DEVELOPMENT OF MOLECULAR IMAGING PROBES FOR POSITRON EMISSION TOMOGRAPHY

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Dedicated to

My dear grandmother, Madam Guizhen L. Zhu
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LIST OF ABBREVIATION

AP, apurinic/apyrimidinic

APE, Apurinic/apyrimidinic endonuclease

BBB, blood-brain barrier

BDB, \((E,E)-1,4\text{-bis}(4'\text{-aminostyryl})-2\text{-dimethoxy-}
       \text{benzene}\)

BER, base excision repair

BMB, \((E, E)-1,4\text{-bis}(p\text{-aminostyryl})-2\text{-methoxy-}
      \text{benzene}\)

BS, brainstem

CC, corpus callosum

cGMP, current good manufacturing practice

CIC, Case Imaging Compound: \(4-((E)-4-(
(E)-4\text{-aminostyryl})-2,5\text{-dimethoxystyryl})\text{-N-}
methylaniline}

CNS, central nervous system

DAS, \((E)-4', 4'\text{-diamino-}
trans\text{-stilbene}\)

DAST, (diethylamino) sulfur trifluoride

DCM, dichloromethane

DIAD, diisopropylazo-dicarboxylate
DLD1 UDG +/+, DLD1 cells with uracil DNA glycosylase

DLD1 UDG -/-, DLD1 cells without uracil DNA glycosylase

DMF, dimethylformamide

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DSBs, double-strand breaks

EDTA, ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

FC₃-BDB, 4, 4’-((I[E, I’E]-2-(3-fluoropropoxy)-5-methoxy-1,4-phenylene)bis(ethane-2,1-diyl))dianiline

F3BX, N-(4,4,4,-trifluorobutanoxy) amine hydrochloride

F3EX, N-(2,2,2-trifluoroethoxy) amine hydrochloride

F3PX, N-(3,3,3-trifluoropropoxy) amine hydrochloride

FBX, N-(4-fluorobutanoxy) amine hydrochloride

FC₃-DAS (E)-4-(4-aminostyryl)-2-(3-fluoropropoxy) aniline

FC₆-DAS (E)-4-(4-aminostyryl)-N-(6-fluorohexyl) aniline

FEX, 2-fluoromethoxyamine

FPX, N-(3-fluoropropoxy) amine
HPLC, high pressure liquid chromatography

HR-ESIMS, high resolution electrospray ionization mass spectra

HRP, horseradish peroxidase

HRMS, high resolution mass spectra

HWE reaction, Horner-Wadsworth-Emmons reaction

i.p., intraperitoneal

K222, 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]

LA, left homology arm

LPC, lysophosphatidyl choline

MBP, myelin basic proteins

MeDAS, 4-amino-4'-methylamino-trans-stilbene

MR, magnetic resonance

MRI, magnetic resonance imaging

MS, multiple sclerosis

MX, methoxyamine

NMR, nuclear magnetic resonance

NSCLC, non-small cell lung cancer
PBS, phosphate buffered saline (0.001 M, pH=7.4, expressed as 1 × PBS)

PCR, polymerase chain reaction

PET, positron emission tomography

PNS, peripheral nervous system

PPh₃, triphenylphosphine

PPTS, pyridinium p-toluenesulfonate

RA, right homology arm

RC, rostral commissure

ROI regions of interest

SSBs, single-strand breaks

St, striatum

SUV standardized uptake value

THF, tetrahydrofuran

THP, tetrahydropyranil

TMZ, temozolomide

TS, thymidylate synthase

dTTP, deoxythymidine triphosphate
UDG, uracil-DNA glycosylase

dUMP, deoxyuridine monophosphate

dUTP, deoxyuridinetriphosphate
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Development of Molecular Imaging Probes for Positron Emission Tomography

WENXIA ZHU

ABSTRACT

Positron emission tomography (PET) is the one molecular imaging modality that can be used in both pre-clinical and human studies. PET provides the most direct diagnoses in oncology with high sensitivity and quantitative analysis. The primary reason is that it can demonstrate dynamic metabolism in animal or human bodies during in vivo molecular imaging. However, PET is not only confined to oncology. It can be used in other physiological or metabolic processes, as long as specific biomarkers are available. Therefore, the development of radiotracers is crucial for in vivo PET imaging. Synthetic chemistry provides a platform to synthesize cold standards (the counterpart of the non-radioactive material of PET radiotracers) and radio-labeling precursors. Once the compounds with high specificity and binding affinity are developed, chemical synthesis can make radiolabeling precursors for $^{11}$C, $^{18}$F or other positron emitting isotopes. In this thesis, two series of molecular probes are synthesized as PET imaging agents. One series of molecules are AP site specific binding agents and the other series of molecules are myelin specific probes. The former one is used for in vivo PET imaging of the AP site formation and can be used as imaging markers to evaluate DNA-targeted chemotherapeutic drugs. The latter type molecules are myelin-binding agents for in vivo imaging of myelination in the central nervous system (CNS). This thesis presents the application of synthetic chemistry in the development of $^{18}$F-labeled probes in two projects: development of $[^{18}$F]-labeled PET tracers for imaging of DNA damage and repair in cancer chemotherapies & development of $[^{18}$F]-labeled PET imaging probes for in vivo studies of myelin pathology.
1.1 Cancer

Cancer refers to a large group of different diseases which are due to unregulated cell growth. The defects in cellular pathways such as DNA damage response, proliferation, and programmed cell death are the main characteristics of various cancer diseases. Meanwhile, all of the cancers have their own unique mechanisms based on the tissues from which they develop. Cancer is one of the major causes of mortality even though researchers have put tremendous efforts for decades into finding effective treatment.

1.2 DNA damage and repair

Chemotherapy is currently one of the two main therapies (the other one is radiotherapy) for cancer treatment. Among most of the effective chemotherapeutic drugs are the DNA-target agents that perturb DNA replication by introducing mutants or producing single- and double-strand breaks (SSBs or DSBs). Since DNA is constantly attacked by endogenous toxins, like free radicals produced by cellular metabolism and exogenous toxins such as carcinogens, it develops a strong capability of maintaining its genetic stability and structural integrity. The hydrolysis of DNA glycosyl bonds occurs at a daily rate of $10^4$ per cell and more than 99.9% of accidental base changes will be repaired by DNA repair pathways. [1, 2]
The repair mechanisms employed by cancer cells induce the direct repair pathway, the base excision repair pathway (BER), the nucleotide excision repair pathway, and the mismatch repair pathway, etc. BER is a fundamental cellular response to the cytotoxicity of chemotherapies.[3-6] During the process of BER, various damage-specific glycosylases cleave the N-glycosidic bond of the damaged bases with high selectivity to form apurinic/apyrimidinic (AP) sites in DNA[7]. (Fig. 1.1) Apurinic/apyrimidinic endonuclease (APE I) can recognize an AP site and cut it, then, a strand incision will be created. The gap is filled with normal nucleotide by DNA polymerase β (polymerase β). The XRCC1/ligase III complex ligates the incised strand.[6, 8] In tumors, the enzymes employed in the BER process have higher expression than normal tissues.[9-11] All of these factors significantly offset the efficacy of DNA-target chemotherapeutic drugs.

**Figure 1.1** Formation of an apurinic/apyrimidinic (AP) site[12]
In order to increase the sensitivity of tumor cells to DNA damage and minimize drug resistance, efforts have been made to inhibit the BER pathway by blocking AP sites, because AP sites are invariably formed as the BER intermediate even though the enzymes involved might vary. In 1985, methoxyamine (MX) was first introduced as a BER inhibitor by Liuzzi et al. [13] Since then, MX has been systematically studied to develop combination therapies aimed at enhancing the efficacy of DNA-targeting chemotherapeutic drugs.[14, 15] The specific action of MX in the disruption of BER is shown in Fig 1.2. MX reacts with the tautomeric open-ring form of deoxyribose after an abnormal base is removed by one of the DNA glycosylases. The reaction of MX with AP sites is fast, even faster than the downstream repair by apurinic/apyrimidinic endonuclease (APE).[16] The inhibitory effect of MX on DNA repair results from the chemical modification of AP sites as APE substrates rather than from poisoning of APE enzyme. [17, 18]

Figure 1.2 Chemical mechanism of blocking an AP site by MX [16]
MX produces less cytotoxicity towards normal cells compared to any protein inhibitor that directly disrupts normal enzymatic or kinase activity. It has been postulated that MX blocks the action of APE in one of two ways: either MX’s chemical structure alters the AP site by forming a covalent linkage with its aldehyde moiety so that the APE cannot recognize the modified MX-AP site, or the MX-AP site renders the phosphodiester bonds adjacent to the AP site refractory to the catalytic activity of the APE even though the enzyme can still bind to the site.

1.3 DNA targeting chemotherapeutical drugs

Among most of the effective DNA-targeted chemotherapeutic drugs are temozolomide, alimta and fluodarabine, etc. All of them trigger BER pathway. MX is a secondary drug to enhance their efficacy. TMZ has low molecular weight, hydrophobicity and is capable of undergoing conversion to active DNA-methylating species at physiological pH level. It shows promising antitumor activity in clinical trials.[19-22] It can also cross the blood-brain barrier (BBB); therefore it is an effective drug for the treatment of brain tumors. The mechanism of the TMZ treatment is that it transports a methylating agent (the methyl diazonium ion) to guanine bases and interferes with the DNA replication as shown in Fig.1.3. That is why the BER process is triggered after the treatment of TMZ.[23] causing drug resistance.[24]
The intermediate AP site formation during the BER pathway is not unique to the treatment of TMZ. Alimta, another important chemotherapeutical drug, also activates the BER pathway. Although the mechanisms of TMZ and alimta are totally different, BER is the same cellular response for their treatment and AP sites are the common intermediates. Alimta induces DNA damage through the inhibition of thymidylate synthase (TS). TS is an important enzyme for the synthesis of thymidine nucleotide which is one of the four nucleotides in DNA. With the inhibition of TS, the concentration of deoxythymidine triphosphate (dTTP) is reduced and the concentration of deoxyuridinetriphosphate (dUTP) is increased as a consequence of deoxyuridine monophosphate (dUMP) accumulation and finally dUTP levels exceed dUPTase activity. While
the polymerase uses dUTP/dTTP at the same rate, dUTP is eventually incorporated into DNA. The mechanism of the incorporation of uracils is shown in Fig. 1.4. With the incorporation of extra uracil base, uracil-DNA glycosylase (UDG) can excise the uracil base from the strands of the DNA, leaving an AP site. Since dUTP/dTTP pools are unbalanced, uracil (dUTP) will be re-incorporated and re-excised leading to DNA strand breaks or cell death. [25-27]

Figure 1.4 Metabolism of dUMP and role of dUTPase with the inhibition of thymidylate synthase by alimta[26]

To monitor the efficacy of DNA-targeting chemotherapeutic drugs, the call for direct imaging and quantitative assessment of AP sites in vivo is great. If patients respond to a given DNA-damaging chemotherapy, in vivo imaging of AP site formation will permit determination of doses and time schedules for maximum efficacy. Meanwhile, BER inhibitors will be most effective if administered at the time when AP site formation peaks. These imaging studies will
also facilitate the development of BER inhibitors to potentiate the efficacy of DNA-damaging drugs. Moreover, a major challenge in cancer chemotherapy is to access patient response soon after initial treatment. If patients do not exhibit elevated AP-site formation in tumor regions then significant DNA damage is likely not induced, and the patient probably will not respond to the given chemotherapeutic drug. In such cases, patients should be switched promptly to a different drug. If patients do exhibit elevated AP site formation in tumor regions, it is likely that significant DNA damage can be induced and detected in tumor regions. This will make it possible to subsequently modulate the sensitivity of cells to DNA damage by blocking the DNA repair pathways. Scientific research already demonstrated that MX can effectively inhibit the BER pathway by blocking AP sites and enhance the efficacy of the treatment by TMZ or alimta.[28] Such combination chemotherapy could be more effective than single-agent chemotherapy and may lead to a significant increase in cancer cure rates. Thus, image-guided prognosis and staging will allow clinicians to find the most effective treatment. This benefit is paramount for early treatment, which is often the key to ultimate healing.

