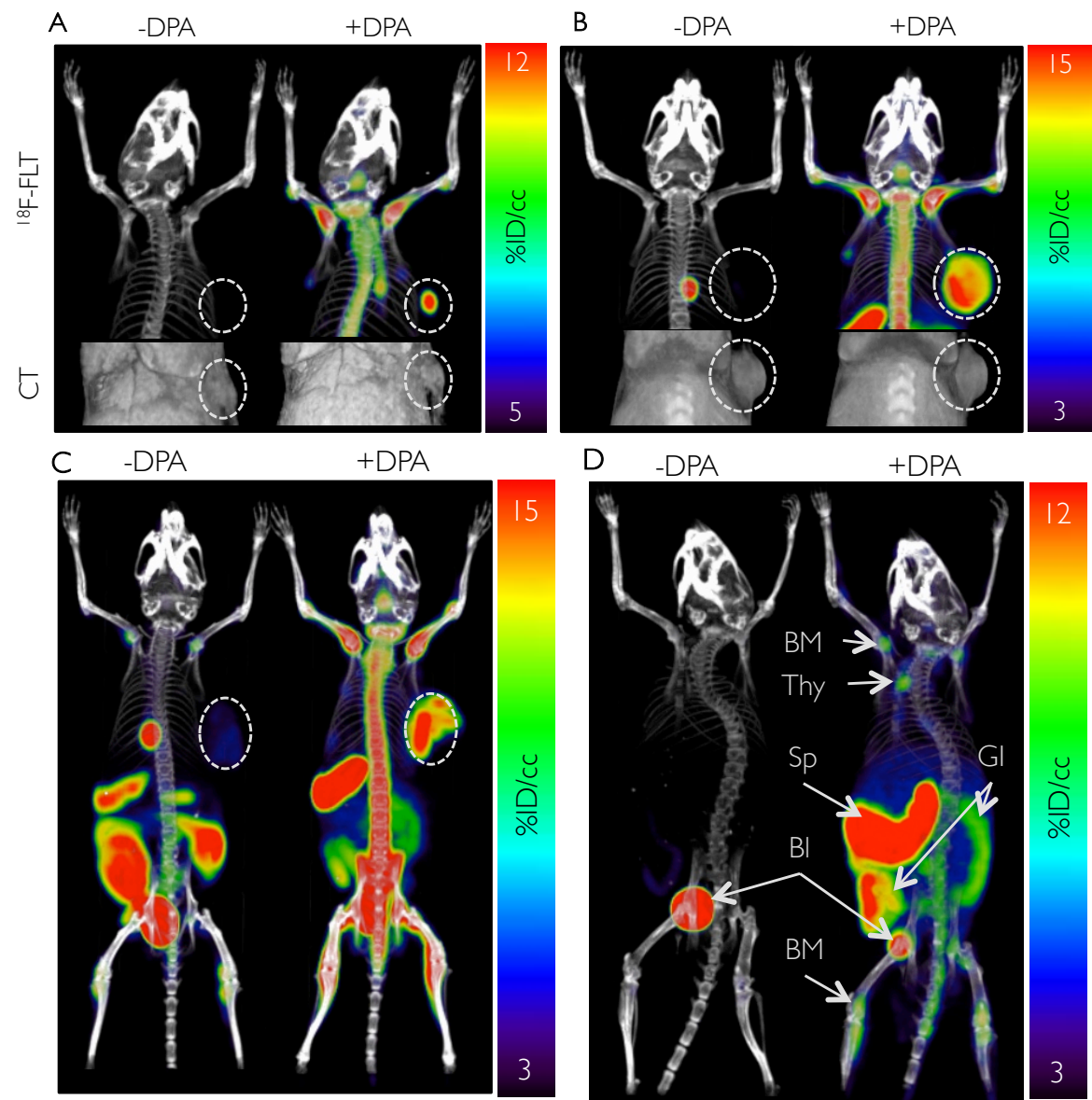
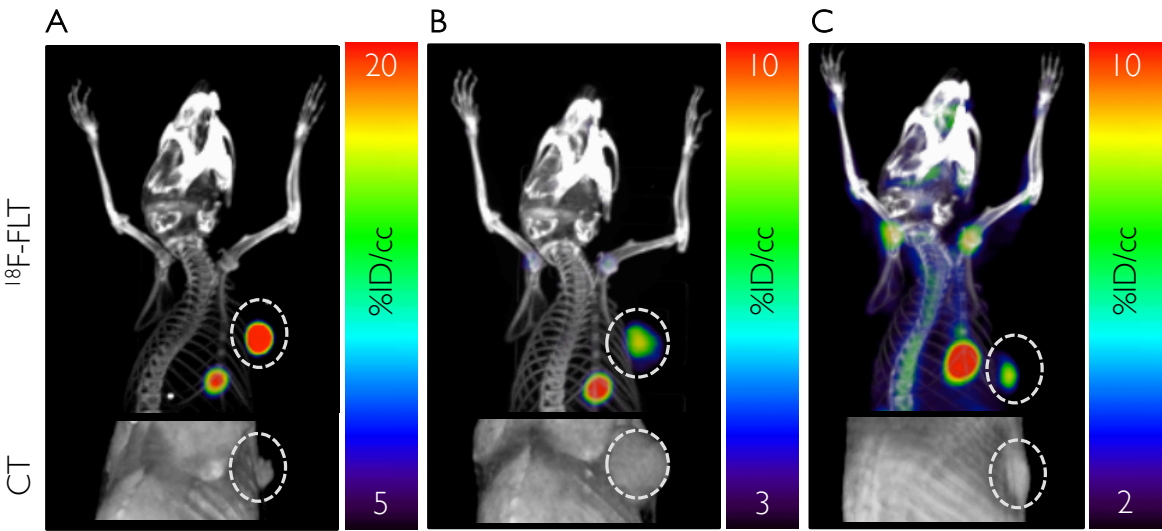


Figure 2.4

^{18}F -FLT PET of human xenografts does not require DPA. Mice were scanned 60 min post-injection of ^{18}F -FLT. (A) A431- (B) LS174T- and (C) U87-bearing SCID mice. n = 3 mice.

FIGURE 2.4



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Chapter 3:
Novel PET Probes Specific for
Deoxycytidine Kinase

Abstract

Deoxycytidine kinase (dCK) is a rate-limiting enzyme in the deoxyribonucleoside salvage pathway and a critical determinant of therapeutic activity for several nucleoside analog prodrugs. We have previously reported the development of ^{18}F -FAC, (1-(2'-deoxy-2'- ^{18}F -fluoro- β -D-arabinofuranosyl) cytosine), a new probe for PET imaging of dCK activity in immune disorders and certain cancers. The objective of the current study was to develop PET probes with improved metabolic stability and specificity for dCK. Towards this goal, several candidate PET probes were synthesized and evaluated *in vitro* and *in vivo*.

Methods: High pressure liquid chromatography was used to analyze the metabolic stability of ^{18}F -FAC and of several newly-synthesized analogs with the natural D-enantiomeric sugar configuration or the corresponding unnatural L-configuration. *In vitro* kinase and uptake assays were used to determine the affinity of the ^{18}F -FAC L-nucleoside analogs for dCK. The biodistribution of selected L- analogs in mice was determined by microPET/CT imaging.

Results: Candidate PET probes were selected using the following criteria: low susceptibility to deamination, high affinity for purified recombinant dCK, high uptake in dCK expressing cell lines and biodistribution in mice reflective of the tissue expression pattern of dCK. Amongst the ten newly-developed candidate probes, 1-(2'-deoxy-2'- ^{18}F -fluoro- β -L-arabinofuranosyl) cytosine (L- ^{18}F -FAC) and 1-(2'-deoxy-2'- ^{18}F -fluoro- β -L-arabinofuranosyl)-5-methylcytosine (L- ^{18}F -FMAC) most closely matched the selection criteria. The selection of L- ^{18}F -FAC and L- ^{18}F -FMAC was validated by showing that these two PET probes can be used to image animal models of leukemia and autoimmunity.

Conclusion: Promising *in vitro* and *in vivo* data warrant biodistribution and dosimetry studies of L- ^{18}F -FAC and L- ^{18}F -FMAC in humans.

Introduction

Deoxycytidine kinase (dCK) catalyzes a rate-limiting phosphorylation step in the deoxyribonucleoside salvage pathway (1). dCK is unique among salvage enzymes due to its ability to provide cells with all four deoxyribonucleoside triphosphates (dNTPs) via direct (dCTP, dATP and dGTP) and indirect (dTTP) mechanisms (Supplemental Figure 3.1, reviewed in (1)). Highest levels of dCK are found in lymphocytes (2, 3), particularly during lymphopoiesis (reviewed in (1)). dCK expression is upregulated in activated T cells (4). dCK is also expressed in most lymphoid and myeloid malignancies and in some solid tumors (reviewed in (1)).

We have recently demonstrated a critical requirement for dCK in normal lymphocyte development *in vivo* (5). This requirement suggests that the rate-limiting function of dCK in deoxyribonucleoside phosphorylation is biologically significant. Moreover, dCK is therapeutically important. dCK phosphorylates and activates cytarabine, fludarabine, gemcitabine, cladribine, decitabine and clofarabine (reviewed in (6)). These nucleoside analogs are mainstay cytotoxic pro-drugs in the treatment of hematologic and solid tumors. Cladribine has completed phase III clinical trials in multiple sclerosis (7). Lamivudine and Emtricitabine, two other dCK-dependent nucleoside analogs, are approved by the FDA for therapy of HIV infections (8).

A common thread amongst dCK-dependent pro-drugs is the high degree of variability in their therapeutic responses. This heterogeneity may reflect genetic variations in deoxyribonucleoside metabolism (9) which confer resistance to therapy. Since decreased dCK activity is a common drug-resistance mechanism (reviewed in (10)), dCK-specific PET probes may enable patient stratification into likely responders and non-responders to dCK-activated pro-drugs.

We have recently described the development of ^{18}F -FAC (Figure 3.1A) (4, 11), a new probe for PET imaging of the deoxyribonucleoside salvage pathway. In mice, ^{18}F -FAC microPET enables imaging of adaptive immunity and of several types of cancer (4). ^{18}F -FAC accumulation in tumor tissues is predictive of responses to gemcitabine (12). Here we analyzed the metabolic stability of ^{18}F -FAC. In mice, ^{18}F -FAC was found to rapidly undergo deamination to 1-(2'-deoxy-2'- ^{18}F -fluoro- β -D-arabinofuranosyl) uracil (^{18}F -FAU). Deamination confounds the specificity of ^{18}F -FAC for dCK and it may decrease its sensitivity in species with high deaminase activity such as humans (13). To bypass tracer deamination, a series of ^{18}F -FAC analogs were synthesized and evaluated. These studies indicated that ^{18}F -FAC analogs with the unnatural L-enantiomeric sugar configuration resist deamination and allow PET imaging of dCK activity *in vivo*.

Materials and Methods

Radiochemical synthesis of ^{18}F -labeled PET probes

^{18}F -FAC was synthesized as previously described (4, 11). The radiochemical syntheses of the new probes are described in the Supplementary Materials and Methods section.

PET probe metabolite analyses

Mice were injected intravenously with ^{18}F -labeled probes and allowed up to 45 min uptake. Tissues were removed and homogenized. Whole blood sampling was collected via retro-orbital eye bleeding, followed by centrifugation to obtain plasma. Tissues and plasma were treated with a mixture of methanol:acetonitrile (1:9) to extract nucleosides. Following centrifugation, supernatants were evaporated at 50°C under nitrogen. The residue is dissolved in 100 mL of mobile phase (5 mM Pentane-1-sulfonic acid, pH 3.1 and methanol (96:4)),

filtered and injected into a Waters microBondapak C18 column with a flow rate of 1.5 ml/min. Peak-radioactivity is measured using a Bioscan coincidence detector.

***In vitro* enzymatic assays**

Production of recombinant human cytidine deaminase (CDA) and dCK is described in the Supplementary Materials and Methods section. The deamination assay solution contained purified CDA (0.05 µg/µL) and 140 µM nucleoside analog in 0.125 M phosphate buffer, pH 7.6. The assay was carried out at 37°C for 1 hour; products were resolved via HPLC using a Waters C18 µBondapak column (3.9 x 300 mm), as previously reported (14). Elution was isocratic using 3% methanol in 0.1 M phosphate buffer (pH 5.5) at a flow rate of 1 mL/min. The rate of dCK-catalyzed phosphorylation was measured continuously at 340 nm using an iEMS Reader MF (LabSystems) system and a modification of a previously described spectrophotometric assay consisting of a coupled lactate dehydrogenase (LDH)/pyruvate kinase (PK) reaction (15). Reactions were carried out in triplicates at 37°C with 50 mM Tris-HCl pH 7.6, 50 mM KCl, 10 mM MgCl₂, 5 mM ATP, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 1 mM DTT, 1.4 µM dCK and PK 10 U/mL (Sigma) and LDH 15 U/mL (Sigma). Kinetic constants were calculated using the GraphPad Prism 5 software.

Cell-based phosphorylation and uptake competition assays using ³H-labeled deoxycytidine (³H-dC)

L1210 cells (12) were lysed by 3 cycles of freeze-thawing in 50 mM Tris-HCl, pH 7.6, 2 mM DTT, 20% glycerol, 0.5% Nonident P40. The lysate was clarified by centrifugation (15,000 g for 10 min) and the protein concentration in the supernatants was determined using the Bradford assay. The kinase reaction mixture contained 4.2 µg of L1210 lysate, 3 µM ³H-dC (Moravek Biochemicals, specific activity 35 Ci/mmol), 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂,

1 mM UTP (Sigma), 2 mM DTT, 10 mM NaF, 1 mM thymidine (to inhibit thymidine kinase 2) and 30 μ M nucleoside analog. The reaction was incubated at 37°C for 20 min and was stopped by the addition of 30 μ L of ice cold water followed by heating at 90°C for 3 min. The reaction was then spotted on Whatmann DE81 filter disks; these were washed 3 times with 4 mM ammonium formate and twice with ethanol. Disks were placed in scintillation vials and counted using a Beckman Liquid Scintillation counter. For uptake assays, 1.05 μ Ci of 3 H-dC were added to L1210 cells (10^5 cells/well in 96-well plates) followed by 3 μ L of 100 μ M stocks of each nucleoside analog (at a final volume of 100 μ L/well). After 1 hour at 37°C, wells were washed 3 times with ice-cold PBS using a vacuum filtration system (Millipore). Plates were oven-dried at 45-50°C for 10 minutes, and 200 μ L of scintillation fluid were added/well. Radioactivity was measured using a Trilux® MicroBeta scintillation counter (Perkin Elmer).

MicroPET/CT imaging studies in mice.

In vivo studies followed the guidelines of the Department of Laboratory Animal Medicine at UCLA and were performed as previously described (4, 12) (see the Supplementary Materials and Methods section). Autoimmune B6.MRL-Fas^{lpr}/J mice were obtained from The Jackson Laboratory (stock number: 000482).

Statistical Analysis

Data are presented as means $\pm$ SD (standard deviation). All *P* values are two-tailed and *P* values of <0.05 are considered to be statistically significant. Graphs were generated using the Prism 5 software.