1.4 Current methods to detect AP sites

1.4.1 ARP bioassay

The binding affinity of MX is measured based on chemiluminescence using aldehyde reactive probe (ARP) bioassay. [10, 11, 26, 27] ARP reacts with AP sites induced by DNA targeting agents and forms a stable ARP-DNA adduct. The biotin part of ARP in APR-DNA adduct reacts with avidin-horseradish peroxidase (HRP), forming a DNA-HRP complex at every biotin residue. The number of the AP sits is calculated by the activity of HRP toward chromogenic substrate in an ELISA (enzyme-linked immunosorbent assay). ARP assay is used
to measure the free AP sites after the binding by MX. Therefore, the less the AP sites are detected by ARP, the higher the binding affinity of the MX or its related derivatives and vice versa. This technique can process 20 to 30 samples within 2 days. Moreover, ARP assay does not need radioactive materials and all of the reagents are commercially available, so this method is very convenient for the estimation of AP sites binding agents. However, ARP assay can only be carried out in vitro.

1.4.2 $[^{11}\text{C}]$MX as an in vivo PET imaging probe for DNA damage and repair by chemotherapy

PET imaging is known for its high sensitivity and can provide quantitative measurement of the concentration of the radioactivity. To get more accurate results, a magnetic resonance imaging (MRI) scanner is used to provide anatomical localization with high spatial resolution, via the co-registration of images by PET and MRI.[29] When diagnostic imaging modalities, such as PET and MRI, are available, the pivotal part of the research is the development of the radiotracers which specifically bind to AP sites with high binding affinity, because radiotracers are the source of positrons which produce γ-rays as the signals for PET imaging reconstruction. Therefore chemistry plays an indispensable role in the realization of clinical diagnosis by PET. The main task of this project is to use chemistry to bridge the biological studies of the diseases and medical imaging, therefore providing a fast screening process for the discovery of more promising diagnostic imaging probes and treatment drugs.

In order to monitor the formation of AP sites in vivo, our laboratory developed radiolabelled MX with positron-emitting isotope $^{11}\text{C}$ and used it as radiotracer for in vivo
positron emission tomography (PET) imaging to directly detect and quantify AP sites.[30] PET imaging can be thought as the pictures taken by a special camera, which captures high energy gamma-rays emitted by the tracers inside of the subject as shown in Fig.1.5. Tracers are usually molecules labeled with positron-emitting isotopes such as $^{15}$O, $^{13}$N, $^{11}$C or $^{18}$F, etc. The positron collides with a nearby electron to produce energy in the form of two 511 KeV gamma-rays which are $\sim 180^\circ$ apart. The gamma-ray detectors record the signals at the same time which can be reconstructed and provide metabolic information in living bodies. The radiotracers injected into animal or human bodies are in trace amounts, therefore will not cause concerns of toxicity.

**Figure 1.5** Principles of positron emission tomography (PET)
(Ref: http://www2.fz-juelich.de/zel/index.php?index=136)
They decay fast and are removed by the kidneys quickly. Advanced PET imaging technology allows for the diagnosis of diseases in early stages.

\(^{11}\text{C}\) labeled MX (\([^{11}\text{C}]\text{MX}\)) is the first AP site imaging radiotracer synthesized in our group (Scheme 1.1). It is also the first in vivo PET imaging agent for in vivo quantification of AP sites ever developed. It has the same structure and molecular formula as MX, while the carbon atom in this molecule is replaced by positron-emitting isotope \(^{11}\text{C}\). \([^{11}\text{C}]\text{MX}\) was successfully synthesized. It reacts with the carbonyl groups of the AP sites to form positron emitting AP site-\([^{11}\text{C}]\text{MX}\) adduct shown in Fig. 1.6.

![Scheme 1.1 Radiosynthesis of \([^{11}\text{C}]\text{MX}\) [30]](image)

By quantitative measurement of the standardized uptake value (SUV) of \([^{11}\text{C}]\text{MX}\), the quantity of AP sites can be obtained according to the formation of \([^{11}\text{C}]\text{MX-AP-DNA}\) adduct, as illustrated in Fig.1.6.
Figure 1.6 Illustration of in vivo quantitative detection of AP sites by $^{[11]C}$MX

In vivo micro-PET studies show that AP site formation is elevated in a melanoma xenograft tumor model following TMZ treatment. In vivo blocking experiments determined that $^{[11]C}$MX binds to AP sites specifically. Correlation of in vivo PET studies with ARP-based biochemical assays suggested that $^{[11]C}$MX-PET is a valuable imaging marker of AP site formation.[30]

1.5 Rationale for the development of $^{[18]F}$fluoro-MX derivatives as AP sites imaging probes for PET

$^{[11]C}$MX is a good AP site imaging agent for PET. However, its half-life is 20 minutes. That means an on-site cyclotron is required for the radio-synthesis. In order to enhance the potential for clinical applications of this imaging technique and to optimize the potency and specificity of binding to AP sites, we have developed a series of fluorinated MX derivatives which can be labeled with $^{18}$F. The half-life of $^{18}$F is 110 minutes which allows practical remote delivery. Fluorinated MX derivatives (fluoro-MX) allow us to monitor the formation and
persistence of AP sites. In the meantime the enhancement of drug–drug synergy is optimized by
the formation of these DNA-bound AP sites after treatment with the combination of
chemotherapeutic agents. Another reason we chose $^{18}$F as the positron emitting isotope is that
fluorinated agents usually possess special biological properties.[31-33] Specific interactions
between some fluorinated compounds and DNA have shown that the introduction of fluorine into
dNA binding agents significantly enhances their binding affinities.[34-36] $[^{18}$F$]$fluoro-MX
derivatives potentially exhibit very promising binding properties and pharmacokinetic profiles.
The relatively longer half-life of $^{18}$F ($t_{1/2}$ 110 min for $^{18}$F; 20 min for $^{11}$C) permits the distribution
of PET tracers to remote imaging facilities that do not have access to an on-site cyclotron. A
series of the fluoro-MX derivatives were designed and synthesized. These fluorinated O-
alcoholhydroxylamine derivatives all have the –ONH$_2$ moiety which is the reactive functional
group to form DNA-adopts. [37]

In the screening of the fluoro-MX derivatives, ARP bioassay was used. The results
showed that 2-fluoromethoxyamine (FEX) is a lead compound with high binding affinity to AP
sites and potential can be labeled by $^{18}$F. After radiolabeling, it can be used to image DNA
damage and repair in vivo by PET/MRI imaging modalities.

FEX was investigated for binding affinity for AP sites after alimta treatment of non-
small cell lung cancer (NSCLC) cells. Then, in vivo PET imaging of the AP site formation was
carried out after alimta treatment in tumor xenograft mouse models. Both ARP bioassays of FEX
to the alimta treated cancer cells and the in vivo PET imaging of the AP site formation by
$[^{18}$F$]$FEX after initial treatment demonstrated that $[^{18}$F$]$FEX is an appropriate imaging maker of
AP site formation for the evaluation of DNA-targeted chemotherapeutic drugs. For example, if
patients do not exhibit elevated AP-site formation in tumor regions then significant DNA
damage apparently is induced. Previous studies showed that UDG is a critical enzyme involved in the BER pathway for DNA repair.[7, 27, 38] The lack of UDG will inhibit the excision of the incorporated uracil bases in DNA, therefore it will hamper AP site formation.[39, 40] In order to evaluate the effectiveness of [18F]fluoro-MX derivatives as imaging markers to monitor the efficacy of alimta on AP site formation, we also detected the AP site formation in the tumor xenograft mouse model in which the UDG gene was knocked out.

The following chapters will report the chemical synthesis of the proposed fluoro-MX derivatives, ARP bioassay for the measurement of AP site binding affinities of these fluorinated compounds, [18F]-radiolabeling of the lead compounds, and [18F]FEX as a PET tracer for the quantitative imaging of AP sites induced by alimta.
Chapter Two

Chemical Synthesis and ARP Bio-assays of Fluoro-MX Derivatives

2.1 Introduction

Fluorinated molecules often possess unique biological properties.[31-33] Specific interactions between some fluorinated compounds and DNA have shown that their binding affinity is significantly enhanced with the incorporation of fluorine into DNA binding agents.[34-36] We thus hypothesize that the introduction of fluorine into MX will facilitate the interaction with DNA and subsequently enhance the efficacy of binding to AP sites. Six fluoro-MX derivatives were synthesized, which vary in the number of methylene groups or fluoro groups. Their binding affinities for AP sites were evaluated by ARP bioassay.

2.2 Design and synthesis

The chemical synthesis of these six fluoro-MX derivatives is shown in Scheme 2.1. For the synthesis of 2-fluoroethoxylamine (6a), N-hydroxyphthalimide was used as a starting material to react with 2-fluoroethanol via Mitsunobu reaction to produce N-(2-fluoroethoxy) phthalimide (2a) in 88% yield. Then, compound 2a reacted with hydrazine monohydrate in dry dichloromethane (DCM) and methanol at room temperature for 2 hours to give 2-fluoroethoxylamine. After being treated with 1 M hydrochloride acid in diethyl ether, it was converted into the form of its hydrochloride salt, in 47% yield. [37, 41] Compounds 2b, 2c, and 2d were prepared similarly to compound 2a. N-(2,2,2-trifluoroethoxy)phthalimide (4) (43%
yield) and N-(3-hydroxypropoxy)phthalimide (3) (77% yield) were made through coupling reaction.

Scheme 2.1 Chemical synthesis of six fluoro-alkoxyamine derivatives

2a) \( \text{R} = (\text{CH}_2)_2 \text{F} \quad 88\% \\
2b) \( \text{R} = (\text{CH}_2)_2 \text{CF}_3 \quad 79\% \\
2c) \( \text{R} = (\text{CH}_2)_4 \text{OH} \quad 81\% \\
2d) \( \text{R} = (\text{CH}_2)_3 \text{CF}_3 \quad 77\% \\
3 \quad 77\%

3 \quad \text{DAST/DCM} \quad -78^\circ\text{C to rt, 16 h}

5 \quad 5a) \( \text{R}' = (\text{CH}_2)_3 \text{F} \quad 67\% \\
5b) \( \text{R}' = (\text{CH}_2)_4 \text{F} \quad 72\%

2a) \quad 6a): \text{HCl} \cdot \text{NH}_2 \text{O(Ch}_2)_2 \text{F} \quad 47\% \\
5a) \quad i) \text{H}_2 \text{NNH}_2 \cdot \text{H}_2 \text{O/DCM/MeOH} \\
6b): \text{HCl} \cdot \text{NH}_2 \text{O(Ch}_2)_3 \text{F} \quad 51\% \\
5b) \quad ii) \text{1 M HCl/ether} \\
6c): \text{HCl} \cdot \text{NH}_2 \text{O(Ch}_2)_4 \text{F} \quad 79\% \\
4 \quad 6d): \text{HCl} \cdot \text{NH}_2 \text{OCH}_2 \text{CF}_3 \quad 44\% \\
2b) \quad 6e): \text{HCl} \cdot \text{NH}_2 \text{O(Ch}_2)_2 \text{CF}_3 \quad 54\% \\
2d) \quad 6f): \text{HCl} \cdot \text{NH}_2 \text{O(Ch}_2)_3 \text{CF}_3 \quad 74\%
under basic conditions. [37, 42] (Diethylamino) sulfur trifluoride (DAST) was used as the fluorination reagent for the fluorination of N-(4-hydroxybutoxy)phthalimide (2c) and N-(3-hydroxypropoxy) phthalimide (3) at -78 °C. [43] The yields of fluorinated products were 67% for N-(3-fluoropropoxy) phthalimide (4a) and 72% for N-(4-fluorobutoxy)phthalimide (4b), respectively.

The other five fluoro-MX derivatives were synthesized similarly with relatively high yields: N-(3-fluoropropoxy)amine hydrochloride (6b), 51%; N-(4-fluorobutanoxy)amine hydrochloride (6c), 79%; N-(2, 2, 2-trifluoroethoxy)amine hydrochloride (6d), 44%; N-(3, 3, 3-trifluoropropoxy)amine hydrochloride (6e), 54%; and N-(4, 4, 4-trifluorobutanoxy)amine hydrochloride (6f) 74%.

Table 2.1 Six fluoro-alkoxyamine derivatives

<table>
<thead>
<tr>
<th>Fluoro Derivatives</th>
<th>Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl·NH₂OCH₂CH₂F</td>
<td>N-(2-Fluoroethoxy) amine hydrochloride</td>
<td>FEX</td>
</tr>
<tr>
<td>HCl·NH₂OCH₂CH₂CH₂F</td>
<td>N-(3-Fluoropropoxy) amine hydrochloride</td>
<td>F3EX</td>
</tr>
<tr>
<td>HCl·NH₂OCH₂CH₂CH₂F</td>
<td>N-(4-fluorobutanoxy) amine hydrochloride</td>
<td>FBX</td>
</tr>
<tr>
<td>HCl·NH₂OCH₂CF₃</td>
<td>N-(2, 2, 2-trifluoroethoxy) amine hydrochloride</td>
<td>F3EX</td>
</tr>
<tr>
<td>HCl·NH₂OCH₂CF₃</td>
<td>N-(3, 3, 3-trifluoropropoxy) amine hydrochloride</td>
<td>F3PX</td>
</tr>
<tr>
<td>HCl·NH₂OCH₂CF₃</td>
<td>N-(4,4,4-trifluorobutanoxy) amine hydrochloride</td>
<td>F3BX</td>
</tr>
</tbody>
</table>
2.3 Materials and synthetic procedures for fluoro-MX derivatives

All of the chemical reagents and solvents were purchased either from Sigma Aldrich or TCI America and used without further purification. The processes of the reactions were monitored by TLC with the solvent system of mixture of hexanes and ethyl acetate. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova AS400 spectrometer at 400 MHz for $^1$H-NMR and 100 MHz for $^{13}$C-NMR. Chemical shifts are presented in ppm on the $\delta$ scale relative to TMS or solvent peak. High resolution mass spectra were recorded using Thermo Scientific LTQ-FT Mass Spectrometry Instrument. (University of Cincinnati Mass Spectrometry Facility).

N-(2-Fluoroethoxy)phthalimde (2a)

To a solution of 2-fluoroethanol (1.28 g, 20 mmol), N-hydroxyphthalimide (3.50 g, 21.5 mmol), and triphenylphosphine (PPh$_3$) (5.98 g, 22.37 mmol) in dry THF (100 mL), diisopropylazo-dicarboxylate (DIAD) was added dropwise, and the reaction flask was put in an ice-water bath. The mixture was stirred from 0 °C to room temperature over 7 h, then concentrated in vacuum and purified on a silica chromatography column (hexanes/ethyl acetate = 9:1, v : v) to give compound 2a. The yield was 88 %, 3.71 g. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 7.86-7.73 (m, $J_1 = 3.2$ Hz, $J_2 = 34$ Hz, 4H), 4.83 (t, $J = 3.6$ Hz, 1H), 4.72 (t, $J = 3.6$ Hz, 1H), 4.50 (t, $J = 3.6$ Hz, 1H), and 4.43 (t, $J = 3.6$ Hz, 1H); $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$: 163.29, 134.57, 128.72, 123.61, 82.23, 80.53, 76.81, and 76.61.
N-(3,3,3-Trifluoropropoxy)phthalimide (2b)

To the solution of 3,3,3-trifluoro-1-propanol (0.73 g, 6.4 mmol), compound 1 (1.043 g, 6.39 mmol) and PPh₃ (1.71 g, 6.41 mmol) in anhydrous THF (30 mL) at 0 °C, DIAD (1.26 mL, 6.40 mmol) was added dropwise. The reaction was stirred for 18 h followed by concentration under vacuum and the product was purified by silica chromatography column (hexanes/ethyl acetate = 9:1, v : v) directly to give compound 2b, 1.30 g (yield: 79%).

¹ H-NMR (400 MHz, CDCl₃) δ: 7.81-7.73 (m, J₁ = 3.2 Hz, J₂ = 34 Hz, 4H), 4.38 (t, J = 6.4 Hz, 2H), and 2.72-2.61 (m, J = 6.4 Hz, 2H), ¹³C-NMR (100 MHz, CDCl₃) δ: 163.22, 134.66, 128.60, 126.69, 123.61, 70.90, 33.38, and 33.09.

N-(4-Hydroxybutoxy)phthalimide (2c)

To a solution of N-hydroxy phthalimide (1.30 g, 8 mmol), 1,4-butanediol (2.16 g, 24 mmol) and PPh₃ (2.31 g, 8.8 mmol) in THF (200 mL) was added with DIAD (1.65 mL, 8.4 mmol) at 0 °C. Then, the whole mixture was stirred at 0 °C for 2 h followed by a workup and purification method similar to that for compound 2a to give 2c, 1.33 g (yield: 81%). ¹ H-NMR (400 MHz, CDCl₃) δ: 7.83-7.70 (m, J₁ = 3.2 Hz, J₂ = 34 Hz, 4H), 4.22 (t, J = 6.0 Hz, 2H), 3.64 (t, J = 6.0 Hz, 2H), 2.07-1.99 (m, J = 6.0 Hz, 2H), and 1.95-1.88 (m, J = 6.0 Hz, 2H); ¹³C-NMR (100 MHz, CDCl₃) δ: 163.46, 134.42, 128.24, 123.40, 77.32, 44.49, and 25.40.

N-(4,4,4-Trifluorbutoxy)phthalimide (2d)
Compound 2d was made by the reaction of 4,4,4-trifluoro-1-butanol (0.75 g, 5.50 mmol) with compound 1 (0.90 g, 5.50 mmol), PPh$_3$ (1.47 g, 5.60 mmol), and DIAD (1.04 mL, 5.50 mmol) in 30 mL anhydrous THF at 0 °C. The reaction was stirred for 16 h followed by a workup and purification method similar to that for compound 2a to give 2d, 1.15 g (yield: 77%). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 7.85-7.748 (m, $J_1 = 3.2$ Hz, $J_2 = 34$ Hz, 4H), 4.28-4.25 (t, $J = 6.4$ Hz 2H), 2.52-2.42 (m, 2H), 2.06-1.99 (m, 2H), $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$:1163.53, 134.60, 128.80, 123.62, 76.57, 30.83, 30.54, 30.24, 29.95, 21.53, 21.22, 21.19.

N-(3-Hydroxypropoxy)phthalimide (3)

Under argon, to a solution of sodium acetate (3.32 g, 40 mmol) in dry DMSO (50 mL), N-hydroxyphthalimide (2.20 g, 13 mmol) was added in one portion. After 1 h, the solution was heated to 70 °C. Then, 3-bromopropan-1-ol (3.61 g, 26 mmol) was added dropwise. The whole mixture was stirred for another 6 h until the color of the reaction mixture turned pale pink. Once the reaction was cooled to room temperature, it was quenched by water (50 mL). DCM (3 × 50 mL) was used to extract the product. The combined organic solution was washed with water (2 × 50 mL), 3 N HCl (30 mL), and brine. After being dried over anhydrous sodium sulfate, the solution was filtered and concentrated. Then the crude product was purified using a flash silica gel column with the eluent of hexanes/ethyl acetate = 1 : 2, v/v to give compound 3, 1.71 g (yield: 77%). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 7.86-7.75 (m, $J_1 = 3.2$ Hz, $J_2 = 34$ Hz, 4H), 4.40-4.37 (t, $J = 6.4$ Hz, 2H), 3.96-3.93 (m, $J = 6.4$ Hz, 2H), 2.03-1.97 (m, $J_1 = 8.4$ Hz, $J_2 = 8.8$ Hz, 2H), $^{13}$C-NMR (400 MHz, CDCl$_3$) $\delta$:153.46, 134.63, 123.66, 107.17, 76.00, 59.06, 30.71.
N-(2,2,2-Trifluoroethoxy)phthalimide (4)

Under argon, to a solution of N-hydroxyphthalimide (1.63 g, 10 mmol) in dry DCM (30 mL) with the presence of N-ethyldiisopropylamine (3.50 mL, 20 mmol), 2,2,2-trifluoroethyl trifluoromethanesulfonate (1.5 mL, 10 mmol) was added dropwise. The mixture was stirred at room temperature for 26 h. The reaction was quenched by water (50 mL). DCM (3 × 50 mL) was used to extract product. The combined organic solution was washed with water (2 × 50 mL), 1 N HCl (2 × 50 mL), and brine. After being dried over anhydrous sodium sulfate, the solution was filtered and concentrated. Then the crude product was purified by flash chromatography using hexanes:ethyl acetate = 1:1 (v:v) as an eluent to give compound 4, 1.05 g (yield: 43%). $^1$H-NMR (400 MHz, acetone-d$_6$) $\delta$: 7.94-7.89 (m, $J_1 = 3.2$ Hz, $J_2 = 34$ Hz), and 4.89-4.82 (q, $J_1 = 8.4$ Hz, $J_2 = 8.8$ Hz, 2H); $^{13}$C-NMR (100 MHz, Acetone-d6) $\delta$: 207.29, 164.09, 136.79, 130.76, 125.25, 74.90, and 74.56.