Results

18 F-FAC is rapidly deaminated *in vivo*

With the exception of the fluorine substitution of the *ara*-2' hydrogen, FAC is identical to deoxycytidine (dC). It is therefore likely that ^{18}F -FAC and dC are competitive substrates and share the same metabolic pathway. According to the model shown in Figure 3.1A, ^{18}F -FAC is either phosphorylated by cytosolic dCK to ^{18}F -FAC-monophosphate (^{18}F -FAC-MP) or is converted to ^{18}F -FAU by extracellular or cytosolic cytidine deaminase (CDA). ^{18}F -FAU, a weak substrate for thymidine kinase 1 (TK1) (16), cannot be phosphorylated by dCK. ^{18}F -FAU can also be produced from ^{18}F -FAC by a CDA-independent mechanism involving cytidylate deaminase (which converts ^{18}F -FAC-MP to ^{18}F -FAU-MP) and 5'nucleotidase (which dephosphorylates ^{18}F -FAU-MP producing ^{18}F -FAU, Figure 3.1A). To validate this model, mice were intravenously injected with ^{18}F -FAC and plasma samples were analyzed by HPLC. ^{18}F -FAU was the only ^{18}F -FAC metabolite detected in plasma by this HPLC method (Supplemental Figure 3.2A). As early as 1 minute post injection, ^{18}F -FAU contributed to ~14% of the total radioactivity in the plasma (Figure 3.1B). Ten minutes later, the contribution of ^{18}F -FAU increased to ~60% and reached ~90% at 45 minutes. The plasma clearance rate of ^{18}F -FAU was significantly slower than that of ^{18}F -FAC (Supplemental Figure 3.2B). Similar to plasma, thymus and liver samples from ^{18}F -FAC-injected mice also contained high concentrations of ^{18}F -FAU (Supplemental Figure 3.2C) at 45 minutes after injection.

2-chloro-2'-deoxy-2'- ^{18}F -fluoro-9- β -D-arabinofuranosyl-adenine (^{18}F -Clofarabine), a potential dCK-specific PET probe that resists deamination

The fast exponential decline of ^{18}F -FAC in plasma and the accumulation of the ^{18}F -FAU metabolite in plasma and tissues reduce the specificity of ^{18}F -FAC. They may also affect the sensitivity of ^{18}F -FAC PET in humans who express twice as much deaminase activity than mice in most tissues (13). This problem can be solved by identifying dCK substrates that are amenable

to ^{18}F labeling and have low affinity for deaminase enzymes. Clofarabine (Supplemental Figure 3.3), is a dCK-dependent pro-drug with excellent metabolic stability (reviewed in (17)). The 2-halogenated aglycone of clofarabine confers resistance to deamination by adenosine deaminase. The 2' fluorine atom blocks cleavage of the glycosidic bond by bacterial purine nucleoside phosphorylase (17). Clofarabine is efficiently phosphorylated by dCK (18) and is amenable to ^{18}F labeling (Supplemental Figure 3.3). Surprisingly, ^{18}F -FAC and ^{18}F -labeled clofarabine (^{18}F -CA) showed distinct biodistribution patterns in immunocompetent (C57BL/6J) mice (Figure 3.2). In contrast to ^{18}F -FAC (4), ^{18}F -CA accumulation in expected target organs such as thymus, bone marrow and spleen was undetectable. Whether the differences in the biodistribution of ^{18}F -FAC and ^{18}F -CA are unique to mice or can also be found in other species, including humans, will be addressed in future studies.

FAC analogs as potential dCK-specific probes

The disappointing performance of ^{18}F -CA in mice indicated that a different approach was required to solve the tracer deamination problem. Towards this end, the FAC pharmacophore was explored to seek pyrimidine analogs matching three criteria: (i) amenability to routine ^{18}F -radiolabeling with high specific activity; (ii) resistance to deamination; (iii), affinity for dCK comparable to that of ^{18}F -FAC. Nine potential dCK-specific probes (Figure 3.3) were designed and synthesized. The rationale for selecting these compounds was twofold. First, modifications of deoxycytidine at position 5 of the nucleobase have been shown to reduce susceptibility to deamination (19). Second, dCK has the unusual property of phosphorylating both D-enantiomers (the natural configuration of nucleosides), *and* unnatural L-enantiomers (20, 21). In contrast, CDA has a strong preference for D- over L- deoxycytidine analogs (20). The susceptibility of FAC analogs to deamination was determined by incubating them with purified recombinant

human CDA followed by HPLC analysis to determine the formation of deaminated products. Figure 3.3 shows that the nucleosides with the D-chirality were completely deaminated while the unnatural L-nucleosides were resistant to deamination. While these modifications solve the deamination problem it is possible that they also reduce the affinity of the FAC analogs for dCK. This possibility was addressed using several assays.

First, a lactate dehydrogenase (LDH)/pyruvate kinase (PK)-coupled kinase reaction (15) was used to determine the kinetic parameters of human recombinant dCK for L-analog candidate probes. Kinetic parameters for three of the five L-analogs were obtained (Table 1). L-FMAC and L-FCAC had similar kinetics with K_m values of 1.0 μM and 0.6 μM , respectively. These K_m values were similar to that of FAC (0.8 μM). L-FMAC and L-FCAC also had specificity constants (k_{cat}/K_m) for dCK that were similar to FAC (0.96-1.2 times that of FAC). However, this was not the case for L-FBAC which had a higher K_m and a lower specificity constant than FAC.

The LDH/PK assay only determines the kinetics for phosphorylation reactions that are faster than the UV-induced decomposition of NADH (22). The inability to obtain the kinetic parameters for L-FAC and L-FFAC using the LDH/PK assay indicated that these nucleoside analogs are either high affinity substrates for dCK with low enzymatic turnover, or that they lack affinity for dCK. To determine if L-FAC and L-FFAC were phosphorylated by dCK, kinase assays (23) were performed using ^{18}F -labeled probes (synthesized according to the scheme shown in Supplemental Figure 3.4) and purified recombinant human dCK as well as lysates from L1210 dCK positive and negative cell lines (12). The kinase assays showed conclusively that L-FAC and L-FFAC are indeed dCK substrates (Supplemental Figure 3.5).

Two cell-based assays were then used to determine whether the L-analogs can compete with the endogenous dCK substrate deoxycytidine (dC). In a kinase assay using whole-cell lysates of the dCK positive murine leukemia cell line L1210 (12), L-FAC and L-FFAC outperformed the other nucleoside analogs by inhibiting ^3H -dC phosphorylation by over 95% (Figure 3.4). The enhanced activity of dCK towards L-FAC and L-FFAC compared to FAC is not entirely surprising as the enzyme has been previously shown to favor the L-conformation of other pyrimidine analogs (21, 24). Confirming the results from the LDH/PK assay, L-FBAC inhibited dC phosphorylation by only 32%. The ability of L-nucleosides to competitively inhibit ^3H -dC uptake by L1210 cells was also determined (Figure 3.4). In addition to dCK-mediated phosphorylation, the uptake assay takes into account the efficiency of transport across the cell membrane. L-FAC and L-FFAC reduced the uptake of ^3H -dC by at least 90%. The inhibition by L-FCAC and L-FMAC was 32-35%.

Biodistribution of the ^{18}F -labeled L-nucleosides in immunocompetent C57BL/6J mice

Results from the *in vitro* phosphorylation and uptake assays narrowed down the selection of candidate L-enantiomer probes to L- ^{18}F -FAC, L- ^{18}F -FMAC, L- ^{18}F -FFAC and L- ^{18}F -FCAC. Similar to ^{18}F -FAC and unlike ^{18}F -CA, all four candidate probes accumulated in the thymus, and to varying degrees in the bone marrow and spleen (Figure 3.5A). Figure 3.5B shows the relative probe uptake values in dCK positive tissues normalized to uptake in the muscle (a dCK negative tissue); absolute probe uptake values are shown in Supplemental Figure 3.6. Amongst the tested L-nucleosides, L- ^{18}F -FCAC had the lowest uptake in lymphoid tissues while L- ^{18}F -FAC had the highest uptake in thymus and spleen. An intermediate profile was observed for L- ^{18}F -FMAC, which had higher uptake than ^{18}F -FAC in the thymus and lower uptake in the spleen.

In addition to bone marrow and thymus, L-¹⁸F-FAC and L-¹⁸F-FFAC are also retained in the liver. Similar to ¹⁸F-3'-deoxy-3'-fluorothymidine (¹⁸F-FLT) (25), these probes accumulate in the liver by a non-specific mechanism (26). Alternatively, the liver uptake may be dCK-specific. A novel dCK knockout (KO) mouse (5) can be used to evaluate these possibilities. L-¹⁸F-FAC microPET/CT scans (Figure 3.5C and Supplemental Figure 3.7) indicate that, with the exception of excretory organs such as gall bladder and bladder, the L-¹⁸F-FAC organ distribution typically observed in wild type mice (Figure 3.5A) was absent in the dCK KO mouse. While these data demonstrate that the liver trapping of L-¹⁸F-FAC requires dCK expression, other mechanisms such as uptake via liver-specific transporters for unnatural L-nucleosides may also play a role.

L-¹⁸F-FAC and L-¹⁸F-FMAC microPET/CT imaging of malignant and autoimmune lymphoproliferative disorders in mice

Biodistribution studies in healthy mice indicate that the deamination-resistant probes L-¹⁸F-FAC, L-¹⁸F-FFAC and L-¹⁸F-FMAC compare favorably with ¹⁸F-FAC. While L-¹⁸F-FMAC had a slightly lower sensitivity than L-¹⁸F-FAC and L-¹⁸F-FFAC, its low liver uptake may be advantageous in certain applications. Since the biodistribution of L-¹⁸F-FFAC was similar to that of L-¹⁸F-FAC, this probe was not selected for further evaluations. L-¹⁸F-FMAC and L-¹⁸F-FAC, the two remaining candidate probes, were compared in two mouse models of cancer and autoimmunity, previously used to evaluate ¹⁸F-FAC (4, 12). The first test was to determine whether L-¹⁸F-FAC and L-¹⁸F-FMAC can distinguish between isogenic murine L1210 leukemia cells that either express dCK (wild type cells) or lack this enzyme (L1210-10K cells) (12). As shown in Figure 3.6A, dCK positive tumors were detected with both L-¹⁸F-FAC and L-¹⁸F-FMAC. Neither of these probes accumulated in the dCK negative L1210-10K tumors. L-¹⁸F-FAC and L-¹⁸F-FMAC were then evaluated in the B6.MRL-Fas^{lpr}/J autoimmune mice (27)(28).

Figure 3.6B shows that both probes detected the cervical, axillary and brachial lymphadenopathies characteristic of the Fas^{lpr} model.

Discussion

Factors that may affect the performance of ^{18}F -FAC, L- ^{18}F -FAC and L- ^{18}F -FMAC in preclinical and clinical applications

Despite its rapid deamination *in vivo* (Figure 3.1B), ^{18}F -FAC performs well in mice (4, 12). A potential limitation of ^{18}F -FAC concerns its muscle background which is the highest amongst tested compounds (Supplementary Figure 3.6). In applications in which muscle background may interfere with specific signals, ^{18}F -FAC could be replaced by L- ^{18}F -FMAC, a probe with a similar biodistribution pattern and sensitivity but with a lower non-specific uptake in the muscle. Regarding L- ^{18}F -FAC, its utility in mice may be limited by the high liver uptake. Furthermore, it is conceivable that, because of their unnatural L-chirality, L- ^{18}F -FAC and L- ^{18}F -FMAC may be transported less efficiently than ^{18}F -FAC across the plasma membrane.

In the context of clinical applications, the deamination resistant probes L- ^{18}F -FAC and L- ^{18}F -FMAC may have an advantage over ^{18}F -FAC given the high deamination activity in certain human tissues (13). Concerning the utility of the FAC probes for treatment stratification, ^{18}F -FAC could be the best probe to use for predicting responses to deamination-susceptible pro-drugs such as cytarabine, gemcitabine and decitabine. In contrast, L- ^{18}F -FAC and L- ^{18}F -FMAC may be more suitable for predicting responses to deamination resistant drugs such as cladribine and clofarabine.

Comparison between TK1- and dCK-specific PET probes

PET imaging of TK1 and dCK, the two rate-limiting enzymes in the deoxyribonucleoside salvage pathway, are now possible due to the development of ^{18}F -FLT by Shields and colleagues in 1998 (26), followed by the identification of ^{18}F -FAC in 2008 (4) and of optimized FAC analogs described herein. Since the high levels of endogenous thymidine in rodent serum compete with ^{18}F -FLT and reduce its sensitivity in mice (29), direct comparisons between ^{18}F -FLT and the FAC series of probes have to be conducted in other species. Nonetheless, it is likely that PET measurements of TK1 and dCK provide non-overlapping information. Thus, while ^{18}F -FLT provides measurements of DNA synthesizing activity and cell proliferation (30), dCK-specific probes may enable PET-guided identification of cancer patients who are more likely to respond to cytotoxic chemotherapy nucleoside analog pro-drugs. Furthermore, a better understanding of the deoxyribonucleoside salvage pathway in terms of its physiological function and potential role in cancer development and progression may expand the utility of TK1 and dCK-specific PET probes beyond their current status of surrogate markers for cell proliferation, immune activation, treatment stratification and monitoring. In this context, recently described mouse genetic models of TK1 (31) and dCK deficiency (5) provide invaluable tools to enable the identification of biological processes that are critically dependent on the activity of these two cytosolic deoxyribonucleoside kinases.

Conclusions

We describe the development of a novel set of PET probes that can be used to measure the metabolic flux through the deoxyribonucleoside salvage pathway. A better understanding of the salvage pathway in terms of its physiologic function and potential role in cancer development and progression may expand the utility of TK1- and dCK-specific PET probes beyond their

current status of surrogate markers for cell proliferation, immune activation, treatment stratification, and monitoring.

Acknowledgements

We thank David Stout, Waldemar Ladno, and Judy Edwards for microPET/CT imaging, Rachel Laing for helping with experiments and the cyclotron group for the production of PET probes. This work was supported by the *In Vivo* Cellular and Molecular Imaging Centers Developmental Project Award NIH P50 CA86306 (to Caius Radu and Jason Lee), and R24CA92865, U.S. Department of Energy Contract DE-FG02-06ER64249 (to Michael Phelps), RT1-01126-1 California Institute for Regenerative Medicine (to Michael Phelps, Owen Witte and Caius Radu) and the Dana Foundation (to Caius Radu). Owen Witte is an Investigator of the Howard Hughes Medical Institute.

Conflict of interest statement

Caius Radu, Owen Witte and Johannes Czernin are among the inventors of the national and PCT patent applications for the FAC technology referred to in the article. That patent application was filed on September 19, 2008. A group of UCLA faculty members including Caius Radu, Johannes Czernin, Michael Phelps and Owen Witte are involved in Sofie Biosciences, a startup company that has licensed this intellectual property.

Figure 3.1

In vivo metabolite analysis of ^{18}F -FAC. (A) Chemical structures of ^{18}F -FAC and ^{18}F -FAU and schematic showing extracellular and intracellular routes by which ^{18}F -FAC can be deaminated to ^{18}F -FAU. (B) Percentage of total detected radioactivity in plasma that is attributable to ^{18}F -FAC over time as determined by HPLC analysis. Analysis is performed after intravenous injection of ^{18}F -FAC in mice. 5'-NT = 5'nucleotidase; DCTD = deoxycytidylate deaminase (catabolic enzymes are shown in red fonts); MP = monophosphate.