N-(3-Fluoropropoxy)phthalimide (5a)

Under argon, to a solution of compound 3 (1.11 g, 5 mmol) in anhydrous DCM (50 mL) at -78 °C, DAST (3.30 mL, 25 mmol) was added dropwise. The mixture was stirred for 16 h and then allowed to warm to room temperature. Progress of the reaction was monitored by TLC. It was quenched with saturated sodium bicarbonate solution at 0 °C until CO$_2$ evolution ceased. The solution was diluted with water (100 mL), then extracted by DCM (3 × 50 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was further purified by flash chromatography using hexanes : ethyl acetate = 1 : 2 (v:v) as an eluent, to give compound 5a, 748 mg (yield: 67 %). $^1$H-NMR (400 MHz, CDCl$_3$), $\delta$: 7.76-7.68 (m, $J_1$
N-(4-Fluorobutanoxy)phthalimide (5b)

To a solution of compound 2c (1.18 g, 5 mmol) in dry DCM (50 mL), DAST (3.26 mL, 2.50 mmol) was added dropwise at -78 °C. The mixture was stirred under argon for 15 h and then allowed to warm up to room temperature. The reaction was monitored by TLC. After quenching with saturated sodium bicarbonate at 0 °C, the solution was added to 50 mL water and extracted by DCM (3 × 100 mL). The combined organic layer was washed by water and brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified by flash silica gel chromatography using hexanes : ethyl acetate from 4:1 to 2:1 (v : v) as eluents, to give compound 5b, 1.27 g (yield: 72 %). 1H-NMR (400 MHz, CDCl3) δ 7.77-7.69 (m, J1 = 3.2 Hz, J2 = 34 Hz, 4H), 4.55-4.52 (t, J = 5.6 Hz, 1H), 4.43-4.41(t, J = 3.6 Hz 1H), 4.21-4.17 (t, J = 4.0 Hz 2H), and 1.97-1.82 (m, J1 = 6.0 Hz, J2 = 25.6 Hz, 2H); 13C-NMR (100 MHz, CDCl3) δ: 163.57, 134.46, 128.84, 123.48, 84.32, 82.68, 77.74, 26.80, 26.60, 24.23, 24.18.

N-(2-Fluoroethoxy)amine hydrochloride (6a)

Hydrazine monohydrate (0.73 mL, 15 mmol) was added dropwise to a solution of compound 2a (1.58 g, 7.5 mmol) in a mixture of dry dichloromethane (DCM) (30 mL) and methanol (2 mL) and the mixture was stirred at room temperature for 2 h. The resulting mixture with precipitate was filtered and the filtrate was washed with saturated sodium bicarbonate (20
mL). The aqueous layer was extracted with DCM (3 × 30 mL) and the organic extraction was
dried over anhydrous sodium sulfate. To the dry solution, 1 M HCl in diethyl ether (30 mL) was
added, and the whole mixture was concentrated under vacuum. The white solid was washed with
dry DCM and dry ether to afford a pure white solid, compound (6a) (419 mg, 47% yield). $^1$H-
NMR (400 MHz, DMSO-d$_6$) $\delta$: 11.17 (br s, 3H), 4.62 (t, $J = 8.0$ Hz, 1H), 4.57 (t, $J = 7.6$ Hz,
1H), 4.30 (q, $J = 6.0$ Hz, 1H), and 4.23 (q, $J = 6.0$ Hz, 1H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$:
81.55, 79.89, 73.36, and 73.18. HR-ESIMS (NH$_2$OCH$_2$CH$_2$F): m/z calculated 80.05062, found:
80.05052.

N-(3-Fluoropropoxy)amine hydrochloride (6b)

Compound (6b) was prepared by a method similar to that used for the preparation of 6a.
The yield was 376 mg. (51%) $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 11.12 (br s, 3H), 4.57-4.54 (t, $J$
= 6.0 Hz, 1H), 4.54-4.42 (t, $J = 6.0$ Hz, 1H), 4.13-4.10 (t, $J = 6.4$ Hz, 2H), and 2.053-1.97 (m, $J_1$
= 6.4 Hz, $J_2 = 26$ Hz 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 81.22, 79.60, 70.29, 28.40, and
28.21. HR-ESIMS (NH$_2$OCH$_2$CH$_2$CH$_2$F): m/z calculated 94.06627, found: 94.06619.

N-(4-Fluorobutanoxy)amine hydrochloride (6c)

Compound 6c was prepared by a method similar to that used for the preparation of 6a.
The yield was 673 mg. (79%) $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 4.52-4.49(t, $J = 5.6$ Hz 1H),
4.41-4.38(t, $J = 6.0$ Hz 1H), 4.06-4.03(t, $J = 6.4$ Hz, 2H), and 1.74-1.64 (m, $J = 3.2$ Hz, 4H); $^{13}$C-
NMR (100 MHz, DMSO-d$_6$) $\delta$: 84.18, 82.57, 26.27, 26.07, 23.22, and 23.17. HR-ESIMS
(NH$_2$OCH$_2$CH$_2$CH$_2$CH$_2$F): m/z calculated 108.08192, found: 108.08189.
N-(2, 2, 2-Trifluoroethoxy)amine hydrochloride (6d)

Compound 6d was prepared by a method similar to that used for the preparation of 6a. The yield was 277 mg. (44%). $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 4.75-4.68 (q, $J = 8.8$ Hz, 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 72.89, 72.57, 70.58, 70.10, 69.76, 69.42, and 69.09. HR-ESIMS (NH$_2$OCH$_2$CF$_3$): m/z calculated 116.03177, found: 116.03178.

N-(3, 3, 3-Trifluoropropoxy)amine hydrochloride (6e)

Compound (6e) was prepared by a method similar to that used for the preparation of 6a. The yield was 269 mg. (51%) $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 4.27-4.24 (t, $J = 5.6$ Hz, 2H), and 2.81-2.70 (m, $J = 6.0$ Hz, 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 127.64, 124.89, 67.12, 67.09, 67.05, 67.02, 32.03, 31.75, 31.47, and 31.18. HR-ESIMS (NH$_2$O CH$_2$CH$_2$CF$_3$): m/z calculated 130.04743, found: 130.04742.

N-(4, 4, 4-Trifluorobutanoxy)amine hydrochloride (6f)

Compound 6e was prepared by a method similar to that used for the preparation of 6a. The yield was 195 mg. (74%) $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 4.09-4.06 (t, $J = 6.4$ Hz, 2H), 2.40-2.27 (m, $J = 3.6$ Hz, 2H), 1.86-1.78 (m, $J = 6.0$ Hz, 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 71.48, 29.33, 29.04, and 20.27. HR-ESIMS (NH$_2$OCH$_2$CH$_2$CH$_2$CF$_3$): m/z calculated 144.06368, found: 144.06304.
2.4 Bioassay based on aldehyde reactive probes (ARP)

The ARP bioassay was performed as previously described with minor modifications.\cite{14, 17, 18, 44} ARP reacted with AP sites induced by TMZ and formed a stable ARP-DNA adduct. The biotin part of ARP in APR-DNA adduct reacted with avidin-horseradish peroxidase (HRP), forming a DNA-HRP complex at every biotin residue. The number of the AP sites was calculated by the activity of HRP toward chromogenic substrate in an ELISA (enzyme-linked immunosorbent assay). ARP bioassay was used to measure the free AP sites after the binding by fluoro-MX derivatives. Therefore, the less the AP sites were detected by ARP, the higher the binding efficacy of the fluoro-MX derivatives, or vice versa. For every compound, the measurement of the binding affinity was based on the TMZ treatment at three different concentrations, 0, 187, and 750 µmole/L. To have a better understanding of the binding efficacy of fluoro-MX derivatives, an MX binding experiment was always done in parallel.

2.4.1 APR binding assay following the treatment of TMZ

Following TMZ treatment for 24 h, melanoma cells (A375) were harvested and cellular DNA was extracted with phenol (Fischer Scientific, Fair Lawn, NJ) and chloroform (Sigma-Aldrich, St Louis, MO). Cellular DNA (15 µg) with or without TMZ treatment was incubated with 15 µl of 1 mM aldehyde reactive probe (ARP, Dojindo Laboratories, Kumamoto, Japan) in 135 µl PBS solution at 37 °C for 15 min. DNA was then precipitated by adding 400 µl ice-cold ethanol (100%) at -20 °C for 20 min and the DNA washed once with 70% ethanol. DNA was dried at room temperature for 30 min and then re-suspended in TE buffer (Tris-EDTA buffer: 10 mmol/L Tris-HCl, 1 mmol/L EDTA, Ph = 7.2) to achieve a final concentration of 0.3 µg/100 µl.
The ARP-labeled DNA was then heat-denatured at 100 ºC for 5 min, quickly chilled on ice, and mixed with an equal amount of 2 M ammonium acetate. The DNA was then immobilized on BA-S 85 nitrocellulose (NC) membrane (Schleicher and Schuell, Dassel, Germany) using a minifold II vacuum filter device (Schleicher and Schuell, Dassel, Germany). Then, the NC membrane was baked at 80 ºC for 1 h and incubated with 0.25% BSA/PBS containing streptavidin-conjugated horseradish peroxidase (BioGenex, SanRamon, CA) at room temperature for 40 min with gentle shaking. ARP-labeled AP sites were visualized by chemiluminescence (Amersham Corp, Piscataway, NJ) followed by quantitative densitometry using NIH Image software.

2.4.2 AP sites formation following TMZ treatment

The binding affinities of the six fluoro-MX derivatives (Fig. 2.1) showed that FEX and FPX have higher binding efficacy for AP sites than MX in the A375 melanoma cell line under the treatment of TMZ. FEX has significantly higher binding efficacy for AP sites at the concentration of both 187 µM TMZ and 750 µM TMZ than that of 0 µM TMZ.

In this project, the biological responses of tumor mouse models to another important chemotherapy drug, Alimta, were also investigated. Although the chemotherapeautic mechanism of Alimta is different from that of TMZ, it also triggers the BER pathway as discussed in chapter one. That is why fluoro-MX derivatives also can be used to test the efficacy of Alimta. As the first step for the biological evaluation of Alimta, ARP bioassay was carried out in non-small-cell lung cancer (NSCL) cells, since alimita is an effective chemotherapy drug for lung cancer.[45-48] The procedures of the ARP binding assays are similar with those done with the treatment of TMZ on A375 melanoma cells.
Figure 2.1 AP sites increased in melanoma cells (A375) after treatment with TMZ in a dose-dependent manner. Using ARP bioassay, the formation of AP sites in A375 melanoma cells were measured. Cells were treated with TMZ (0–750 μM) alone or TMZ plus MX (3 mM) for 24 h for a dose-dependent assay, or treated with TMZ and fluorinated methoxyamine (3 mM) for 24 h. Co-treatment with MX or some of the fluorinated MX derivatives significantly reduced the ARP-detected AP sites. This suggests that fluorinated MX derivatives behave the same as MX and compete with ARP by binding to AP sites induced by TMZ.

2.4.3 AP site formation following Alimta treatment
The results of ARP-based bioassay with the treatment by TMZ indicated that FEX was the most promising probe. Therefore it was selected to evaluate the efficacy of Alimta. For the evaluation of the efficacy of alimita, NSCL cells were treated with different doses of Alimta. AP site formation was then measured by the ARP-based bioassay. NSCLC cells (H460) were treated with Alimta alone (0-400 μM), Alimta plus MX (6 mM), and Alimta plus FEX (6 mM). The treatment time for every set was 24 hours.

The ARP bioassay was performed as previously described with minor modifications. Briefly, following Alimta treatment for 24 hours, NSCLC cells were harvested and cellular DNA was extracted by phenol (Fischer Scientific, Fair Lawn, NJ) and chloroform (Sigma-Aldrich, St Louis, MO). Cellular DNA (10 μg) was incubated with 15 μl of 1 mM aldehyde reactive probe (ARP, Dojindo Laboratories, Kumamoto, Japan) in 135 μl PBS solution at 37 ºC for 15 min. DNA was then precipitated by adding 400 μl ice-cold ethanol (100%) at -20 ºC for 20 min and washed once with 70% ethanol. DNA was dried at room temperature for 30 min and then resuspended in TE buffer to achieve a final concentration of 0.3 μg/100 μl. The ARP-labeled DNA was then heat-denatured at 100 ºC for 5 min, quickly chilled on ice and mixed with an equal amount of 2 M ammonium acetate. The DNA was then immobilized on a BA-S 85 nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a Minifold II vacuum filter device (Schleicher and Schuell, Dassel, Germany). The membrane was baked at 80 ºC for 1 h and incubated with 0.25% BSA/PBS containing streptavidin-conjugated horseradish peroxidase (BioGenex, SanRamón, CA) at room temperature for 40 min with gentle shaking. ARP-labeled AP sites were visualized by chemiluminescence (Amersham Corp, Piscataway, NJ) followed by quantitative densitometry using NIH Image software.
As shown in Figure 2.2, the formation of AP sites was induced by different doses of Alimta. Co-treatment with MX or FEX (6 mM) significantly reduced the density of unbound AP sites. Using a dose of 200 µM Alimta, co-treatment with MX (6 mM) reduced the amount of unbound AP sites by 56%, while co-treatment with FEX reduced the amount of unbound AP sites by 52%. Using a dose of 400 µM Alimta, co-treatment with MX (6 mM) reduced the amount of unbound AP sites by 43% while co-treatment with FEX reduced the amount of unbound AP sites by 36%. Our preliminary AP site binding results showed that FEX potently bound to AP sites in a way similar to MX.

**Figure 2.2** AP sites increased in NSCLC cells after treatment with Alimta in a dose-dependent manner. By using ARP bioassay, the formation of AP sites in NSCLC cells was measured. Cells were treated with Alimta (0 µM, 200 µM, and 400 µM), Alimta (0 µM, 200 µM, and 400 µM) plus MX (6 mM), and Alimta (0 µM, 200 µM, and 400 µM) plus FEX (6 mM) for 24 hrs. Co-treatment by Alimta with MX or FEX reduced the the amount of unbound AP sites detected by ARP bioessays under the same conditions, suggesting that FEX, just like MX, competed with ARP in binding to AP sites induced by Alimta.
The results of APR bio-assays by the treatment of Alimta also demonstrated that NSCLC cells have similar sensitivity to Alimta by producing more AP sites at higher doses. Meanwhile FEX effectively competes with ARP in binding to AP sites. In summary, FEX is an effective probe to evaluate the efficacy of Alimta in cancer cells. With such promising data, the radio-synthesis of $[^{18}\text{F}]$FEX was proposed and the in vivo biological evaluation of Alimta by PET/MRI imaging with quantitative analysis in xenograft tumor mouse models was conducted.
Chapter Three

Radiosynthesis of $[^{18}\text{F}]$FEX & $[^{18}\text{F}]$FPX and In Vivo PET/MRI Studies of AP Sites Formation Induced by Alimta on Cancer Cell Xenograft Models

3.1 Introduction

With the promising results of the fluoro-MX derivatives’ binding efficacy for AP sites induced by the treatment of TMZ and Alimta, the in vivo biological evaluation of Alimta by PET using $[^{18}\text{F}]$FEX or $[^{18}\text{F}]$FPX as a tracer was conducted. Although FPX was not investigated by the treatment of Alimta, it shows high binding affinity to AP sites induced by TMZ. Furthermore, it can be labeled with $[^{18}\text{F}]$. This is why $[^{18}\text{F}]$FPX radio synthesis was initiated. As discussed in chapter one, UDG is the enzyme used to break the carbon-nitrogen bond in nucleotides to remove the incorporated uridines, thereby producing AP sites. For comparison, both DLD1 UDG +/+ and DLD1 UDG -/- melanoma xenograft mouse models were prepared. Differences of AP site formation in the wild type xenograft tumor mouse and the UDG knockout xenograft tumor mouse were expected both before and after Alimta treatment.

The radiolabeling precursors of FEX and FPX are N-(2-tosylethoxy)phthalimide and N-(3-tosylpropoxy)phthalimide, respectively. N-(2-tosyloxyethoxy)phthalimide was synthesized by tosylation of N-(2-hydroxyethoxy)phthalimide with tosyl chloride and pyridine.[49] N-(3-thosylpropoxy)phthalimide was synthesized under the same conditions. The radiosynthesis of $[^{18}\text{F}]$FEX took two steps. The first step of the radiolabeling was to substitute the tosylate group with $[^{18}\text{F}]$.[50, 51] The second step was to deprotect the amino group with hydrazine monohydrate. [34] The whole synthetic route is shown in Scheme 3.1.
Scheme 3.1 Chemical synthesis and radiosynthesis of FPX and FEX
3.2 The chemical synthesis of radio-labeling precursors and radiosynthesis of \([^{18}\text{F}]\text{FEX}\) and \([^{18}\text{F}]\text{FPX}\)

**N-(3-Tosyloxypropoxy)phthalimide (7)**

To a mixture of compound 3, N-(3-hydroxypropoxy)phthalimide, (0.22 g, 1 mmol) and \(p\)-toluenesulfonyl chloride (0.20 g, 1.05 mmol), was added dropwise with pyridine (4 mL, 0.491 mmol) at 0 °C. Then, the whole mixture was stirred from 0 °C to room temperature over 15 h. The reaction mixture was transferred to a separatory funnel (250 mL) and was extracted three times using 75 mL of diethyl ether. The organic layers were washed with 100 mL cold 1 N HCl, 100 mL water, and 100 mL brine sequentially. The combined solution was dried over anhydrous sodium sulfate. After concentration under reduced pressure, the tosylate product was used without further purification. (0.38 g, 89 % yield). \(^1\)H-NMR (400 MHz, CDCl\(_3\)) \(\delta\): 7.84-7.74 (m, 6H), 7.37-7.35 (m, 2H), 4.34-4.31 (m, \(J = 6.0\) Hz, 2H), 4.28-4.25 (m, \(J = 6.0\) Hz, 2H), 2.45 (s, 3H), and 2.16-2.10 (m, \(J = 6.4\) Hz, 2H); \(^{13}\)C-NMR (100 MHz, CDCl\(_3\)) \(\delta\): 163.41, 144.79, 134.55, 132.68, 129.84, 128.66, 127.86, 123.49, 73.99, 66.70, 27.99, and 21.57.

**N-(2-Hydroxyethoxy)phthalimide (8)**

To a solution of \(N\)-hydroxyphthalimide (1.63 g, 10 mmol) and potassium carbonate (5.52 g, 40 mmol) in 20 mL dry DMSO under argon, was added with 2-bromethanol (2.00 g, 1.60 mmol). Then the whole mixture was stirred for 1.50 h at 70 °C until the red solution turned almost colorless. Heating was then stopped and the reaction mixture was cooled to room temperature. Water (200 mL) was added to the mixture followed by extraction using DCM (3 ×
50 mL). The combined organic extract was washed with 2 N 50 mL HCl, 50 mL water, and 50 mL brine. It was then dried over anhydrous sodium sulfate and concentrated under reduced pressure using rotary evaporator. Purification was achieved by silica column chromatography (hexanes/ethyl acetate = 1.5:1, v:v), yielding 1.55 g of the product, compound 4 (75%). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 7.82-7.73 (m, $J_1 = 3.2$ Hz, $J_2 = 34$ Hz, 4H), 4.42-4.39 (t, $J = 6.4$ Hz, 2H), and 3.81-3.78 (m, $J = 6.4$ Hz 2H); $^{13}$C-NMR (100MHz, CDCl$_3$) $\delta$: 163.24, 134.59, 128.58,123.56, 77.29, and 40.24. HR-ESIMS: m/z calculated 207.14238, found: 207.18280.

**N-(2-Tosyloxyethoxyl)phthalimide (9)**

Compound 8 (110 mg, 0.53 mmol) was dissolved in 10 mL anhydrous pyridine at 0 °C. After stirring for 2 h, $p$-toluenesulfonyl chloride (133 mg, 6.90 mmol) was added and the ice bath was removed. After the reaction was stirred for another 8 h at room temperature, the reaction mixture was transferred to a separatory funnel (250 mL) and was extracted three times using 75 mL of diethyl ether. The combined organic extract was washed sequentially with cold 1N HCl (100 mL), water (100 mL), and brine (100 mL). The solution was dried over anhydrous sodium sulfate. After concentration under reduced pressure, the tosylate product was used for the next step. (187 mg, 98%). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 7.82-7.74 (m, 6H), 7.34-7.31 (m, 2H), 4.43-4.38 (m, $J = 5.6$ Hz, 4H), and 2.43 (s, 3H); $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$: 163.29, 145.023, 134.62, 132.46, 129.85, 128.66, 128.02, 123.64,75.06, and 67.09. (HR-ESIMS: m/z calculated 381.05123, found: 381.15146).

### 3.3 Radiosynthesis of $[^{18}$F$]FPX$
Fluoride was generated by an on-site Scanditronix MC-17 cyclotron. The $[^{18}\text{F}]F$ was trapped on an anion Sep-pak column, then rinsed into a vial by a mixture of 15 mg of 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] (K222) in 0.8 mL acetonitrile and 5 mg of K$_2$CO$_3$ in 0.2 mL water. After azeotropic distillation 3 times with 1 mL acetonitrile for each time, N-(3-tosyloxypropoxyl)phthalimide (7) (2 mg dissolved in 0.4 mL anhydrous acetonitrile) was added into the reaction vial. The reaction mixture was then stirred for 10 min at 100 °C to produce N-(3-$[^{18}\text{F}]$fluoropropoxy)phthalimide (5a). This was confirmed by HPLC with the same retention time of unlabeled N-(3-fluoropropoxy)phthalimide at 8.78 min. The mobile phase was CH$_3$CN:H$_2$O = 85:15. The $[^{18}\text{F}]$F-labeled phthalimide was then deprotected by hydrazine monohydrate in methylene chloride for 10 min at room temperature. The reaction mixture was extracted by ethyl acetate (2 × 2 mL), and the organic layer was washed with H$_2$O (2 × 1 mL). After concentration to less than 2 mL, the crude product was further purified by high pressure liquid chromatography (HPLC) on a semi-preparative HPLC column (Luna 5 µm C18 250 mm × 10 mm), at a flow rate of 2 mL.min$^{-1}$ and with a retention time of 6.71 min. The peak of the $[^{18}\text{F}]$F labeled FPX was characterized and confirmed by HPLC through co-injection with the cold standard compound N-(3-fluoropropoxy)amine (6b). However, after concentration of the HPLC fractions, only free $[^{18}\text{F}]F$ was detected. Free $[^{18}\text{F}]F$ might be eliminated due to the ring closure of $[^{18}\text{F}]N$-(3-fluoropropoxy) amine at 100 °C. (Shown in Scheme 3.2) The same phenomenon happened even when the temperature for the concentration was lowered to 70 °C. Therefore, the radio-synthesis of $[^{18}\text{F}]$FPX was not successful.