FIGURE 3.1

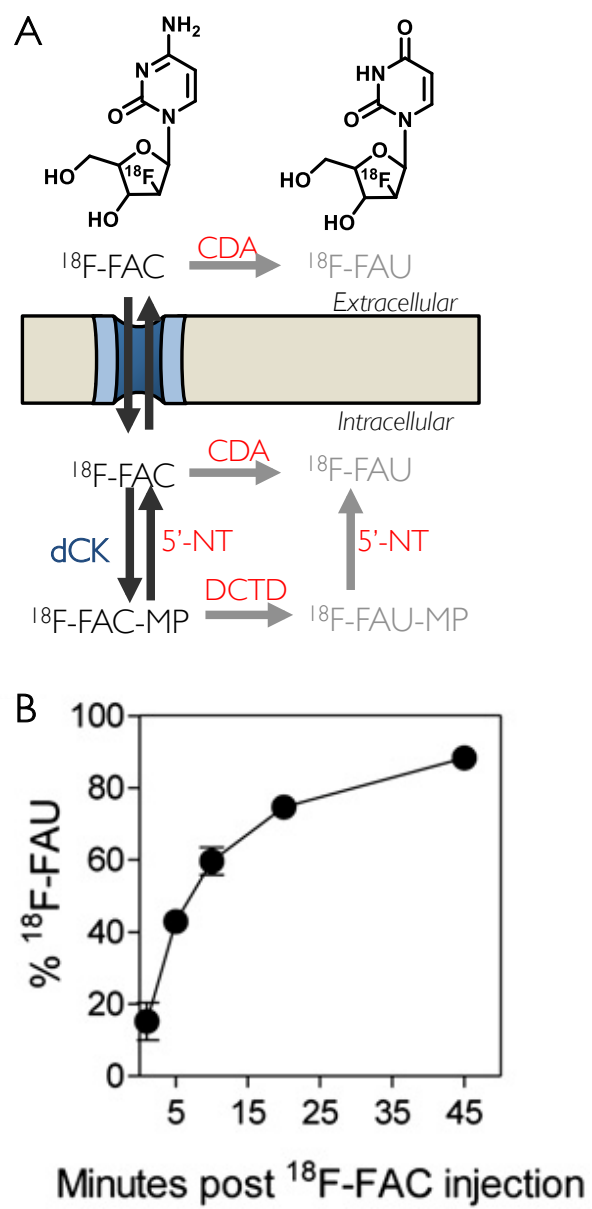


FIGURE 3.2

^{18}F -FAC and ^{18}F -clofarabine (^{18}F -CA) small-animal PET/CT scans of C57BL/6J mice (images are representative of pattern observed in 3 mice scanned with each probe). %ID/g = percentage injected dose per gram of tissue; Bl = urinary bladder; BF = brown fat; BM = bone marrow; GI = gastrointestinal tract; K = kidney; L = liver; S = spleen; Thy = thymus.

FIGURE 3.2

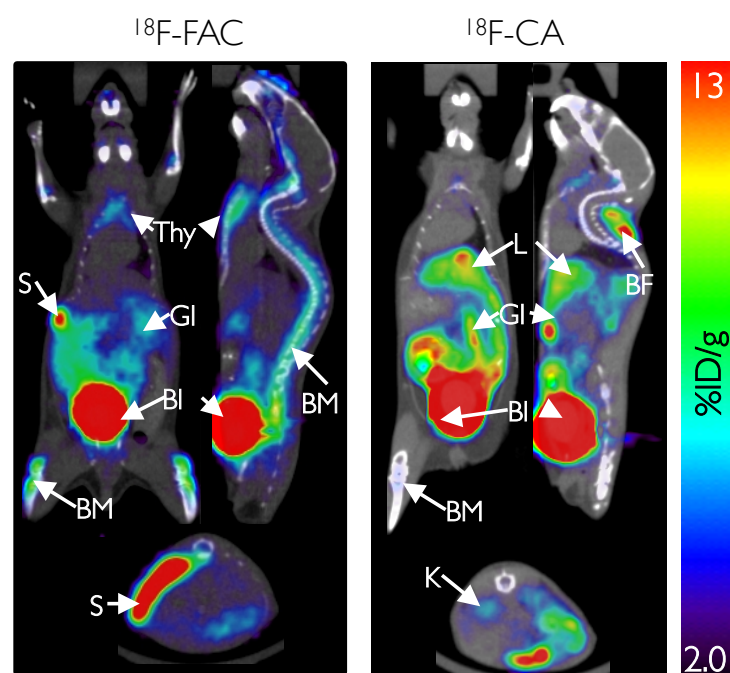


Figure 3.3

Candidate dCK-specific PET probes that resist deamination. (A) Chemical structures of deoxycytidine analogs amenable to ^{18}F -labeling. (B) *In vitro* deamination assay. Following incubation at 37°C for 1 hour in the presence (blue dashed traces) or absence (red solid traces) of recombinant purified CDA, candidate probes were analyzed on HPLC. D-analogs are shown in the top row and the L-analogs are shown in the bottom row. abs = absorbance. R = H (FAC); R = CH₃ (FMAC); R = F (FFAC); R = Cl (FCAC); R = Br (FBAC).

FIGURE 3.3

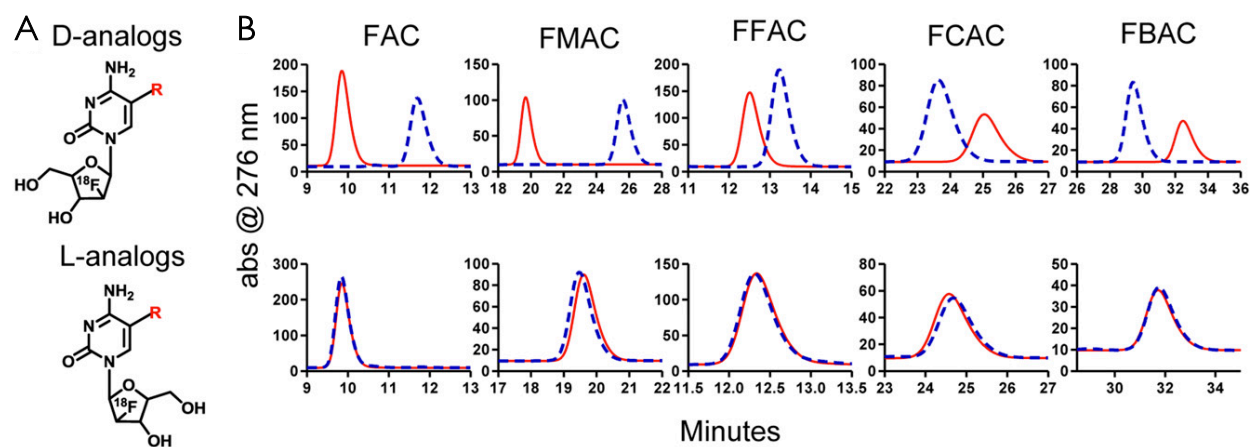


TABLE 1. Kinetic Parameters for FAC and Its Analogs with L-Chirality

Probe	K_M (μM)	Relative k_{cat}	Relative k_{cat}/K_M
FAC	0.76 ± 0.15	1.0	1.0
L-FMAC	1.02 ± 0.15	1.0	0.96
L-FCAC	0.61 ± 0.09	0.96	1.20
L-FBAC	6.54 ± 0.99	2.70	0.31

k_{cat} and specificity constant (k_{cat}/K_M) were determined using purified recombinant human dCK, and values are given relative to FAC.

Figure 3.4

Analyses of affinity of L-analogs for dCK. L-analogs were tested for their ability to competitively inhibit phosphorylation (red bars; left axis) and uptake (blue bars; right axis) of tritium-labeled deoxycytidine (^3H -dC) using dCK expressing L1210 cells. Results represent 2 independent experiments. P values are calculated relative to water ($n = 3$). * $P < 0.05$. dC = deoxycytidine.

FIGURE 3.4

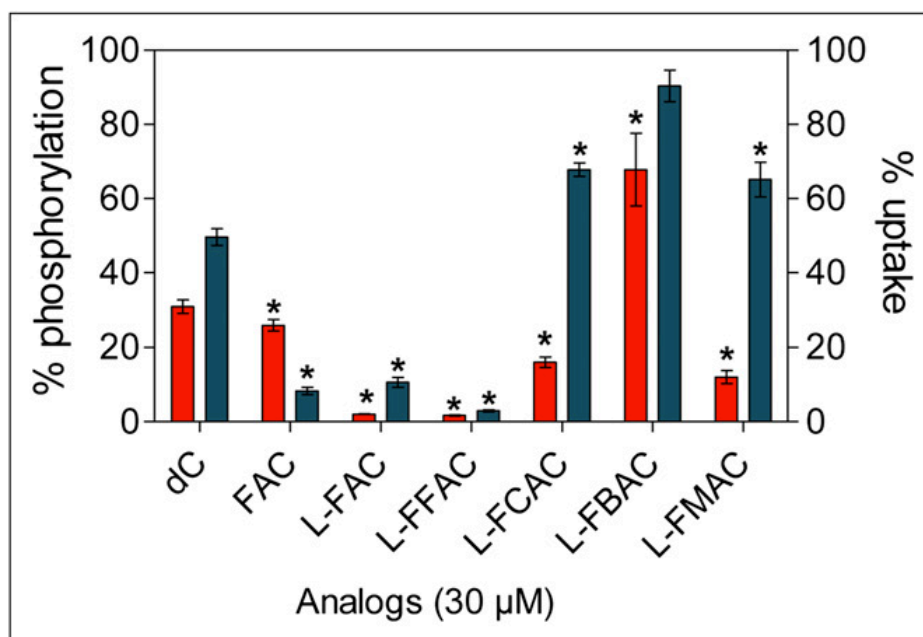


Figure 3.5

Biodistribution of ^{18}F -labeled unnatural nucleosides in mice. (A) Small-animal PET/CT images of C57BL/6J mice. (B) Quantification of PET data. Probe uptake was normalized to muscle background (absolute uptake values are shown in Supplemental Fig. 3.6). (C) $\text{L-}^{18}\text{F}$ -FAC small-animal PET/CT scan of dCK knockout mouse. P values were calculated relative to FAC for each specific tissue. * $P < 0.05$; $n = 5$ (^{18}F -FAC), $n = 3$ ($\text{L-}^{18}\text{F}$ -FAC), $n = 3$ ($\text{L-}^{18}\text{F}$ -FMAC), $n = 2$ ($\text{L-}^{18}\text{F}$ -FFAC), and $n = 2$ ($\text{L-}^{18}\text{F}$ -FCAC). %ID/g = percentage injected dose per gram of tissue; Bl = urinary bladder; B. Marrow = bone marrow; BM = bone marrow; GI = gastrointestinal tract; KO = knockout; L = liver; S = spleen; SG = salivary gland; Thy = thymus.

FIGURE 3.5

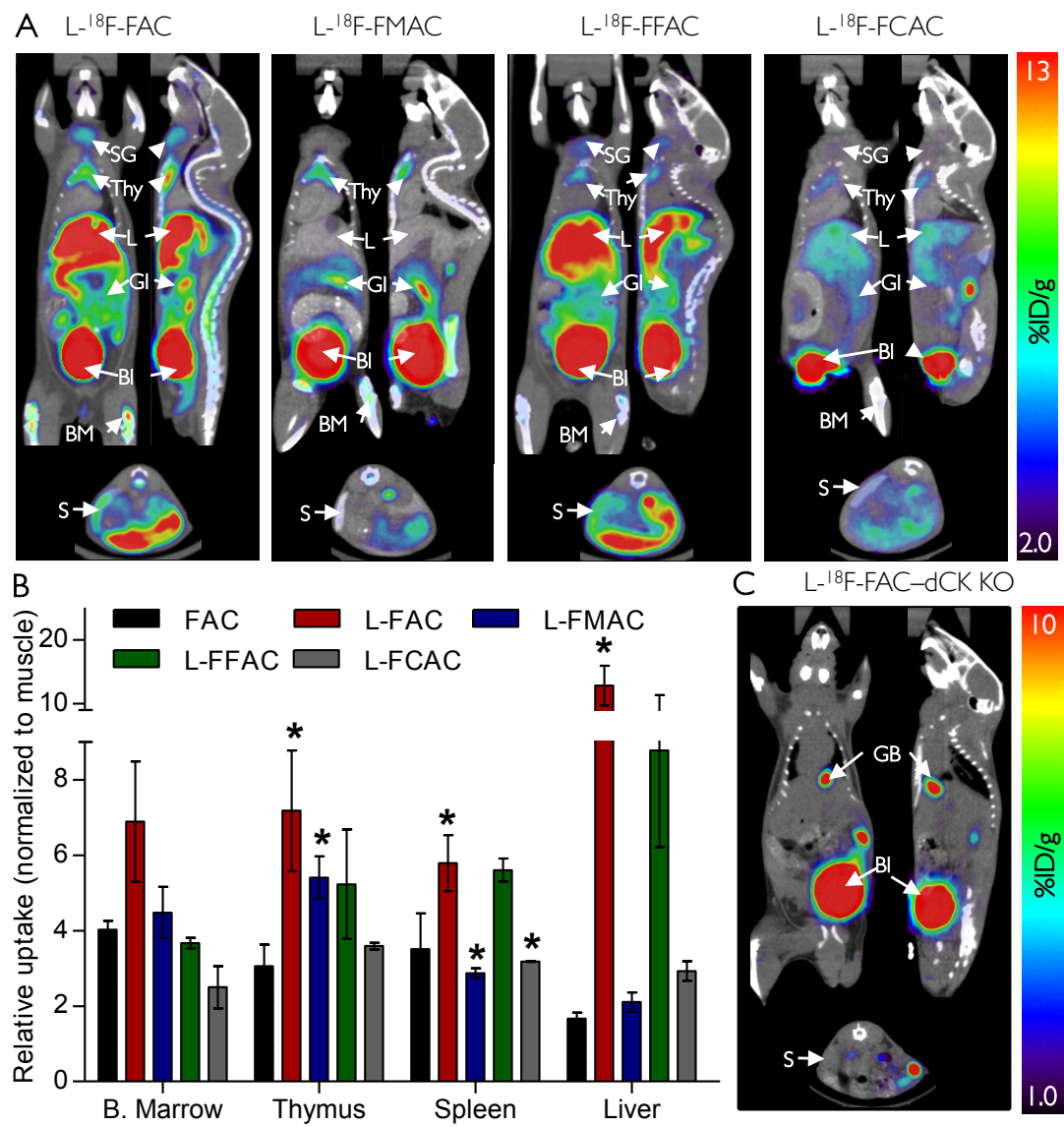
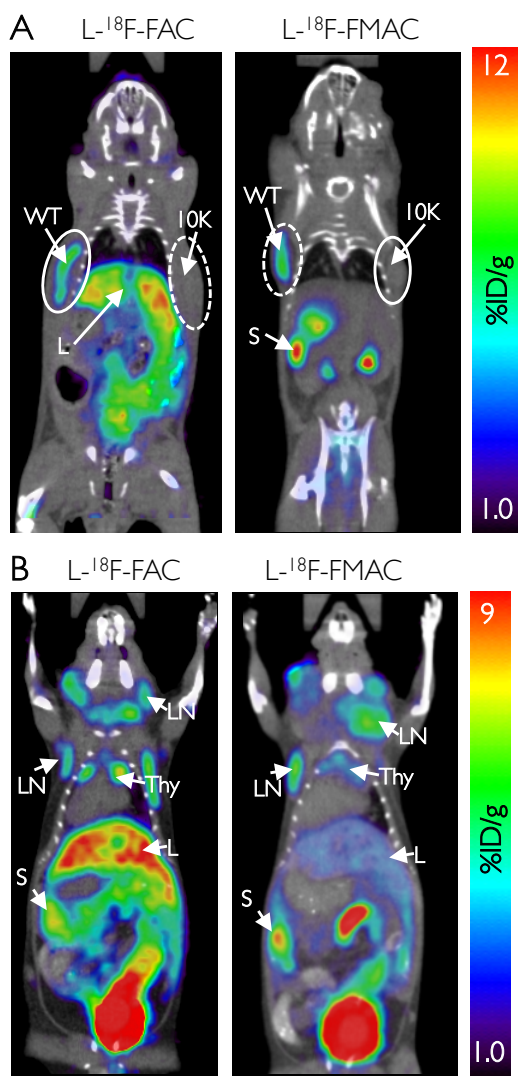


Figure 3.6

L-¹⁸F-FAC and L-¹⁸F-FMAC microPET/CT scans of malignant and autoimmune lymphoproliferative disorders. (A) L-¹⁸F-FAC and L-¹⁸F-FMAC microPET/CT imaging of L1210 lymphoma tumors. The L1210 parental cell line (WT, solid-lined circle) and the dCK-deficient variant L1210-10K (10K, dash-lined circle) were injected subcutaneously under the left and right shoulder of the mouse, respectively. Only the parental cell line accumulated both probes; (B) L-¹⁸F-FAC and L-¹⁸F-FMAC microPET/CT imaging of autoimmune B6.MRL-Fas^{lpr/J} mice. Both probes detected cervical, axillary, and brachial lymphadenopathy in these mice. %ID/g = percentage injected dose per gram of tissue; L = liver; LN = lymph nodes; S = spleen; Thy = thymus; WT = wild-type. Number of mice per probe ≥ 3.