3.4 Radiosynthesis of $[^{18}\text{F}]FEX$
[\(^{18}\text{F}\)]Fluoride was generated by an on-site Scanditronix MC-17 cyclotron. The [\(^{18}\text{F}\)]F was trapped on an anion Sep-pak column, then rinsed into a vial filled with a mixture of 15 mg of K222 in 0.8 mL acetonitrile and 5 mg of K\(_2\)CO\(_3\) in 0.2 mL H\(_2\)O. After azeotropic distillation with acetonitrile (3 × 1 mL), 3 mg N-(2-tosyloxyethoxy)phthalimide (9) in 0.3 mL anhydrous acetonitrile was added into the vial containing \(^{18}\text{F}/\text{K222}\). The reaction mixture was then stirred for another 10 min at 100 °C to produce N-(2-\[^{18}\text{F}\]fluoroethoxy)phthalimide ([\(^{18}\text{F}\]2a), which was confirmed by HPLC with the same retention time of unlabeled N-(2-fluoroethoxy) phthalimide (2a). The phthalimide was then de-protected by hydrazine monohydrate in methylene chloride for 10 min at room temperature. The reaction mixture was extracted by ethyl acetate (2 × 2 mL), and the organic extract was washed with H\(_2\)O (2 × 1 mL). After concentration to less than 2 mL, the crude product was purified by HPLC on a semi-preparative HPCL column (Luna 5 µm C18 250 mm × 10 mm). The mobile phase was CH\(_3\)CN/H\(_2\)O = 75%:25%. At a flow rate of 3 mLmin\(^{-1}\), the retention time of 6a was 4.68 min. The peak of the F-18 labeled FEX was characterized and confirmed by HPLC through co-injection with the cold standard, compound 6a. The total synthesis takes about 75 min with an average final radiochemical yield of 11.6% after decay correction [49].

3.5 Preparation of the animal models and PET/MRI in vivo biological evaluation of the efficacy of Alimta

3.5.1 Preparation of the animal models through intracranial tumor implantation

Tumor cells (5 × 10\(^6\)) were injected into the flanks of female athymic HSD nude mice at the age of 6-8 weeks. The tumors were measured with calipers using the National Cancer
Institute formula: \( V = L \text{ (mm)} \times I^2 \text{ (mm)} / 2 \) where \( L \) is the largest diameter and \( I \) is the smallest diameter of the tumor. When the volume of tumor nodules reached 100 - 150 \( \text{mm}^3 \), the tumor-bearing mice were used for experiments.

### 3.5.2 Preparation of UDG-knockout model

The tumor xenografts were derived from paired cell lines DLD1 UDG \(+/-\) and DLD1 UDG-\(-/-\) cells. UDG null cells were generated by gene disruption via homologous recombination. Briefly, rAAV targeting vectors were constructed by inserting the left homology arm (LA) and the right homology arm (RA) into the rAAV-Neo-LoxP vector. Targeted rAAV viruses were packaged in 293T cells. The virus was harvested and used to infect target cells. Cells were then treated with G418 (geneticin) to select for resistant clones. These clones were then screened for homologous recombination by PCR using primers complementary to sequences in the neomycin resistance gene and upstream of the left homology arm (indicated as LA-F and LA-R). Confirmative PCR was also performed on G418 resistant clones using primers complementary to the neomycin resistance gene and to a sequence downstream of the RA (indicated as RA-F and RA-R). The neomycin gene cassette was excised by infecting the target clones with adenovirus expressing Cre-recombinase. Genomic PCR was used for the final screening to amplify 178 bp fragments surrounding the LoxP insertion site (PC-F and PC-R).

### 3.5.3 Alimta treatment on xenograft tumor mice

Alimta treatment on xenografts tumor mice was carried out by intraperitoneal (i. p.) injection with 500 µL of 15 gL\(^{-1}\) for every mouse, both wild type and UDG knockout tumor mouse. The i. p. injections were done 24 h prior to PET/MRI scans on the animals.
3.5.4 MicroPET/MRI imaging

MicroPET studies were conducted using a Concord R4 microPET scanner under anesthesia with 2.0% isoflurane carried by oxygen. The dynamic PET image acquisition was performed immediately after tail vein injection of $[^{18}\text{F}]\text{FEX}$ (~300μCi for each mouse) over 90 min in a list mode of emission acquisition. This was followed by 10 min transmission acquisition using $^{57}\text{Co}$ which was used for attenuation correction. A heating lamp was used to maintain the animals’ body temperature around 37 ± 2 °C. Image reconstruction was made into a matrix of 128 × 128 with 0.8 mm in thickness of the slices.

MRI scans were performed immediately after the microPET scans while the animals were kept still under anesthesia to keep their positions unchanged. MR image acquisition was done on a Bruker Biospin horizontal magnet (7T; 30 cm bore) with a transmit/receive rat volume coil. Two mice were mounted on the holder for each scan under the anesthesia consisting of 2% isofluorane and 98% oxygen. The sequence was T2-weighted turbo spin echo (TR/TE = 3000/60 ms, resolution = 1 mm × 200 μm × 200 μm). During acquisition, the respiration rates of the mice were monitored and regulated within the range of 50-70 breath per minute by adjusting the percentage of isofluorane, while the animals’ body temperature was kept around 37 ± 2 °C by thermal control of warm air directly from the magnet core. Multi-slices of the region of interest were selected in three directions: axial, coronal and sagittal.

3.5.5 Quantitative imaging analysis

Quantitative image analysis was based on the co-registration of tumor regions defined by MRI images and the radiotracer uptake determined by microPET scans. The in vivo
pharmacokinetic profile of $^{18}$F-FEX in tumor regions was analyzed in terms of $^{18}$F-FEX uptake and retention according to the co-registered images. The radioactivity concentration in the tumor tissues was represented by means of standardized uptake value (SUV) [(μCi/cc)/(μCi/g)] as a function of time.

3.6 RESULTS

3.6.1 In vivo micro PET/MRI imaging co-registration

For quantitative analysis of FEX uptake, both microPET and MRI were conducted and the images were co-registered. The tumor tissues in the xenografts as detected by high resolution MRI images were defined as regions of interest (ROI). As shown in Fig. 3.1, co-registration of MRI and PET images using COMKAT permits quantification of standardized uptake value (SUV) of $^{18}$F-FEX.[52] For the Alimta treatment, the dosage was 0.3 g/kg for each mouse through i. p. injection 24 h prior to PET/ MRI scans.

Figure 3.1 Coronal view of melanoma xenografts by 7T MRI and Micro-PET imaging co-registration: (A) An in vivo PET image by $^{18}$F-FEX, (B) PET-MRI fusion images, (C) An in vivo MRI image.
3.6.2 Imaging of AP sites formation in DLD1 UDG +/- xenograft tumor mice

To examine the binding specificity of $[^{18}\text{F}]$FEX in vivo and the efficacy of Alimta, micro-PET imaging studies in a DLD1 UDG +/- melanoma cell xenograft model was conducted 24 h after the treatment with Alimta via i.p. injection. After micro-PET and MRI image acquisition and coregistration, $[^{18}\text{F}]$FEX uptake in the tumor regions was quantified and compared with non-treated controls. In the treated xenografts, $[^{18}\text{F}]$FEX uptake was almost twice of the nontreated mouse model. The uptake of the tracer is proportional to the amount of AP sites. The results are well correlated with the treatment effect of Alimta, which induces AP site formation. The trend of SUV over time and average quantitative uptake of $[^{18}\text{F}]$FEX in treated and nontreated xenograft tumor mice are shown in Fig. 3.2.

Figure 3.2 The treated mouse received Alimta treatment 24 h prior to PET imaging. (A) Average SUV time curves of Alimta treated versus non-treated wild type cancer cell xenograft model. (B) 60 minute average SUV values for Alimta treated and non-treated wild type cancer cell xenograft model.
3.6.3 Imaging of AP site formation in the DLD1 UDG -/- tumor mice

For a better comparison, imaging studies in DLD1 UDG -/- melanoma xenografts were also investigated. One mouse was treated with Alimta 24 hours prior to the PET scans and the other was untreated. The results are illustrated in Fig 3.3. There was almost no difference in the uptake of $[^{18}\text{F}]\text{FEX}$ in terms of SUV between the Alimta-treated and nontreated DLD1 UDG -/- mouse model. That indicates that the enhanced $[^{18}\text{F}]\text{FEX}$ uptake in the alitmta treated wildtype mouse is due to additional AP sites formed by BER owing to the action of UDG. Without UDG, BER cannot be triggered; therefore there are no additional AP sites formed in the Alimta treated DLD1 UDG -/- mouse model than that of the background.

![Figure 3.3](image)

**Figure 3.3.** Treatment and non-treatment with Alimta on UDG knockout cancer cell xenograft model. The treated mouse received Alimta treatment 24 h prior to PET imaging. (A) Average SUV time curves of Alimta treated versus non- treated UDG knockout cancer cell xenograft model. (B) 60 minute average SUV values for treated and non-treated UDG knockout cancer cell xenograft model.
3.6.4 Comparison of AP site imaging between DLD1 UDG +/+ and DLD1 UDG -/- tumor models treated by Alimta

To further understand AP site formation, a comparison study between treated DLD1 UDG +/+ and DLD1 UDG -/- cell xenograft models was conducted. Results showed that AP sites formed in the DLD1 UDG +/+ cell xenograft model were 21% higher than that in the DLD1 UDG -/- cell xenograft model as illustrated by the quantitative analysis in Fig. 3.4. The difference in SUV is sufficiently significant to show that Alimta effectively induces AP sites, which means it successfully inhibits the synthesis of thymidine in the body of tumor mice and triggers BER as discussed in Chapter One.

Figure 3.4. Alimta treatment in both DLD1 UDG +/+ and DLD1 UDG -/- cell xenograft models. (A) Average SUV time curves of treated DLD1 UDG +/+ xenograft tumor mouse versus treated DLD1 UDG -/- xenograft tumor mouse. (B) 60 minute average SUV values of treated DLD1 UDG +/+ xenograft tumor mouse versus treated DLD1 UDG -/- xenograft tumor mouse.
3.7 Discussion

For fluorinated compounds and DNA interaction can be enhanced owing to the effect of fluorine,[34-36] fluorinated agents usually possess unique biological properties.[31-33, 53] Longer half-life of $^{18}$F (110 min) is another advantage of $[^{18}F]$ fluoro-MX derivatives. We thus hypothesized that the introduction of fluorine into MX might enhance their efficacy of binding to AP sites by facilitating interaction with DNA.

One or more fluorine atoms were incorporated into MX through the linkage of methylene groups. When the number of methylene groups is 3, the elimination of free $[{^{18}F}]$F$^{-}$ from the $[^{18}F]$F$^{-}$ labeled compounds was observed. One reasonable explanation is the formation of a 5-membered ring as a result of intramolecular cyclization. Therefore, $[^{18}F]$FEX was the only radiotracer to be radiosynthesized among those six fluoro-MX derivatives. Moreover, during the $[^{18}F]$F$^{-}$ radiolabeling, the amino group has to be protected in order to carry out the nucleophilic substitution reaction of $[^{18}F]$F$^{-}$ with the tosylate precursor in the presence of potassium carbonate. For this purpose, we found that phthalimide was an effective protecting group which could be readily removed when treated with hydrazine monohydrate. The efficacy of FEX for binding to AP sites was evaluated in an ARP-based binding assay that we previously established.[54] Using Alimta to induce AP sites in the NSCLC cell line, FEX effectively blocked the induction of AP sites.

Encouraged by these results, we conducted in vivo micro-PET imaging studies in xenograft tumor mouse models bearing either DLD1 UDG +/- or DLD1 UDG +/- tumor tissues. For quantification of $[^{18}F]$FEX uptake in tumor tissues, high resolution MRI images were also acquired immediately after microPET studies to define the tumor regions. Co-registration of MRI images and PET images using COMKAT[52] allowed us to quantify the radioactivity
concentration in terms of average SUV as a function of time. Each type of tumor xenograft mouse model was imaged before and after Alimta treatment for comparison.

The primary goal was to evaluate the effectiveness of $[^{18}\text{F}]$FEX as an imaging agent to monitor AP site formation. In order to define the in vivo specificity, we used xenograft tumor mice bearing either DLD1 UDG $^{+/+}$ or DLD1 UDG $^{-/-}$ tumor tissues. Previous studies showed that UDG is a critical enzyme involved in the BER pathway for DNA repair. Lacking UDG inhibits the excision of uracil bases, therefore hampers formation of AP sites.

When the DLD1 UDG $^{+/+}$ cell xenograft mice were treated with Alimta, $[^{18}\text{F}]$FEX uptake was significantly increased in the tumor tissues compared to that in non-treated tumor tissues. The increase of FEX uptake suggested that formation of AP sites was drastically elevated (Figure 3.1). This is the first time that Alimta-induced AP-site formation was detected in vivo, which was consistent with previous in vitro studies. Alimta induced DNA damage through the inhibition of thymidylate synthase (TS). Alimta treatment results in the increase in the ratio of dUMP to dTMP. As a consequence of dUMP accumulation, the level of dUTP exceed dUPTase activity. Because the polymerase uses dUTP and dTTP at the same rate, dUTP is eventually erroneously incorporated in DNA. With the incorporation of uracil bases, UDG can excise them from the strands of the DNA, thereby triggering BER pathways and forming more AP sites.$^{[27, 55, 56]}$ The increase of $[^{18}\text{F}]$FEX uptake from the quantitative SUV data clearly showed the effect of Alimta in the tumor mouse models.

In UDG knockout tumor tissues, no difference of $[^{18}\text{F}]$FEX uptake was observed before and after Alimta treatment. This suggests that Alimta did not induce AP-site formation in the absence of UDG. This also showed that the enhanced $[^{18}\text{F}]$FEX uptake was due to the formation
of additional AP sites. This is consistent with prior reports that knockout of UDG hampers AP-site formation.[48, 57, 58]

The comparison of AP site formation between the treated DLD1 UDG +/+ and treated DLD1 UDG -/- cell xenograft models showed 21% percent more AP sites formed in the former case. This comparison again suggests a strong correlation between the formation of AP sites and $[^{18}F]$FEX uptake. Therefore, the studies above showed $[^{18}F]$FEX can effectively evaluate the efficacy of DNA-targeted chemotherapeutic drugs such as Alimta.

3.8 Conclusions

In order to enhance the potential for routine clinical studies, particularly in medical facilities without on-site cyclotron, more $^{18}$F labeled MX analogs with high specificity and binding efficacy needed to be developed. For this purpose, six fluorinated MX derivatives were synthesized and screened for AP site binding efficacy in cancer cells. FEX exhibited similar binding efficacy to MX. FEX was also successfully radiolabeled with $^{18}$F for in vivo imaging studies. Subsequent in vivo PET/MRI imaging studies with $[^{18}F]$FEX as a radiotracer in both DLD1 UDG +/+ and DLD1 UDG -/- tumor cell xenograft models demonstrated its effectiveness as an imaging marker of DNA damage and repair. Therefore, $[^{18}F]$FEX can evaluate the efficacy of DNA-targeted chemotherapy drugs.
4.1 Myelin pathology and multiple sclerosis

Myelin sheath is a spiral extension of glial plasma membrane and appears as a compact and multilayered stack of membranes. Glial cells which cover the axons of neurons exist both in the central nervous system (CNS) and the peripheral nervous system (PNS). The myelin in the CNS is composed of the membranes of oligodendrocytes while that in the PNS is composed of

Figure 4.1 Myelin sheath in CNS and PNS[59]
Schwann cells. Myelin sheath does not cover the entire axon. Small sections ranging from 0.2 to 2 millimeters called nodes of ranvier are uncovered by myelin sheath, as shown in Fig. 4.1. The myelin sheath offers electrical insulation due to its composition of bilayer lipids, high cholesterol content and cellular membrane proteins.[60, 61] Therefore, myelin sheath limits the leakage of ions as well as promotes rapid, effective, repetitive communication along axons and also modulates the axons’ survival and maturation. Without the myelin sheath, the action potential fades within a very short distance during the propagation, and axons will atrophy or even disappear.

The damage of the myelin sheath, also called demyelination, causes numerous neurological diseases including multiple sclerosis (MS) illustrated in Fig. 4.2.[62, 63]
MS, the most common demyelination disease, causes neurologic disability in young adults. Clinical symptoms of MS patients are ataxia, depression, slow sensation, cognitive impairment and loss of vision, etc. The average age of patients is 32 years old. There are around 2 million MS patients in the world, 350,000 in the United States (US).[64, 65] The US is one of the high frequency areas (prevalence 30+ per 100,000) which includes Canada, northern US, Europe, Israel, New Zealand and the southern Mediterranean basin.[66]

In the brains of MS patients, there are abundant demyelinated lesions (plaques) and partially remyelinated lesions (shadow plaques). Remyelination is a process in which demyelinated axons regain newly-generated myelin sheaths. If remyelination occurs, new myelin sheaths will grow, but they are usually thinner and shorter than the original ones. However, these thin and short regenerated myelin sheaths help to restore a sufficient conduction along the axons and enable some functional recovery.[67, 68] Therefore, current research on MS treatments focuses on promoting endogenous myelin repair and/or transplanting an exogenous source of glia cells and genes to the demyelinated areas to facilitate remyelination.[69-72]

4.2 The development of myelin imaging agents

In order to evaluate the efficacy of the treatment, quantification of myelination is crucial. There were studies of the contribution of activated microglia to myelin destruction and stage progression by $^{[11]}$C PK11195, which is designed as a PET imaging probe to detect the increased peripheral benzodiazepine receptor (PBR) expressed by microglia cells. However the results of $^{[11]}$C PK11195 PET imaging did not directly show specific correlation with demyelination and myelin changes in CNS white matter.[73]
In order to develop myelin-specific PET imaging probes, myelin-binding ligands can be labeled with positron-emitting isotopes. Chemical synthesis and radiolabeling are thus important steps during the development of myelin sheath probes for PET imaging. A series of myelin-binding agents based on luxol fast blue and Congo red have been synthesized in our laboratory. Four analogs which are either mono-stilbenes or bis-stilbenes are lead myelin imaging probes: \((E,E)-1,4\text{-bis}(p\text{-aminostyryl})\text{-}2\text{-methoxy-benzene}\) (BMB, 1), 4-amino-4\text{'-}methylamino-trans-stilbene (MeDAS, 2), \((E,E)-1,4\text{-bis}(4\text{'-}aminostyryl)\text{-}2\text{-dimethoxy-benzene}\) (BDB, 3), and 4-((E)-4-((E)-4-aminostyryl)-2,5-dimethoxystyryl)-N-methylaniline (also called Case imaging compound, CIC, 4) (Scheme 4.1).

Scheme 4.1 The structures of 1 (BMB), 2 (MeDAS), 3 (BDB) and 4 (CIC)

All of these probes showed specific and quantitative staining of myelin sheath in vitro, ex vivo or in vivo.\cite{74, 75} Tissue staining has been a very important classic method to image
neurons and nervous systems since the era of Cajal and Golgi. Before the incorporation of positron isotopes into the imaging probes, the binding affinity and specificity of myelin imaging probes were first screened by in vitro and ex vivo brain tissue staining shown in Fig. 4.3.

Figure 4.3 (A) Ex vivo CIC staining of myelin sheaths in the region of corpus callosum (CC) in wild type mouse brain[77] (B) Ex vivo BDB staining of normally myelinated corpus callosum in the control mouse brain[75] (C) MeDAS ex vivo staining in corpus callosum (CC) region of wild type mouse brain[78] (D) Ex vivo BMB staining of wild type mouse brain. Note the strong fluorescence in myelinated structures such as the corpus callosum (CC), rostral commissure (RC), myelinated bundles within the striatum (St) and brainstem (BS)[74]

In vitro staining by BMB showed selective binding to myelinated regions in the mouse brain (Fig. 4.3 (D)). BMB ex vivo staining also demonstrated that BMB can penetrate the brain
blood barrier (BBB) and bind to myelin sheaths. Only the wild type (normal) mouse that had a normal myelin sheath showed strong fluorescence. No fluorescent BMB was detected in the shiverer mouse which is deficient in myelin basic proteins (MBP) in the CNS.[74] Ex vivo brain tissue staining by BDB showed similar high selectivity and specificity in myelinated area as shown in Fig. 4.3 (B).[75] The compound of CIC also can pass the BBB after a tail vein injection. The fluorescent images of corpus callosum demonstrated that CIC selectively binds to myelinated areas in the mouse brain in Fig. 4.3 (A). Similar ex vivo brain tissue staining results also were found with MeDAS in Fig. 4.3 (C).

Encouraged by the results of ex vivo tissue staining, we radiolabeled the myelin-imaging agents with $^{11}$C for in vivo PET imaging studies.

4.3 In Vivo PET imaging of myelin by $^{11}$C labeled probes

[$^{11}$C]BMB and [$^{11}$C]CIC were successfully synthesized in our laboratory. The in vivo PET imaging of brain myelin by [$^{11}$C]BMB was carried out in a non-human primate. The in vivo quantitative results showed that the uptake in myelin-rich white matter areas is significantly higher than that in myelin-deficient gray matter areas in the baboon brain.[74]

[$^{11}$C]CIC can also quantify myelination in rodents. Quantitative analysis of animal model data showed a strong correlation between the content of myelination and the standardized uptake values of the tracers.

[$^{11}$C]CIC as the PET tracer for in vivo imaging of myelin changes in the vertebrate nervous system has been reported by our lab.[77] For in vivo [$^{11}$C]CIC-PET imaging, the first step is to obtain the labeling precursor through multi-step synthesis and obtain the [$^{11}$C]CIC
labeled product through radiosynthesis. Longitudinal microPET imaging studies monitored normal myelination, demyelination, and remyelination in a rat model. The demyelination was induced by lysolethcin, a neurotoxin which is derived from phosphatidylcholine. It induces demyelination that mimics MS.[79] The quantitative analysis was conducted based on the co-registration PET/MRI imaging studies as shown in Fig.4.4.