FIGURE 3.6



Supplemental Methods

MicroPET/CT Imaging

Mice were kept warm, under gas anesthesia (2% isoflourane) and injected with 200 μ Ci of ^{18}F -labeled probes (i.v.). 1 hr interval for uptake was allowed between probe administration and microPET/CT scanning. Data were acquired using a Siemens Preclinical Solutions (Knoxville, TN) microPET Focus 220 and a MicroCAT II CT instrument. MicroPET data were acquired for 10 min and was reconstructed using statistical maximum a posteriori probability algorithms (MAP) into multiple frames (32). The spatial resolution of PET is ~ 1.5 mm, 0.4 mm voxel size. CT images are at low dose 400 μ m resolution acquisitions, with 200 μ m voxel size. MicroPET and microCT images were co-registered using a previously described method (33). 3D regions of interest (ROI) were drawn using AMIDE software (34). Color scale is proportional to tissue concentration with red being the highest and lower values in yellow, green & blue.

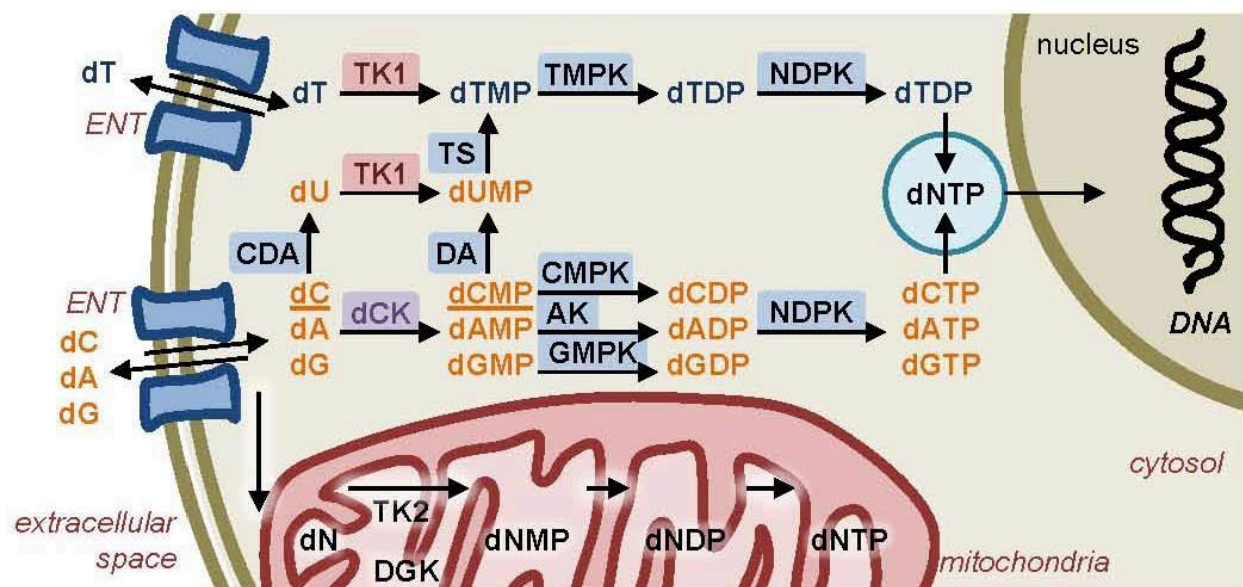
Production of recombinant CDA and dCK

Human CDA and dCK (plasmids were gifts from Dr. Margaret Black at Washington State University) were expressed in *E. coli*. Protein induction was carried out at 37°C with 1 mM IPTG (Fisher Scientific) for at least four hours. The cells were lysed via sonication followed by three cycles of freeze-thawing using liquid nitrogen and a 37°C water bath. Poly-His-tagged proteins were purified using HisPur™ Cobalt Resin (Pierce); purity was checked by SDS-PAGE.

Supplemental Figure 3.1

dCK regulates a rate-limiting step in the deoxyribonucleoside salvage pathway. dCK is the only salvage enzyme that can supply cells with all 4 precursors of DNA, 3 of them through direct phosphorylation and trapping of dC, dA and dG and the fourth through metabolic processing of dCMP to dTMP. ENT, equilibrative nucleoside transporter; CDA, cytidine deaminase; DA, dCMP deaminase; TS, thymidylate synthase; AK, adenylate kinase; GMPK, guanylate kinase; CMPK, cytidylate kinase; TMPK, thymidylate kinase; NDPK, nucleotide diphosphate kinase.

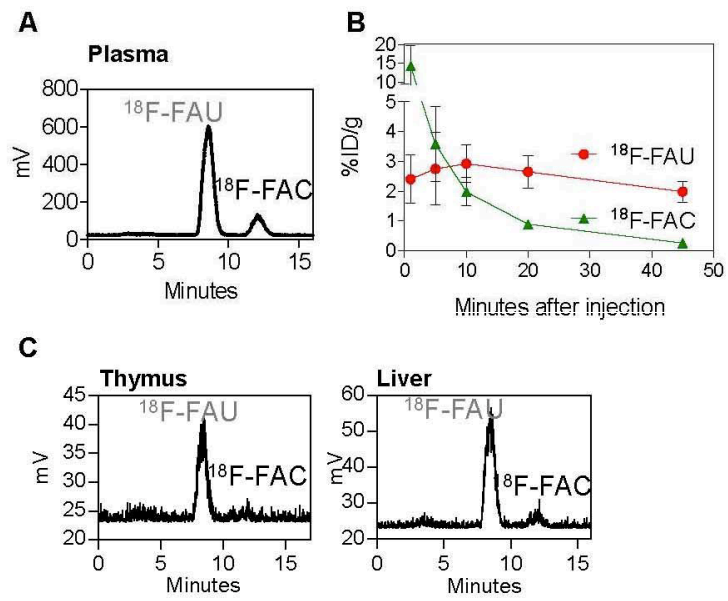
SUPPLEMENTAL FIGURE 3.1



Supplemental Figure 3.2

***In vivo* metabolite analysis of ^{18}F -FAC.** (A) HPLC analysis of plasma samples 45 min following ^{18}F -FAC injection. (B) Plasma clearance rates of ^{18}F -FAC and ^{18}F -FAU analyzed by HPLC (assuming no other radioactive metabolites present in blood); (C) HPLC analysis of the deamination metabolite of ^{18}F -FAC, ^{18}F -FAU, accumulation in thymus and liver. Traces show accumulation of the probes at 45 minutes after i.v. injection of ^{18}F -FAC into C57BL/6J mice. Results are representative of $n \geq 2$ independent experiments.

SUPPLEMENTAL FIGURE 3.2



Supplemental Figure 3.3

Radiochemical synthesis of 2-Chloro-9-(2'-deoxy-2'-¹⁸F-beta-D-arabinofuranosyl) adenine

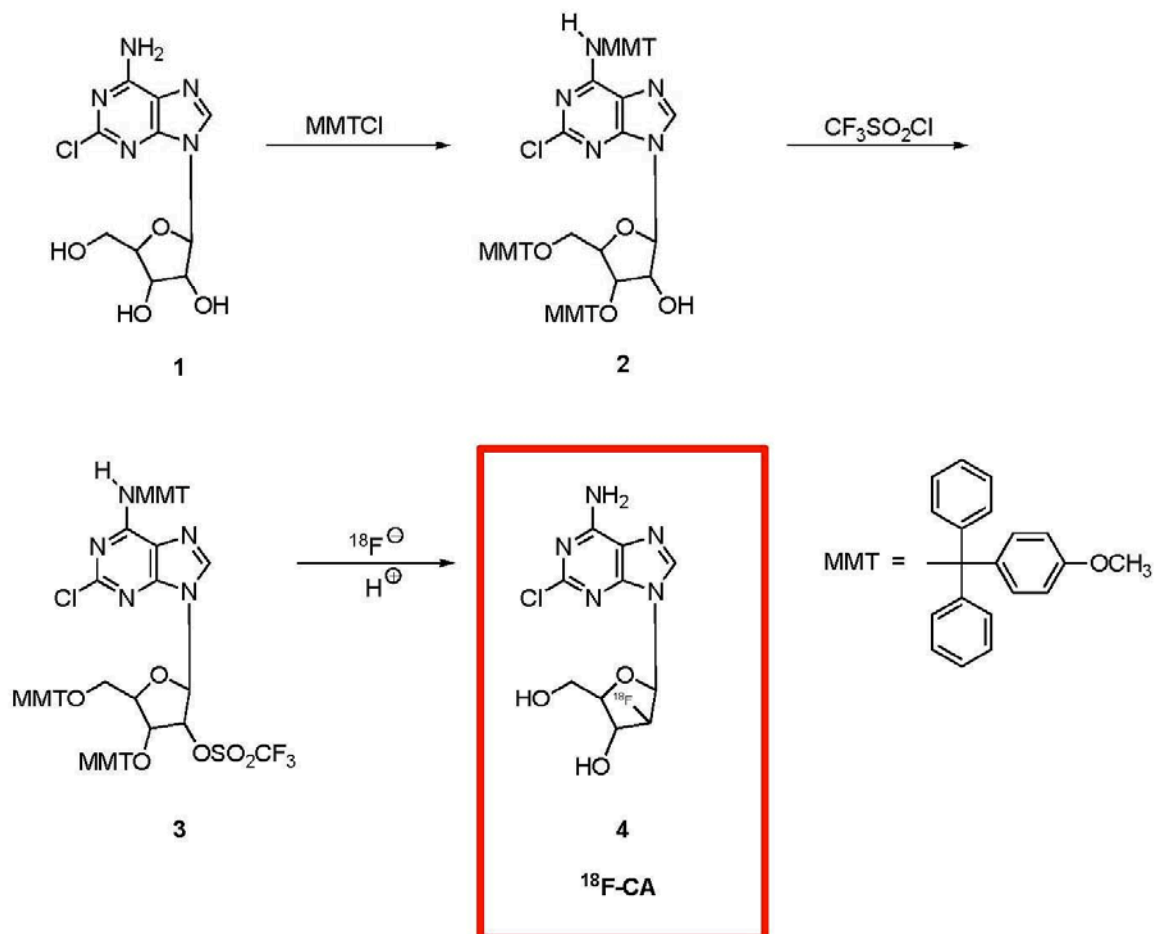
– ¹⁸F-CA (¹⁸F-labeled Clofarabine). The trityl protected chloroadenosine derivative **2** was prepared by a general procedure developed previously (35). Thus, 2-chloroadenosine (**1**) (9.2 mmol), 4-dimethylaminopyridine (9.2 mmol) and monomethoxytrityl chloride (32.4 mmol) were placed in a dry 250 mL round bottom flask under argon and 80 mL of dry pyridine was added. The mixture was stirred at 90°C for 18 hr. Pyridine was evaporated in rotary evaporator and the last traces of it were azeotropically removed with toluene. The residue was dissolved in dichloromethane and washed with water. The organic layer was dried with Na₂SO₄, filtered and evaporated. The crude product was subjected to silica gel column chromatography with 25% ethyl acetate in hexane as the eluent to isolate pure hydroxy product **2**. The triflate **3** was prepared from the corresponding hydroxy derivative **2** as follows: The hydroxy compound **2** (0.1 mmol) was dissolved in 3 mL of dichloromethane under argon and 4-dimethylaminopyridine (0.18 mmol) was added. The solution was cooled in an ice bath at 0°C for 10 min. Triflyl chloride (0.02 mL) was then added and the reaction mixture was gradually warmed to room temperature and stirred for 3 hr. The reaction mixture was diluted with 10 mL of dichloromethane and washed with water. The organic layer was dried with Na₂SO₄. Evaporation of dichloromethane gave an oily residue, which was purified by silica gel column chromatography using 30% ethyl acetate in hexane as eluent provided the pure triflate derivative **3**.

No-carrier-added ¹⁸F-fluoride ion was produced by 11 MeV proton bombardment of 98% enriched ¹⁸O-water in a silver target body using a RDS-112 cyclotron. The aqueous ¹⁸F-fluoride

ion was treated with a solution of K_2CO_3 (1 mg) and Kryptofix 2.2.2 (10 mg) dissolved in water (0.04 mL) and acetonitrile (0.75 mL) mixture. The solution was evaporated at 115°C with a stream of nitrogen gas. The residue was dried by the azeotropic distillation with acetonitrile (3 x 0.5 mL). The triflate precursor **3** (10 mg) dissolved in 1 mL of acetonitrile was added to the dried $K^{18}F$ /Kryptofix complex and reacted at 110°C for 25 min. The reaction mixture was cooled to room temperature and passed through a small cartridge of silica gel. The cartridge was eluted with 4 x 2 mL of ethyl acetate. The ethyl acetate was evaporated to dryness and the residue was then dissolved in 0.5 mL of acetonitrile. One mL of 1M HCl was added to the acetonitrile solution and heated at 100°C for 5 min. The reaction mixture was diluted to a total volume of 3 mL with a solution of 15% ethanol and 85 % 25 mM ammonium acetate in water and injected into a semi-preparative HPLC column (Phenomenex Gemini C-18 column; 25 x 1 cm) and eluted with a mobile phase of 15% ethanol and 85% 25 mM ammonium acetate in water at a flow rate of 5.0 mL/min. The effluent from the column was monitored with an UV detector ($\lambda = 263$ nm) and a gamma radioactive detector. The chemically and radiochemically pure ^{18}F -labeled product **4** with retention time between 11 and 13 min isolated in 10 – 15% radiochemical yield was made isotonic by dilution with sterile saline solution which also decreased the concentration of ethanol to < 10%. The solution was then sterilized by passing through a Millipore sterilizing filter (0.22 μ m) into a sterile multi-dose vial.

SUPPLEMENTAL FIGURE 3.3

Synthesis of ^{18}F -Clofarabine (^{18}F -CA)



Supplemental Figure 3.4

Radiochemical synthesis of ^{18}F -labeled D- and L-analogs. The reaction schemes to synthesize these compounds were similar to that previously reported for ^{18}F -FAC synthesis (4, 11). Typical procedures for the syntheses of D-isomers (**A**) and L-isomers (**B**) of ^{18}F -FAC are provided. The synthesis of all the other FAC family of probes utilized the methods outlined herein using the appropriate 5-substituted cytosine silyl analogs.

A

OCOPh
PhOCO OSO₂CF₃ $\xrightarrow{^{18}\text{F}^{\oplus}}$ OCOPh
PhOCO $\xrightarrow{\text{HBr}}$ OCOPh
PhOCO Br $\xrightarrow{\text{Me}_3\text{SiO}-\text{NHSIMe}_3-\text{R}}$ OCOPh
PhOCO $\xrightarrow{\text{NaOCH}_3}$ OCOPh

¹⁸F-FAC (R = H);
¹⁸F-FMAC (R = CH₃);
¹⁸F-FBAC (R = Br);
¹⁸F-FCAC (R = Cl);
¹⁸F-FFAC (R = F).

B

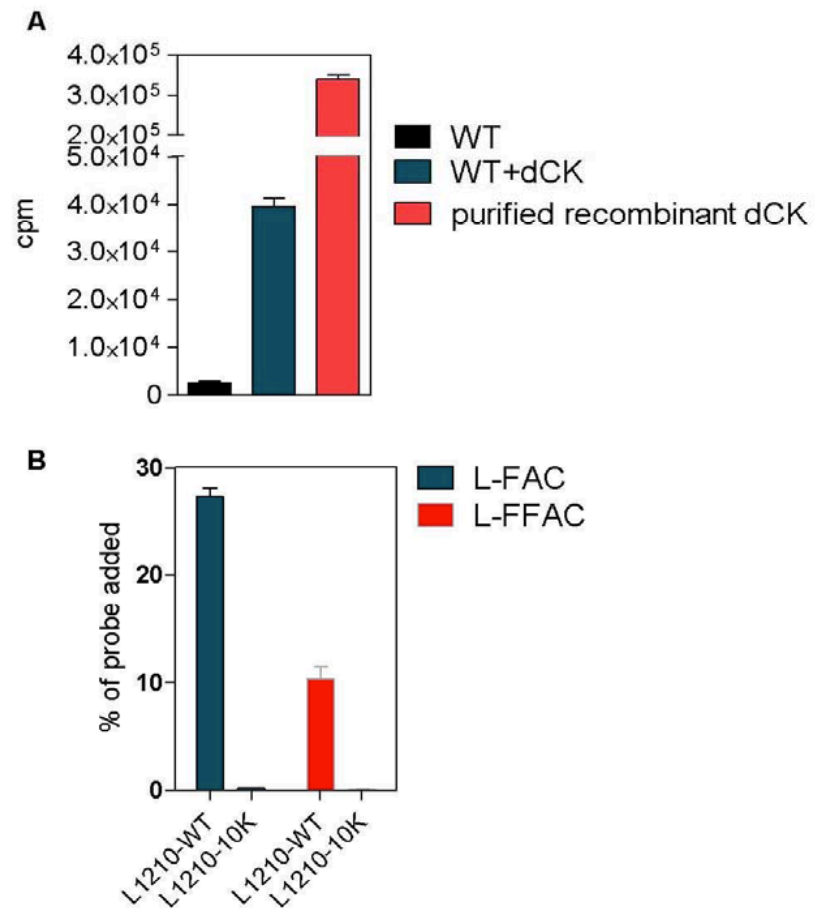
PhOCO PhOCO
F₃CO₂SO OCOPh $\xrightarrow{^{18}\text{F}^{\oplus}}$ PhOCO PhOCO
OCOPh $\xrightarrow{\text{HBr}}$ OCOPh
Br $\xrightarrow{\text{Me}_3\text{SiO}-\text{NHSIMe}_3-\text{R}}$ OCOPh
OCOPh $\xrightarrow{\text{NaOCH}_3}$ OCOPh

L-¹⁸F-FAC (R = H);
L-¹⁸F-FMAC (R = CH₃);
L-¹⁸F-FBAC (R = Br);
L-¹⁸F-FCAC (R = Cl);
L-¹⁸F-FFAC (R = F).

Supplemental Figure 3.5

L-¹⁸F-FAC and L-¹⁸F-FFAC. To determine if L-FAC was phosphorylated by dCK, a kinase assay was performed with the purified recombinant human dCK and the ¹⁸F-labeled L-FAC (**A**). As controls, lysates from wild-type *E. coli* (WT) and *E. coli* induced to overexpress the human protein (WT+dCK) were included. Kinase assays were also performed using lysates from dCK-positive L1210 and dCK-negative L1210-10K cells (**B**) and either L-¹⁸F-FAC or L-¹⁸F-FFAC. For all the kinase assays shown, 1 µg of total protein was used for each sample. The kinase assays were carried out at 37°C for 20 minutes.

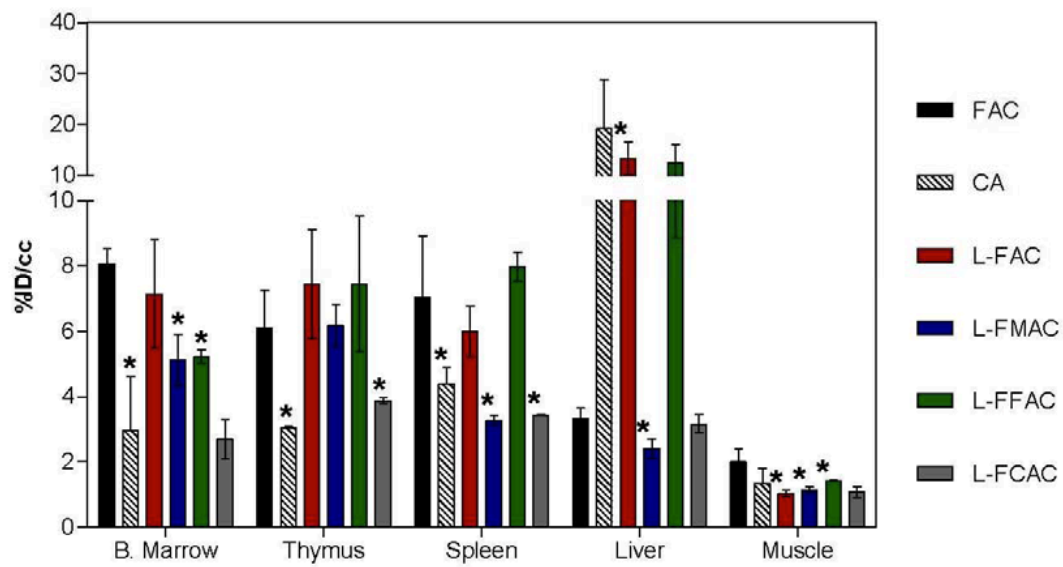
SUPPLEMENTAL FIGURE 3.5



Supplemental Figure 3.6

Quantification of microPET raw data shown in Figure 4. The L-analog probes were injected intravenously into C57BL/6J mice and images were acquired 60 min post injection ($n \geq 3$; mean with SD error bars). (P values are calculated relative to FAC for each specific tissue. * = $P < 0.05$, $n=5$ (^{18}F -FAC), $n=3$ ($\text{L-}^{18}\text{F}$ -FAC), $n=3$ ($\text{L-}^{18}\text{F}$ -FMAC), $n=2$ ($\text{L-}^{18}\text{F}$ -FFAC), $n=2$ ($\text{L-}^{18}\text{F}$ -FCAC)).

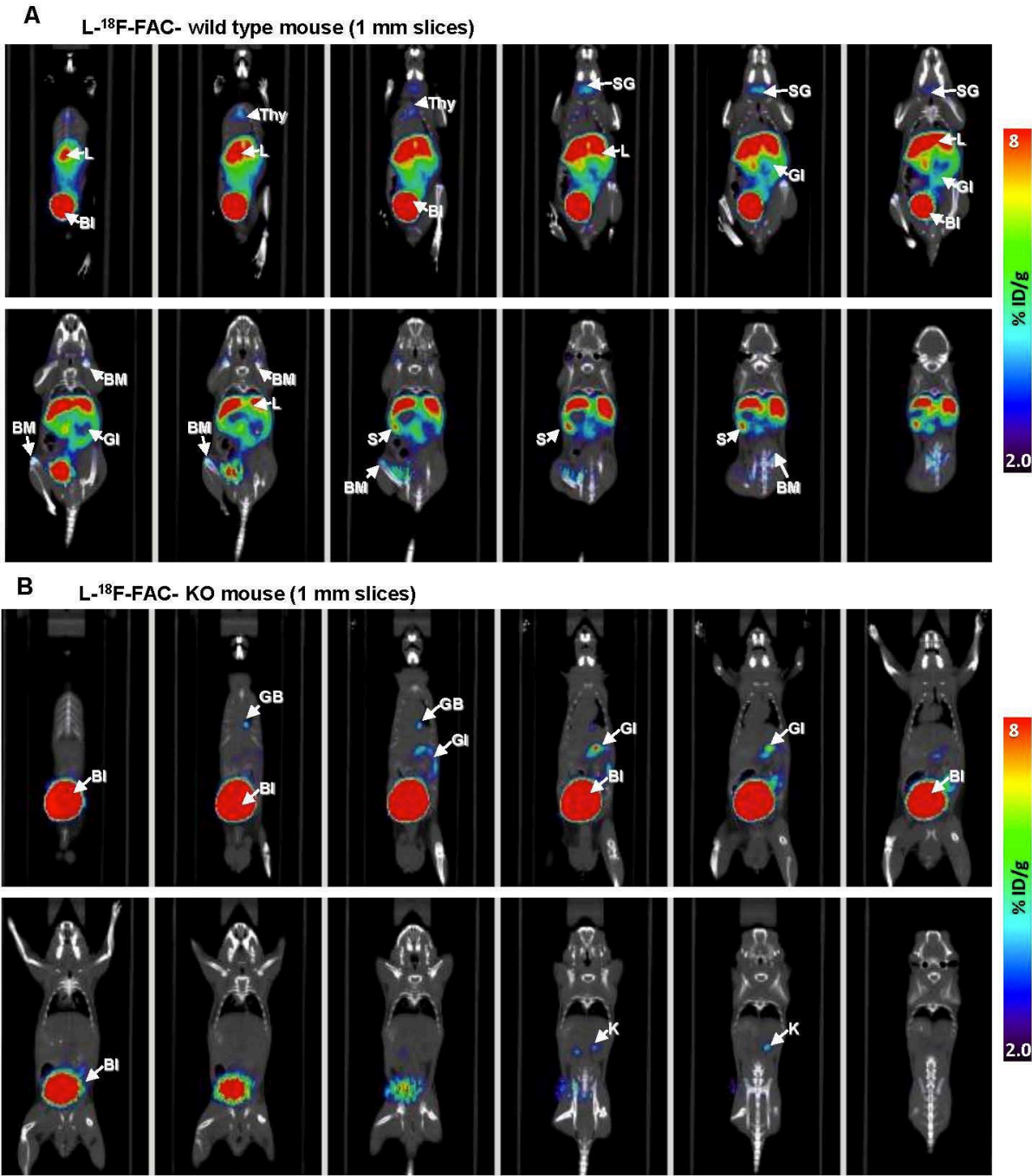
SUPPLEMENTAL FIGURE 3.6



Supplemental Figure 3.7

Lack of *in vivo* uptake of ^{18}F -L-FAC in the dCK KO mice. 1-mm coronal slices of (A) WT and (B) dCK KO mice imaged 60 min post i.v. administration of 200 μCi of ^{18}F -L-FAC. Thy, thymus; BM, bone marrow; S, spleen; L, liver; GI, gastrointestinal tract; K, kidney; Bl, bladder.

SUPPLEMENTAL FIGURE 3.7



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Chapter 4:

Stratification of Nucleoside Analog Chemotherapy Using 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-D-Arabinofuranosyl)Cytosine and 1-(2'-Deoxy-2'-¹⁸F-Fluoro-β-L-Arabinofuranosyl)-5-Methylcytosine PET

Abstract

The ability to measure tumor determinants of response to nucleoside analog (NA) chemotherapy agents such as gemcitabine and related compounds could significantly impact the management of several types of cancer. Previously we showed that the accumulation in tumors of the new PET tracer 1-(2'-deoxy-2'-¹⁸F-fluoro-β-D-arabinofuranosyl)cytosine (¹⁸F-FAC) is predictive of responses to gemcitabine. ¹⁸F-FAC retention in cells requires deoxycytidine kinase (dCK), a rate-limiting enzyme in the deoxyribonucleoside salvage metabolism and in gemcitabine conversion from an inactive prodrug to a cytotoxic compound. The objectives of the current study were (i) to determine if ¹⁸F-FAC tumor uptake is also influenced by cytidine deaminase (CDA), a determinant of resistance to gemcitabine, (ii) to develop a new PET assay using ¹⁸F-FAC and the related probe 1-(2'-deoxy-2'-¹⁸F-fluoro-β-L-arabinofuranosyl)-5-methylcytosine (L-¹⁸F-FMAC) to profile tumor lesions for both dCK and CDA enzymatic activities and (iii) to determine if this PET assay can identify the most effective NA chemotherapy against tumors with differential expression of dCK and CDA.

Methods: Isogenic murine leukemic cell lines with defined dCK and CDA activities were generated by retroviral transduction. A cell viability assay was used to determine the sensitivity of the isogenic cell lines to the dCK-dependent NA prodrugs gemcitabine and clofarabine. *In vitro* enzymatic and cell-based tracer uptake assays and *in vivo* PET imaging using ¹⁸F-FAC and L-¹⁸F-FMAC were used to predict tumor responses to gemcitabine and clofarabine.

Results: dCK and CDA activities measured by kinase and tracer uptake assays correlated with the sensitivity of isogenic cell lines to gemcitabine and clofarabine. Co-expression of CDA decreased the sensitivity of dCK-positive cells to gemcitabine treatment *in vitro* by 15-fold, but did not affect responses to clofarabine. Co-expression of CDA decreased ¹⁸F-FAC, but not L-¹⁸F-

FMAC, phosphorylation and uptake by dCK-positive cells. ^{18}F -FAC and L- ^{18}F -FMAC PET estimates of the enzymatic activities of dCK and CDA in tumor implants in mice were predictive of responses to gemcitabine and clofarabine treatment in vivo.

Conclusion: These findings support the utility of PET-based phenotyping of tumor nucleoside metabolism for guiding the selection of NA prodrugs.

Introduction

Nucleoside analog (NA) prodrugs are indicated in many types of cancer, but generally have low response rates and can induce significant side effects. For example, the response rates to gemcitabine in pancreatic, ovarian and lung cancer rarely exceed 20% (1, 2) while grade 3 or 4 toxicity occurs in up to 38% of patients. Nonetheless, for each malignancy a subset of patients respond well to gemcitabine. The ability to identify likely responders and non-responders before treatment would be important in the management of cancers treated with nucleoside analogs. In previous work we have developed a new PET probe that may enable patient stratification in malignancies in which gemcitabine is indicated as first or second line of treatment. This PET probe, designated 1-(2'-deoxy-2'-fluoroarabinofuranosyl) cytosine (^{18}F -FAC), closely resembles the chemical structure of gemcitabine (3). ^{18}F -FAC has a high affinity for deoxycytidine kinase (dCK), the rate-limiting enzyme in the activation of gemcitabine and related chemotherapeutic agents (Table 1). Pre-treatment ^{18}F -FAC PET imaging of a murine leukemia/lymphoma tumor model identified dCK-positive and -negative tumors and predicted responses to gemcitabine (4). However, in addition to decreased dCK activity, other mechanisms of resistance to gemcitabine have been identified. Examples include down-regulation of nucleoside transporters and overexpression of the ribonucleotide reductase subunit M1 (RRM1) and of cytidine deaminase (CDA) (reviewed in (5)). CDA catalyzes the deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. In humans, CDA activity is primarily found in human liver, spleen and plasma, whereas in mice it is mainly in the kidneys (6). The enzyme has been associated with resistance to various nucleoside analog therapies such as gemcitabine (7). In this follow-up study we focus on the development of a PET assay that can identify resistance to chemotherapy due to high tumor CDA activity. While dCK and CDA act on the same substrates,

the outcomes of their enzymatic activities are different: dCK phosphorylates and activates gemcitabine while CDA deaminates and inactivates this prodrug, thereby opposing the action of dCK (**Supplemental Fig. 4.1A**) (5). The ability to identify tumors that co-express dCK and CDA may enable chemotherapy stratification by indicating that such tumors will likely be resistant to gemcitabine but may still be sensitive to other dCK-dependent chemotherapeutics (Table 1). Amongst the dCK-dependent agents, clofarabine ranks highest as a potential alternative to gemcitabine in selected cancers that co-express dCK and CDA. Clofarabine is indicated for pediatric acute lymphoblastic leukemia, has excellent metabolic stability, resists deamination by CDA and has broad cytotoxicity in xenograft models of human colon, renal, non-small cell lung and prostate cancers as well as leukemias (8). Identification of dCK-positive tumors that co-express CDA would require PET probes that can be phosphorylated and trapped by dCK, but similar to clofarabine, resist deamination by CDA. We have recently developed a series of L-enantiomers of ^{18}F -FAC with these properties (9). Among the L-enantiomers, 1-(2'-deoxy-2'- ^{18}F -fluoro- β -L-arabinofuranosyl)-5-methylcytosine (L- ^{18}F -FMAC) had the most desirable biodistribution (9). L- ^{18}F -FMAC biodistribution in mice was similar to that of ^{18}F -FAC with the added advantage of lower non-specific retention in muscle for the L-enantiomer.

In the current study, we describe the utility of ^{18}F -FAC and L- ^{18}F -FMAC PET to differentiate cell subtypes by their relative dCK and CDA activity levels. We analyzed the value of ^{18}F -FAC and L- ^{18}F -FMAC PET in predicting differential tumor responses to gemcitabine and clofarabine and we determined their ability to guide treatment decisions in murine cancer models. Our data support the use of PET to predict treatment responses to NA chemotherapeutics in murine models of cancer and potentially in cancer patients.

Materials and Methods

Cell lines

L1210 cell lines (positive for dCK and negative for CDA, designated WT cells) and 10K (negative for both dCK and CDA) (10), were a gift from Charles Dumontet (Université Claude Bernard Lyon I, Lyon, France). Cells were cultured at 5% CO₂ and 37°C in RPMI 1640, supplemented with 5% FCS and 2 mM L-glutamine. Murine stem cell virus-based helper-free retroviruses encoding human CDA (huCDA; gift from Dr. Margaret Black at Washington State University), an internal ribosomal entry site (IRES) and the yellow fluorescent protein (YFP) were produced by transient co-transfection of the amphotrophic retrovirus packaging cell line Phoenix (American Type Culture Collection, SD 3443) (11). WT cells underwent spinfection with the pMSCV-*huCDA*-IRES-*YFP* retrovirus with 2 µg/mL polybrene (1000g, 120 min, 37°C) and were sorted by flow cytometry to ensure a pure population of CDA-expressing cells.

Drugs and IC₅₀ assays

Gemcitabine (570287, AK Scientific Inc.) stock solutions were prepared in water. Clofarabine (C7495, Sigma-Aldrich Co.) was prepared in dimethyl sulfoxide (DMSO). Tetrahydrouridine (584222, EMD Chemicals) was prepared in water. Cells were seeded in 384-well plates (1 x 10³ cells/well in 30 µL culture media) and allowed to settle for 4 hours. Serial drug dilutions (4x) were performed in drug solvent to ensure equal concentrations of solvent for all dilutions and diluted with culture media; 10 µL of this dilution were added to cells. Results were normalized to vehicle control.

In vitro kinase and uptake assays using ¹⁸F-FAC and L-¹⁸F-FMAC

Kinase and cell-based uptake assays were performed as previously described (9) using 185 kBq (5µCi) ¹⁸F-FAC or L-¹⁸F-FMAC and without addition of a competing NA. The

radiochemical purities of ^{18}F -FAC and L- ^{18}F -FMAC were >99% and the specific activity were >1000 Ci/mmol. Briefly, for kinase assays, 5×10^6 cells growing in exponential phase were lysed by three rounds of freeze-thaw. Supernatant containing purified protein was incubated with the radiolabeled probe for 20 min at 37°C and spotted on positively-charged DE-61 Whatman filters which bind negatively-charged phosphorylated products. Filters were washed, allowed to dry and analyzed for radioactivity. In uptake assays, cells were plated for 4-5 hours in growth media followed by incubation with the radiolabeled probe. For ^{18}F -based uptake assays, L1210 cells (2.5×10^5 cells per well in 24-well plates) were incubated in 1 mL culture media supplemented with 185 kBq (5 μCi) ^{18}F -labeled probe. After 1 h at 37°C and 5% CO_2 , samples were washed 3 times and the cell pellet was resuspended in ice-cold phosphate buffered saline. Samples were measured for radioactivity using a Wallac Wizard 3" 1480 Automatic Gamma Counter (PerkinElmer).

In vivo microPET/CT imaging and treatment model

Animal studies were approved by the UCLA Animal Research Committee and were carried out according to the guidelines of the Division of Laboratory Animal Medicine at UCLA. On day -7, SCID mice were injected subcutaneously in the right flank with 1×10^6 cells resuspended in 50% phosphate-buffered saline and 50% MatrigelTM (354234, BD Biosciences). On day -2, mice underwent ^{18}F -FAC microPET/CT imaging (Inveon, Siemens Medical Solutions USA Inc.; microCAT; Imtek Inc.). On day 0, prior to treatment, mice underwent L- ^{18}F -FMAC microPET/CT imaging. Mice were then randomized into treatment groups. Gemcitabine (360mg/kg/dose) (10) was injected i.p. on days 0 and 4. Clofarabine (60mg/kg/dose) was administered i.p. on days 0-4. Vehicle control mice received 5.4% DMSO in saline on days 0-4. Mice were sacrificed when tumors reached an upper limit of 1.5 cm as required by DLAM

regulations. ^{18}F -FAC and L- ^{18}F -FMAC were synthesized and used for microPET/CT imaging studies as described in the patent (12) and previously elsewhere (3, 9). The radiochemical purity of the probes was >99% and the specific activity was >1000 Ci/mmol. Static microPET images were acquired for 600 sec, followed by 3D histogramming and reconstruction with a zoom factor of 2.1 using 3D-OSEM with 2 iterations followed by MAP with 18 iterations (beta=0.1). Whole-body MicroCAT images were acquired with the X-ray source based at 70 kVp and 500 μ A and exposure time of 480 sec. A Feldkamp reconstruction algorithm was applied. Images were analyzed using OsiriX Imaging Software version 3.8.

Statistical Analyses

Data are presented as mean $\pm$ SD. All *p* values were determined with unpaired, two-tailed T tests and values less than 0.001 were considered to be statistically significant. GraphPad Prism 5 software was used to calculate statistics and generate graphs.

Results

Co-expression of dCK and CDA confers differential sensitivity to nucleoside analog chemotherapeutics.

To investigate the roles of dCK and CDA in resistance to NA chemotherapy we generated a panel of L1210 isogenic cell lines that correspond to three metabolic subtypes: dCK-positive, CDA-negative (WT); dCK-positive, CDA-positive (WT+CDA); and dCK-negative (10K, these cells also lack CDA expression). To validate the isogenic cell lines we performed *in vitro* kinase assays using tritiated deoxycytidine (^3H -dCyd), which is a substrate for both dCK and CDA. WT cells were 13-fold more efficient than WT+CDA cells at phosphorylating ^3H -dCyd. This difference was abolished in the presence of tetrahydrouridine (THU), a potent

inhibitor of CDA (**Supplemental Fig. 4.1B**). 10K cells did not phosphorylate ^3H -dCyd, as previously shown (4), and this was unaffected by THU (**Supplemental Fig. 4.1B**). The results of the kinase assays were confirmed using a cell-based ^3H -dCyd uptake assay (**Supplemental Fig. 4.1C**).

The differential uptake and phosphorylation of ^3H -dCyd by the isogenic cell lines were consistent with their differential responses to the dCK-dependent NA prodrugs gemcitabine (which is deaminated by CDA) and clofarabine (which is resistant to deamination) (**Table 1**). WT cells were 15-fold more sensitive to gemcitabine than cells co-expressing dCK and CDA (WT+CDA). WT+CDA cells were >350 times more sensitive than the dCK-negative 10K cells (**Table 4.2**). In contrast, WT+CDA cells were marginally more sensitive than WT cells to clofarabine (not statistically significant). WT cells were >290 times more sensitive to clofarabine than 10K cells (**Table 4.2**), reflecting the dependence of clofarabine activation on dCK activity.

A new PET assay to stratify tumor dCK/CDA activities

We previously reported on the ability of ^{18}F -FAC and L- ^{18}F -FMAC (**Supplemental Fig. 4.2**) to differentiate dCK-positive and -negative tumors (9). Here, we investigated whether these two PET tracers may allow additional measurements of CDA activity. In cell-based uptake assays both ^{18}F -FAC and L- ^{18}F -FMAC showed high retention in WT cells and low retention in 10K cells, as previously reported (4, 9) (**Figs. 4.1A** and **4.1B**). Relative to WT cells, WT+CDA cells showed drastically reduced ^{18}F -FAC accumulation. ^{18}F -FAC accumulation was restored in the presence of THU (**Fig. 4.1A**). In contrast, L- ^{18}F -FMAC uptake was similar between WT and WT+CDA cells and was independent of THU (**Fig. 4.1B**).

We next sought to determine whether PET imaging with ^{18}F -FAC and L- ^{18}F -FMAC could predict treatment responses in the L1210 tumor model. Prior to treatment, each tumor-

bearing mouse was scanned with ^{18}F -FAC and L- ^{18}F -FMAC on days -2 and 0, respectively. ^{18}F -FAC uptake was significantly higher in WT tumors compared to WT+CDA or 10K tumors, with the latter two being indistinguishable by PET (**Figs. 4.2A, 4.2B and 4.2C**, respectively). In contrast, L- ^{18}F -FMAC PET detected WT and WT+CDA tumors equally (**Figs. 4.2A and 4.2B**) and distinguished these from the dCK-deficient 10K tumors (**Fig. 4.2C**). The tumor-to-muscle ratio was approximately 5-fold higher for L- ^{18}F -FMAC than for ^{18}F -FAC, as expected due to the higher nonspecific muscle uptake of ^{18}F -FAC (9) (**Fig. 4.2D**).

PET imaging with ^{18}F -FAC and L- ^{18}F -FMAC predicts treatment responses in vivo.

To determine if the ^{18}F -FAC/L- ^{18}F -FMAC PET assay is predictive of tumor responses in vivo, SCID mice bearing established subcutaneous tumors were treated with gemcitabine, clofarabine or vehicle control. Daily caliper measurements were performed to determine tumor growth and animals were sacrificed when tumors reached 1.5 cm in the largest diameter. Growth curves for each tumor subtype are shown in **Fig. 4.3A**. WT tumor volumes decreased significantly in response to both gemcitabine and clofarabine compared to vehicle control treated mice. Clofarabine-treated WT tumors relapsed earlier (approximately day +8) than the gemcitabine-treated WT tumors (approximately day +12), which parallels the *in vitro* sensitivities of WT cells to these drugs (**Fig. 4.3A and Table 4.2**, respectively). Whereas CDA overexpression in WT+CDA tumors significantly diminished the response to gemcitabine, it enhanced the response to clofarabine. Neither drug significantly affected the growth of dCK-negative 10K tumors compared to vehicle controls. Tumor growth profiles paralleled differences in survival (**Fig. 4.3B**).

Discussion

The ability to non-invasively estimate tumor dCK and CDA activities in vivo has therapeutic implications. Several NA prodrugs including gemcitabine require activation by dCK and are susceptible to inactivation by CDA, while others such as clofarabine are phosphorylated and activated by dCK but are not susceptible to deamination (5). Here we show that PET imaging using ^{18}F -FAC and L- ^{18}F -FMAC can be used to estimate dCK and CDA activities in tumor lesions and that these measurements can guide treatment stratification. Low ^{18}F -FAC uptake in tumors indicates several possibilities such as poor tumor vascularization, inefficient transport across the cell membrane, low dCK phosphorylation as well as high levels of CDA activity. Subsequent PET imaging of these tumors with L- ^{18}F -FMAC may identify tumors in which CDA-mediated deamination represents the main mechanism of resistance to gemcitabine. These tumors are good candidates for treatment with dCK-dependent, CDA-insensitive prodrugs such as clofarabine.

Clinical studies have demonstrated the prognostic significance of low dCK and/or high CDA activities for poor patient outcome (13-15). The current study assesses these enzymes at the upper and lower ranges of expression. Our data in a panel of 50 human lymphoma cell lines indicate dCK mRNA levels vary as much as 40-fold compared to control (**Supplemental Fig. 4.3A**) and correlate with dCK enzymatic activity (**Supplemental Fig. 4.3B**). These findings are further supported by the variable dCK activities across human ovarian cancer cell lines (**Supplemental Fig. 4.3C**). Collectively, these data suggest cancer cells are metabolically distinct from one another in regards to the activity of the deoxyribonucleoside salvage pathway. It will be important to profile the panel of lymphoma and ovarian cancer cell lines for CDA activity and determine whether the ^{18}F -FAC/L- ^{18}F -FMAC PET assay developed using the murine L1210 leukemia model can be generally applicable to human tumors of different

histological types. Ongoing clinical studies are evaluating the relationship between dCK activity measured on tumor biopsies and corresponding ^{18}F -FAC/L- ^{18}F -FMAC PET signals in lymphoma, ovarian and pancreatic cancer patients. It might be possible to estimate phosphorylation versus deamination activities with dynamic ^{18}F -FAC/L- ^{18}F -FMAC PET studies and we are, therefore, developing a tracer kinetic model to better describe these parameters. In addition to low dCK activity and increased deamination, reduced expression of nucleoside transporters such as SLC29A1 (16) and overexpression of the ribonucleotide reductase (RR) subunit M1 (RRM1) (17) have also been associated with NA chemoresistance. We have previously demonstrated that ^{18}F -FAC is a substrate for SLC29A1 (3). The order of magnitude difference in probe uptake between ^{18}F -FAC and L- ^{18}F -FMAC may reflect differences in transport between natural D- and unnatural L-enantiomers and transporters other than SLC29A1 may also be involved. The contribution of RRM1 activity to the uptake of ^{18}F -FAC and analogs remains to be determined. In theory, overexpression of RRM1 in tumors should expand their dCTP pools, which in turn may reduce the activity of dCK by feedback inhibition. Furthermore, extrinsic factors such as poor tissue perfusion may also contribute to a small therapeutic index of gemcitabine (18) and may also limit PET probe delivery. It is likely that PET alone will be insufficient to identify all mechanisms of resistance and that complementary imaging modalities such as contrast-enhanced endoscopic ultrasound or magnetic resonance imaging (MRI) have to be employed.

Conclusions

Measurements of tumor nucleoside metabolism are clinically relevant for cancer diagnosis, prognosis and assessment of therapy response (19-21). Our findings indicate that PET imaging

using ^{18}F -FAC and L- ^{18}F -FMAC may be useful for guiding the selection of NA chemotherapeutic agents. A more in depth understanding of the advantages and limitations of the ^{18}F -FAC and L- ^{18}F -FMAC PET probes together with the use of other imaging modalities such as MRI will further the role of imaging in personalized, predictive medicine.

Acknowledgments

We thank Larry Pang and Dr. Liu Wei for animal and imaging expertise; the cyclotron group for the production of PET probes; Drs. Oliver Dorigo and Sven de Vos for providing the human ovarian and lymphoma cell lines, respectively; Dr. David Gjertson for review of biostatistics; Dr. Chintda Santiskulvong for ovarian cancer samples preparation; Amanda Armijo, Gerald Toy, Evan Shih, Michelle Tom and Jeremy Work for help in general assay assistance. This work was supported by the *In Vivo* Cellular and Molecular Imaging Centers Developmental Project Award NIH P50 CA86306 (to Caius G. Radu and Jason T. Lee) and the Dana Foundation (Caius G. Radu).

Conflict of Interest Statement

Caius G. Radu, Nagichettiar Satyamurthy and Johannes Czernin are among the inventors of the national and PCT patent applications for the FAC technology referred to in the article. A group of UCLA faculty members including Caius G. Radu and Johannes Czernin are involved in Sofie Biosciences, a startup company that has licensed this intellectual property.

TABLE 4.1. Panel of nucleoside analog prodrugs

dCK-dependent drug	Nucleobase	CDA substrate
Cytarabine	pyrimidine	yes
Decitabine	pyrimidine	yes
Gemcitabine	pyrimidine	yes
Cladribine	purine	no
Clofarabine	purine	no
Fludarabine	purine	no

TABLE 4.2. IC₅₀ of nucleoside analog prodrugs

	Gemcitabine (μM)	Clofarabine (μM)
WT	0.00730 ± 0.00597	0.386 ± 0.202
WT+CDA	0.109 ± 0.0880	0.0894 ± 0.0516
10K	38.5 ± 28.6	112 ± 68.1

Values are given ± SD. Results are representative of three independent experiments at 72 hours drug incubation.

Figure 4.1

Detection of dCK/CDA-defined metabolic phenotypes. In vitro (A) ^{18}F -FAC and (B) L- ^{18}F -FAC enzymatic (left) and cell-based uptake (right) assays using the L1210 cell lines, with or without THU (100 μM). Results are normalized to protein concentration for kinase assays or cell number for uptake assays, and representative of three independent experiments. THU – tetrahydrouridine.

FIGURE 4.1

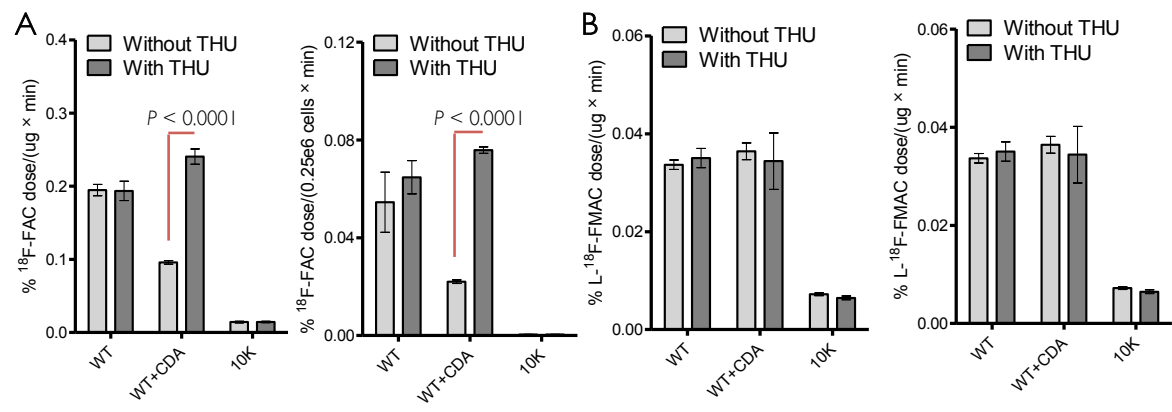


Figure 4.2

***In vivo* detection of dCK/CDA-dependent metabolic phenotypes using ^{18}F -FAC and L- ^{18}F -FMAC microPET/CT.** (A-C) Representative microPET/CT scans of L1210 tumor subtypes and (D) quantification of tumor microPET signals normalized to muscle. The same mouse was imaged on day -2 with ^{18}F -FAC and day 0 with L- ^{18}F -FMAC, prior to day 0 treatment. Images have been scaled differently between probes to offset the higher muscle background of ^{18}F -FAC. CT-only images (panels below the microPET/CT images) are displayed with volume rendering. Each tumor group consisted of $n \geq 5$ mice. Results are representative of three independent experiments.

FIGURE 4.2

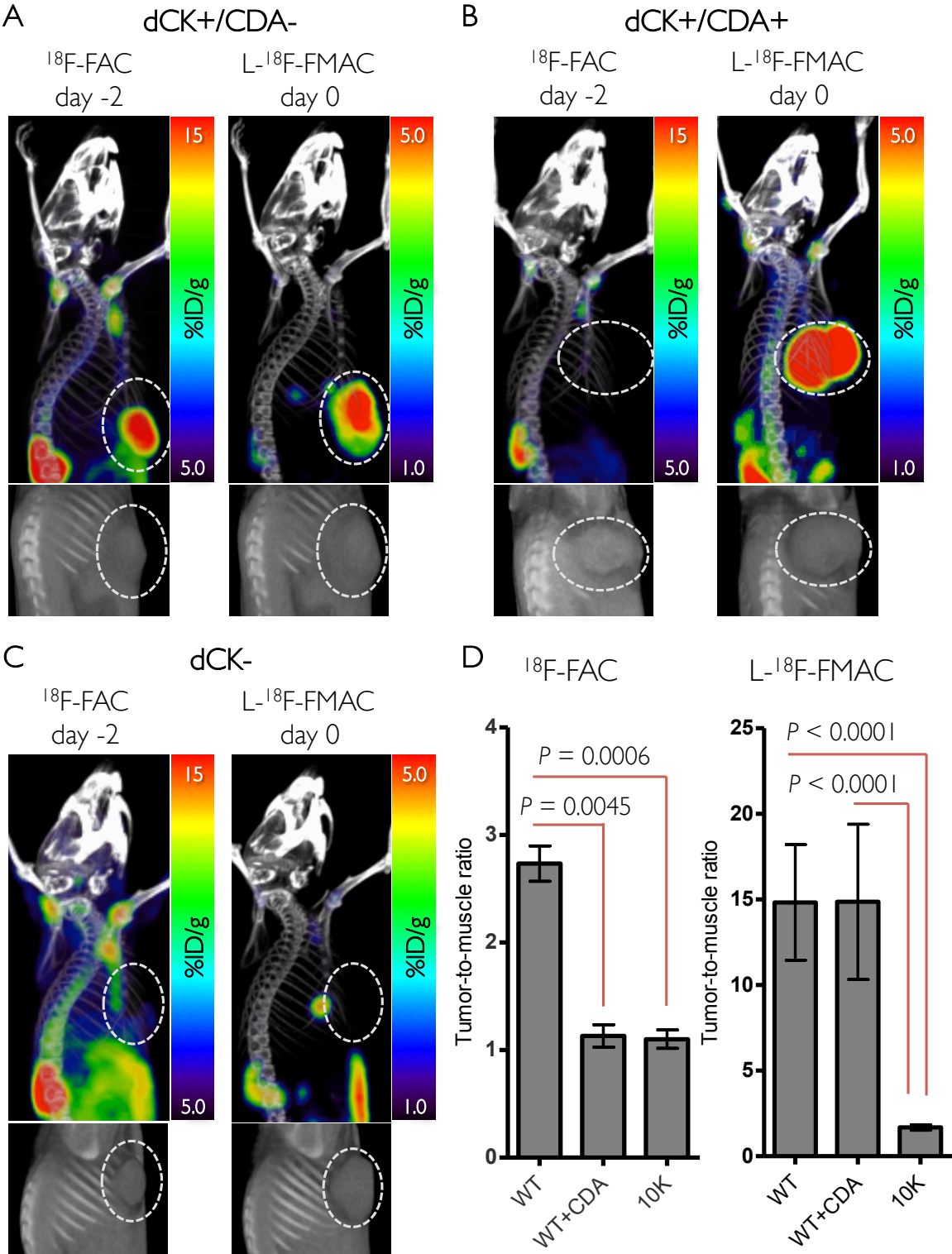
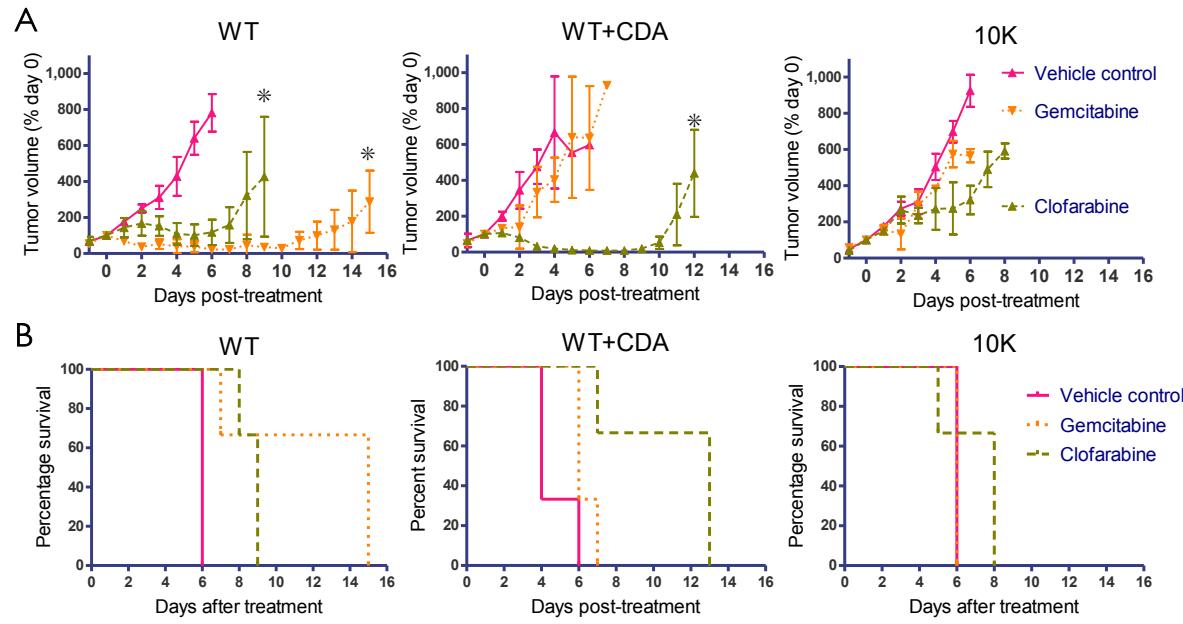


Figure 4.3

dCK and CDA-defined metabolic phenotypes correspond to differential responses to nucleoside analog pro-drugs *in vivo*. (A) *In vivo* tumor growth curves in response to treatment as determined by caliper measurements and normalized to tumor volume prior to start of treatment. * - statistically significant ($p < 0.001$) compared to vehicle control. (B) Kaplan-Meier survival curves as defined by regulatory restrictions on tumor size of tumor-bearing mice treated with gemcitabine, clofarabine or vehicle control. $n = 3$ for all groups. Results are representative of two independent experiments.

FIGURE 4.3



Supplemental Methods

In vitro kinase and uptake assays using ^3H -dCyd, ^3H -FAC and ^{18}F -FAC

Kinase and cell-based uptake assays were performed as previously described in the main text using 0.9 μM ^3H -dCyd (2'-deoxycytidine, [$5\text{-}^3\text{H}(\text{N})$], MT673, Moravek Biochemicals; specific activity, 0.833 TBq [22.5 Ci] /mmol), 1.5 μM ^3H -FAC (2'-deoxy-2'-fluoro-D-arabinofuranosylecytosine, [^3H]-, MT1858, Moravek Biochemicals; specific activity, 0.503 TBq [13.6 Ci] /mmol) or 185 kBq (5 μCi) ^{18}F -FAC. The radiochemical purities of ^{18}F -FAC were >99% and the specific activity were >1000 Ci/mmol. Briefly, 5×10^6 cells growing in exponential phase were lysed by three rounds of freeze-thaw. Supernatant containing purified protein was incubated with the radiolabeled probe for 20 min at 37°C and spotted on positively-charged DE-61 Whatman filters which bind negatively-charged phosphorylated products. Filters were washed, allowed to dry and analyzed for radioactivity. In uptake assays, cells were plated for 4-5 hours in growth media followed by incubation with the radiolabeled probe. In uptake assays, cells were plated for 4-5 hours in growth media followed by incubation with the radiolabeled probe. Samples were measured for radioactivity using a Wallac Wizard 3" 1480 Automatic Gamma Counter (PerkinElmer) for ^{18}F and a Beckman Liquid Scintillation counter for ^3H . For uptake assays, 38.85 kBq (1.05 μCi) of ^3H -dCyd were added to L1210 cells (1×10^5 cells per well in 96-well plates). After 1 h at 37°C, wells were washed 3 times with ice-cold phosphate-buffered saline using a vacuum filtration system (Millipore). Plates were oven-dried at 45–50°C for 30 min and 200 μL of scintillation fluid were added per well. Radioactivity was measured using a Trilux MicroBeta scintillation counter (Perkin Elmer). Tetrahydrouridine (THU, 584222, EMD Chemicals) was prepared in water.

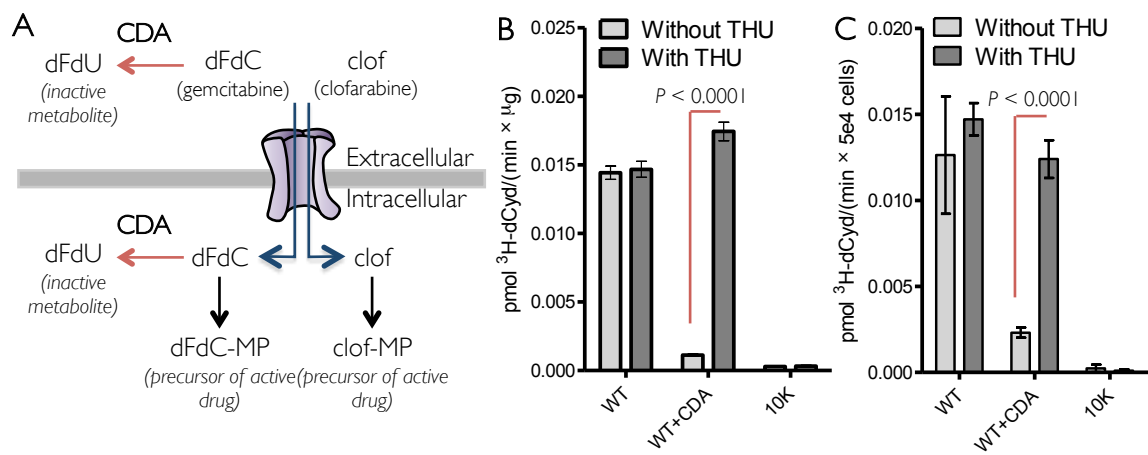
Quantitative Real-Time PCR gene expression analysis (qPCR)

Total RNA was purified from tissues using the Qiagen RNeasy Mini kit. 1.5 µg of RNA was then used to synthesize cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Pre-designed TaqMan Gene Expression assays for dCK (Assay ID: Hs01040726_m1) and GAPDH (Applied Biosystems, Part: Hs02758991_g1) as an endogenous control for quantification were used for qPCR analysis. The samples were run on a 48-well StepOne Real-Time PCR System (Applied Biosystems) and were analyzed with the StepOne Software v2.0 (Applied Biosystems) using the comparative C_t method ($\Delta\Delta C_t$). The qPCR mixture (50 µL) contained 15 ng cDNA, TaqMan Universal PCR Master mix and the appropriate TaqMan assay. Each assay included cDNA template in triplicates.

Supplemental Figure 4.1

Roles of dCK and CDA in gemcitabine and clofarabine metabolism. (A) Gemcitabine and clofarabine are phosphorylated and activated by dCK while only gemcitabine is deaminated and inactivated by CDA. *In vitro* ^3H -dCyd (B) enzymatic and (C) cell-based uptake assays using the L1210 cell lines, with or without the CDA inhibitor, tetrahydrouridine (THU, 100 μM). Results are normalized to protein concentration (B) or cell number (C), and representative of three independent experiments. dFdC – gemcitabine, clof – clofarabine, dCK – deoxycytidine kinase, CDA – cytidine deaminase, dFdU – deaminated gemcitabine, MP – monophosphate, THU – tetrahydrouridine.

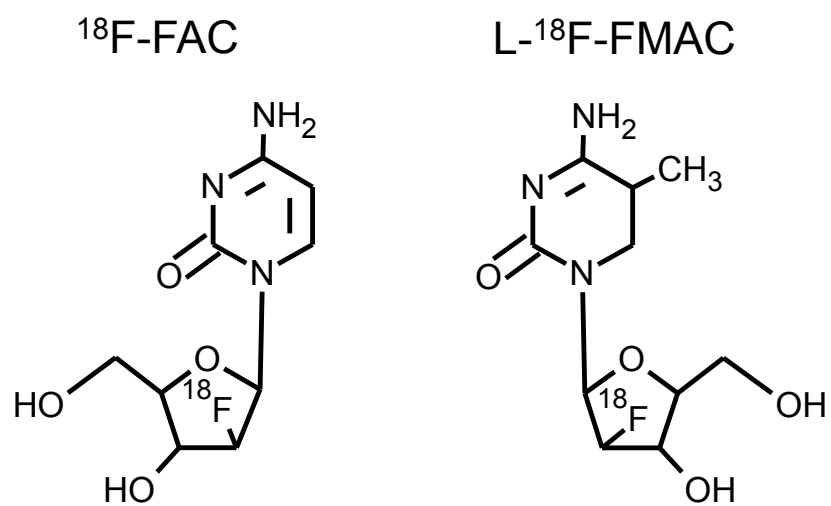
SUPPLEMENTAL FIGURE 4.1



Supplemental Figure 4.2

Structures of ^{18}F -FAC and L- ^{18}F -FMAC, two PET probes that are phosphorylated and trapped by dCK. ^{18}F -FAC is also a substrate for CDA while L- ^{18}F -FMAC is resistant to deamination.

SUPPLEMENTAL FIGURE 4.2

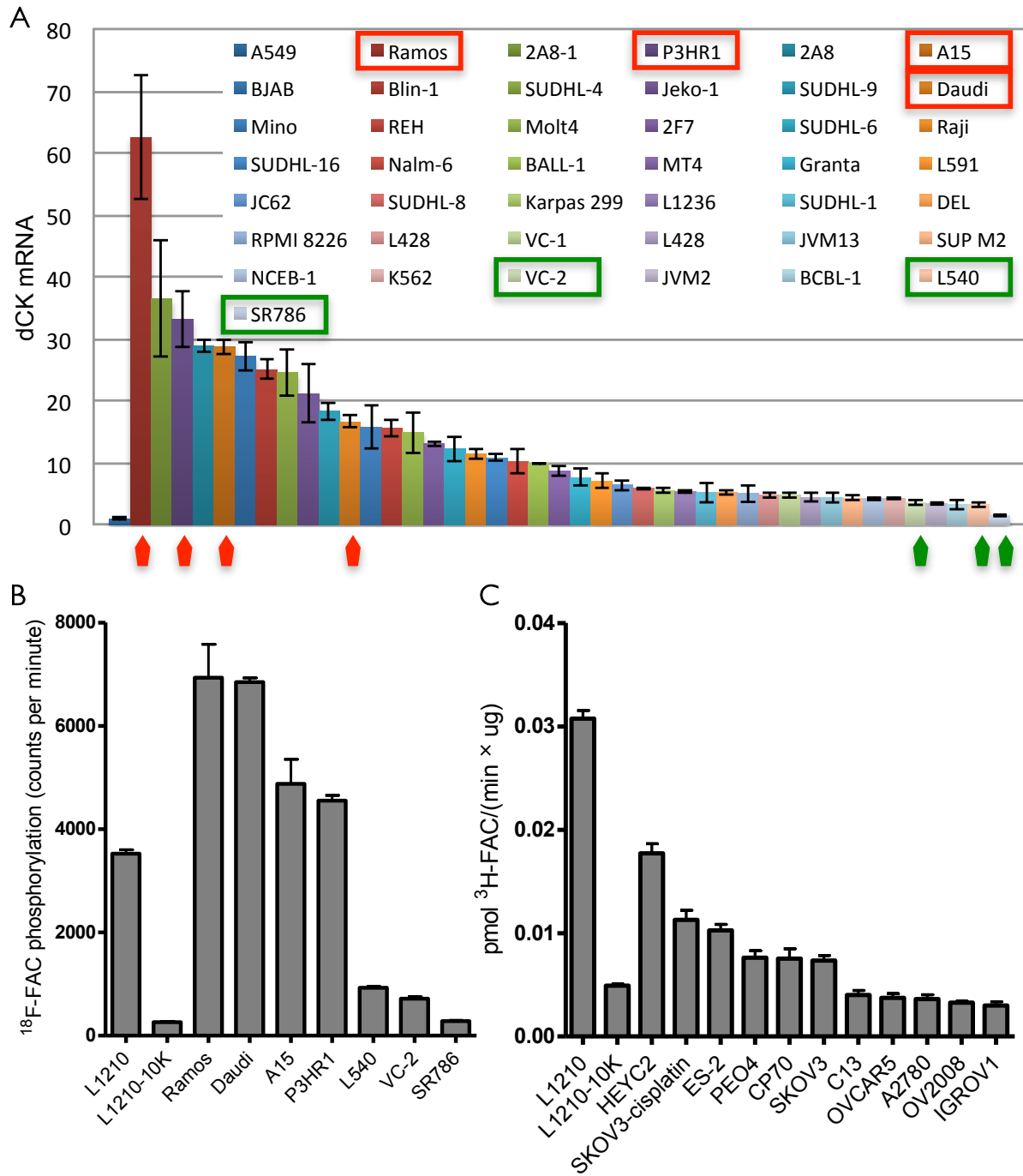


Supplemental Figure 4.3

Variation in dCK levels in panels of human lymphoma and ovarian cancer cell lines.

(A) Quantative real-time PCR assay of dCK mRNA levels relative to A549 and (B) ^{18}F -FAC enzymatic assay of dCK activity in a panel of human lymphoma cell lines. Arrows indicate cell lines common to both assays. (C) ^3H -FAC enzymatic assay of dCK activity in a panel of human ovarian cancer cell lines. Results are normalized to protein concentration.

SUPPLEMENTAL FIGURE 4.3



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Chapter 5:

Conclusions

Overview

PET provides a unique platform to assess the molecular basis of disease and its therapeutic intervention. It is extensively used in preclinical research to monitor the development and progression of cancer in laboratory animals and can be integrated into the development process of therapeutic agents (1). Clinical PET, and in particular ^{18}F -FDG PET, has substantial impact on patient management in cancer diagnosis, staging, re-staging and monitoring response to therapy (2, 3). My dissertation focuses on the improvement and understanding of two PET tracers, ^{18}F -FAC and ^{18}F -FLT, for studying nucleoside salvage metabolism in cancer. ^{18}F -FAC and its analog, L- ^{18}F -FMAC, were further investigated as predictive markers for stratification of tumors to chemotherapy.

Improvement of ^{18}F -FLT for imaging murine models of cancer

^{18}F -FLT has limited use in imaging rodent models of disease. Toward this end, we developed a pharmaceutical intervention to amplify ^{18}F -FLT signals in normal and malignant mice tissues. Administration of dipyridamole (DPA), a nucleoside transport inhibitor, 10 minutes prior to injection of ^{18}F -FLT substantially improved probe biodistribution consistent with the expected tissue pattern of proliferation. Highly proliferative immune tissues of bone marrow, thymus and spleen, previously undetectable by ^{18}F -FLT, were clearly visible with this new approach. We confirmed that signal amplification was TK1 dependent by validating the method in mice genetically deficient in one or both copies of TK1. DPA allowed the visualization by ^{18}F -FLT PET of previously undetectable murine models of leukemia/lymphoma, melanoma and lung cancer.

Future Directions

Mechanism of DPA amplification

The mechanism of DPA amplification has not been determined. Understanding this is critical to the future use of DPA in ^{18}F -FLT PET imaging to study cancer because DPA may alter the biology one seeks to measure. Additionally, as a nucleoside transport inhibitor, DPA may potentiate toxicity of drugs targeting nucleoside metabolism (4).

Furthermore, while ^{18}F -FLT is extensively used as a marker of proliferation, its metabolism is complex (5). This may limit the information that can be obtained from ^{18}F -FLT PET imaging and, in some cases, be misleading: we observed that slow growing human xenografts exhibited magnitudes higher ^{18}F -FLT signal than more proliferative murine tumors. This underscores possible species differences in nucleoside metabolism that preclude ^{18}F -FLT as a direct surrogate of proliferation. Theoretically, it may be possible to delineate these variables by applying mathematical techniques such as tracer kinetic modeling, assuming the specific factors that impact ^{18}F -FLT tumor uptake are known and quantified.

Imaging TK1 in cancer

While the biochemistry of TK1-mediated metabolism is well studied, the role of TK1 in cancer remains unknown. Because of the high regulation of the enzyme throughout the cell cycle and its involvement in nucleotide synthesis, its role has largely been attributed to providing dTTP in tumor proliferation. Studies in our group have shown that TK1 has a significant role in regulating replication stress in hematopoietic and cancer cells and that this has therapeutic implications in cancer (unpublished). The ability to image TK1 activity with ^{18}F -FLT provides a unique opportunity to study the biological relevance of this enzyme *in vivo* and stratify treatments targeting thymidine metabolism.

Optimization of ^{18}F -FAC for imaging dCK and application to predictive dCK-dependent therapy

^{18}F -FAC is an analog of deoxycytidine and the prodrug gemcitabine. The latter two are highly susceptible to deamination by CDA and, as monophosphates, by deoxycytidylate deaminase. Using HPLC we demonstrated that ^{18}F -FAC is rapidly deaminated to the uracil analog, ^{18}F -FAU, the latter accounting for approximately 90% of total plasma radioactivity at 45 min after injection of ^{18}F -FAC in C57/BL6 mice. Thus, we developed two ^{18}F -FAC analogs, L- ^{18}F -FAC and L- ^{18}F -FMAC, that are more specific for dCK using the following criteria: low susceptibility to deamination, high affinity for purified recombinant dCK, high uptake in dCK-expressing cell lines, and biodistribution in mice reflective of the tissue expression pattern of dCK. L- ^{18}F -FAC and L- ^{18}F -FMAC were both validated in mice models of leukemia and autoimmunity.

^{18}F -FAC was useful for predicting tumor response to gemcitabine based on dCK expression (6). Deamination is a reported mechanism of gemcitabine (and other NA) resistance. With the development of the deamination-insensitive L- ^{18}F -FMAC, we expanded on the ^{18}F -FAC study to further stratify tumors for both dCK and CDA activities. Isogenic leukemic cell lines expressing differential levels of dCK and CDA correlated with sensitivity to gemcitabine and the dCK-dependent, deamination-resistant prodrug, clofarabine. Tumors co-expressing dCK and CDA were indistinguishable from dCK-low tumors by ^{18}F -FAC PET. In contrast, L- ^{18}F -FMAC PET was able to detect dCK-positive tumors independent of CDA expression. In summary, imaging with both ^{18}F -FAC and L- ^{18}F -FMAC stratified tumors for dCK and CDA expression and this was predictive of tumor response to gemcitabine and clofarabine.

Future Directions

Determining rates of probe metabolism by tracer kinetic modeling

PET tracer kinetic modeling allows quantitative evaluation of regional blood flow, regulation of key enzymes and chemotherapy response. Application of this method to ^{18}F -FAC/L- ^{18}F -FMAC may provide noninvasive measurements for rates of dCK-related salvage metabolism. In our initial attempt, traditional blood sampling validated the left ventricle PET region-of-interest for describing the plasma input function, and HPLC was used to determine rates of probe deamination in various tissues for modeling validation. Both ^{18}F -FAC and L- ^{18}F -FMAC kinetics were best fitted to a 3-compartment model based on the Akaike information criterion and, therefore, we were unable to delineate by modeling the deamination rate of ^{18}F -FAC. While we were unsuccessful, future efforts to determine corrections to our model assumptions that may result in more realistic kinetic parameters are warranted.

The role of dCK in cancer

Our group has shown that dCK is important in the development of T and B lymphocytes (7). Its biological role in cancer remains to be determined. The degree of dCK upregulation may be in response to DNA replication stress present in cancer cells, which, in turn, may be due to oncogenic stimulation of proliferation in the presence of insufficient supply of nucleotides or reduced *de novo* dCTP synthesis due to excess thymidine salvage from high levels of TK1. Thymidine has been used to treat solid tumors in mice, but had limited clinical value (8). A possible explanation for the low response may be attributed to the presence of dCK, which can rescue the thymidine-induced replication stress. Thus, we hypothesize that therapy involving thymidine will require inhibition of dCK. ^{18}F -FAC analog PET can guide the development of

novel dCK inhibitors *in vivo* as a pharmacodynamic biomarker, stratify tumors for dCK activity, and validate target inhibition in treatment studies. This also suggests complementary imaging of TK1 activity with ^{18}F -FLT PET may be useful.

Further improvements to ^{18}F FAC analogs

The development of L-analogs that are resistant to deamination improves specificity for dCK. However, the affinity of L- ^{18}F -FMAC for dCK was reduced and L- ^{18}F -FAC exhibits high uptake in dCK-low tissue such as liver. Therefore, future work will address the following issues: optimal metabolic stability, affinity for dCK, and simplified radiochemical synthesis.

Clinical translation

An initial survey in human lymphoma and ovarian cancer cell lines reveal great variations in levels of dCK mRNA and enzymatic activity. Investigational new drug (IND) applications have been granted for ^{18}F -FAC, L- ^{18}F -FMAC and L- ^{18}F -FAC to determine translatability of preclinical findings. Correlating PET imaging with measurements of dCK activity from tumor biopsies and response to therapeutic interventions could demonstrate predictive benefit of imaging.

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