The control rat with intact myelin sheaths has the highest uptake of [\(^{11}\text{C}\)]CIC among normal myelination, demyelination and remyelination stages. After the induction of demyelination by lysolethicin, [\(^{11}\text{C}\)]CIC uptake was significantly decreased accordingly. After recovery, [\(^{11}\text{C}\)]CIC uptake in the same rat brain was higher than that of demyelinated rat brain, but lower than that in the normal stage as shown in Fig. 4.5. Therefore, [\(^{11}\text{C}\)]CIC selectively bound to myelin sheaths in the rat brain and worked as an imaging marker to monitor the changes of myelination in vivo. [\(^{11}\text{C}\)]MeDAS has also been successfully synthesized and showed a similar ability to image myelination in vivo.[74, 78]
**Figure 4.4** Axial views corpus callosum of control and demyelinated rat model. Demyelination was induced via injection of lysolethicin directly into the left hemisphere of the corpus callosum as shown by the arrow. (A) MRI image of control rat. (B) Fusion of MRI and PET images of corpus callosum of control rat model. (C) MRI image of corpus callosum of demyelinated rat model. (D) Fusion of MRI and PET images of corpus callosum of demyelinated rat model.[77]
Figure 4.5 Images of microPET scans of rat models in the order of decreasing myelin content, control (A), remyelinated (B), demyelinated (C). (D) Average radioactivity concentration in terms of standardized uptake value (SUV) as a function of time in intact control region (blue) versus remyelinated region (green) versus demyelinated region (red) in the right hemisphere of corpus callosum. (E) Average SUV of intact control (blue), remyelination (green), and demyelinated (red) regions throughout 60 min of scan with values of $\rho = 0.011$ for demyelinated versus remyelinated in corpus callosum.[77] N = Number of mice averaged.

4.4 Design of $^{18}$F labeled myelin imaging probes for in vivo PET studies
The half-life of $^{11}$C is 20 minutes. Thus, an on-site cyclotron is required for the radio-synthesis. To enhance the potential of these myelin makers in clinical studies, our lab set out to develop $[^{18}\text{F}]$-labeled derivatives of these lead probes. We have developed several fluorinated derivatives which can be labeled with $^{18}\text{F}$. The half-life of $^{18}\text{F}$ is 110 min. Therefore $^{18}\text{F}$ labeled imaging probes are eligible for remote delivery. Another important reason is that with a longer half-life, the influence of nonspecific retention could be minimized by prolonging PET image acquisition and quantifying the data during the latest frames.

To accomplish this goal, we must develop viable synthetic approaches to myelin imaging probes. No tissue staining or in vivo PET imaging can be achieved without the synthesis of the probes. The following chapters will describe the chemical synthesis of the fluorinated MeDAS and BDB derivatives which can penetrate the BBB and bind to myelin sheaths with high affinity and specificity. In vitro and ex vivo brain tissue staining were conducted for screening these myelin imaging probes. Those probes with promising results in brain tissue staining were then radio-labeled with $^{18}\text{F}$ for further in vivo PET/MRI imaging for quantitative characterization of myelin content in living rodents.
5.1 Introduction

In studies of neurological diseases, molecular imaging characterizes the biological processes at the cellular and molecular level, and therefore bridges the gap between brain functions and neurohistology. There are three established modalities in molecular imaging: MRI, PET, and near-infrared optical imaging. PET is unique in that it can be used for experimental studies in both cells and humans.[80] PET and MRI complement each other in terms of sensitivity and special resolution.[81] MRI and PET co-registration is very important. The development of PET tracers is pivotal for the in vivo study of myelin pathology.[82] $^{18}$F-labeled probes are more adaptable for automated synthesis of PET tracers under a cGMP manufacturing condition, which is a time-constraint step in drug production for regional distribution. This chapter presents the chemical synthesis of fluorinated myelin imaging probes and the biological evaluation of compounds.

Three fluorinated derivatives of myelin imaging probes were designed as shown in Scheme 5.1. Compound (8) is a derivative of BDB. Compounds (11) and (15) are two types of fluorinated MeDAS molecules.
Scheme 5.1 Structures of fluorinated myelin imaging probes

5.2 Chemical synthesis

The structures of fluorinated BDB and MeDAS are listed in Scheme 5.1. The synthesis of the fluorinated BDB, (FC$_3$-BDB) (8) is shown in Scheme 5.2 with 2,5-dimethylphenol as a starting material.[83] After oxidation by Jones reagent and conversion to 4-methoxy-2,5-dimethylphenol, a side chain with a fluorine atom was introduced by a nucleophilic coupling reaction under a basic condition.[84] N-bromosuccinimide was used as a bromination reagent to make the intermediate for the synthesis of phosphonate compound (6).[85] Then this intermediate reacted with 4-nitrobenzaldehyde to give bi-stilbene compound (7) via a Horner–Wadsworth–Emmons (HWE) coupling reaction.[86, 87] Compound (8) was obtained after reduction of compound (7) by tin (II) chloride under acidic conditions.[88, 89]
Scheme 5.2 Chemical synthesis for FC₃-BDB

For the synthesis of fluorinated analogs of MeDAS, two types of molecule were designed. One was introduced in a side chain on the benzene ring, and another was introduced in a side chain to the nitrogen of one amino group. The side chain is introduced before the HWE coupling to make (E)-4-(4-aminostyryl)-2-(3-fluoropropoxy)aniline (11), (FC₃-DAS) in which the side chain contains 3 methylene groups. In the case of (E)-4-(4-aminostyryl)-N-(6-fluorohexyl)aniline (15) (FC₆-DAS) in which the side chain contains six methylene groups, the side chain was introduced after the formation of a stilbene structure,
Scheme 5.3 Chemical synthesis for FC₃-DAS

Scheme 5.4 Chemical synthesis for FC₆-DAS
because the fluorination step by DAST also converted the benzyl aldehyde group into difluoromethylene group, therefore precluding the HWE coupling.[92] Once the side chain with a hydroxyl group on the end was introduced, DAST was used to convert the hydroxyl group into a fluoro group at -76 °C.[43] The detailed synthetic routes for these two molecules are shown in Scheme 5.3 and Scheme 5.4 respectively.

2,5-Dimethyl-1-benzoquinone (1)

To a solution of 2,5-dimethylphenol (15 g, 12.50 mmol) in 200 mL diethyl ether was added with Jones reagent drop-wise over 2 h. The total amount of Jones reagent was made by sodium dichromate dehydrate (83 g, 27.50 mmol), sulfuric acid (95 %, 60 mL) and cold water (70 mL). The reaction flask was immersed in an ice-water bath. The reaction mixture was stirred for 24 h at room temperature. Then the resulting mixture was extracted with ether (3 × 200 mL). The combined extracts were washed with saturated sodium bicarbonate solution (2 × 100 mL) followed by water (2 × 100 mL) and brine (100 mL). The ether layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to deliver 10 g of 2,5-dimethyl-1-benzoquinone, compound (1). The yield was 61%. (10.38 g) ¹H-NMR (400 MHz CDCl₃) δ: 2.03 (s, 6H, -CH₃), and 6.60 (s, 2H, aromatic protons).

4-Methoxy-2, 5-dimethylphenol (3)

In a three-necked flask, 2,5-dimethyl-1,4-benzoquinone (1) (8 g, 28 mmol) and trimethylphosphate (9.70 g, 78 mmol) were dissolved in toluene (80 mL). The reaction mixture was heated to 140 °C and continually stirred for 24 h. The reaction mixture was then concentrated after
cooling to room temperature. A solution of potassium hydroxide (7 g, 1.24 mmol) in 95% ethanol (100 mL) was added to the concentrated residue. The mixture was then stirred for another 16 h followed by acidification with a 2 N hydrochloric acid solution and extraction with ether (3 × 100 mL). The combined extracts were washed with 1 N sodium hydroxide solution, water and brine. After being dried over anhydrous sodium sulfate, the ether layer was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel by the eluent of hexanes:ethyl acetate = 9:1 to give 4-methoxy-2,5-dimethylphenol (3), 4.74 g. (yield: 53 %). 1H-NMR (400 MHz CDCl3) δ: 2.11 (s, 3H, -CH3), 2.32 (s, 3H, -CH3), 3.78 (s, 3H, -OCH3) and 6.61 (s, 2H, aromatic protons).

1-(3-Fluoropropoxy)-4-methoxy-2,5-dimethylbenzene (4)

Under argon, a mixture of 4-methoxy-2,5-dimethylphenol (3) (3.05 g, 20 mmol) and anhydrous potassium carbonate (10.62 g, 80 mmol) in dry DMF (100 mL) was heated to 80 ºC and stirred for 2 h at this temperature. Then, 1-bromo-3-fluoropropane (3.43 g, 23 mmol) was added to the reaction mixture dropwise. After being stirring for another 5 h, the color of the reaction mixture turned to pale pink. After it was cooled to room temperature, the suspension was filtered and the filtrate was concentrated to produce crude 1-(3-fluoropropoxy)-4-methoxy-2,5-dimethylbenzene which was used for the next step without purification.

1,4-Bis(bromomethyl)-2-(3-fluoropropoxy)-5-methoxybenzene (5)
Under argon, to compound (4), 1-(3-fluoropropoxy)-4-methoxy-2,5-dimethylbenzene (3.18 g, 15 mmol) and N-bromosuccinimide (5.69 g, 42 mmol) in CCl₄ (100 mL) was added with benzoyl peroxide (70 mg). The suspension was heated under reflux for 6 h and then cooled to room temperature. The solid was filtered off and the filtrate was concentrated to deliver crude product. The crude compound (5) was recrystallized in DCM to give pure 1,4-bis(bromomethyl)-2-(3-fluoropropoxy)-5-methoxybenzene (5) 4.98 g, 90% in yield. ¹H-NMR (400 MHz, CDCl₃) δ: 6.89 (s, 1H), 6.86 (s, 1H), 4.78-4.75 (t, 1H), 4.67-4.64 (t, 1H), 4.52 (s, 2H), 4.51(s, 2H), 4.15-4.11(t, 2H), 3.87 (s, 3H), and 2.28-2.16 (m, 2H); ¹³C-NMR (100 MHz, CDCl₃) δ: 151.32, 150.37, 127.52, 114.69, 113.67, 81.57, 79.94, 64.30, 56.20, 30.56, 30.36, 28.57, and 28.48.

Tetraethyl ((2-(3-fluoropropoxy)-5-methoxy-1,4-phenylene)bis(methoxy))bis(phosphonate) (6)

Under argon, a mixture of 1,4-bis(bromomethyl)-2-(3-fluoropropoxy)-5-methoxybenzene (5) (4.45 g, 12 mmol) and triethyl phosphite (5 mL, 29 mmol) was refluxed at 170 °C for 8 h. After it was cooled to room temperature, the excess triethyl phosphite was removed by evaporation under vacuum and the residue was used for the next step without further purification.

4, 4’-((1 E, 1 ‘E)-(2-(3-Fluoropropoxy)-5-methoxy-1,4-phenylene)bis(ethane-2.1-diyl))bis(nitrobenzene) (7)

Under argon, compound (6) (4.69 g, 10 mmol) was dissolved in anhydrous THF (100 mL). Sodium hydride (1.43 g, 30.70 mmol) was added to the solution in portions. One hour later,
4-nitrobenzaldehyde (3.03 g, 20 mmol) was added at room temperature. Then, the suspension was refluxed at 70 °C for 2 h. After cooling to room temperature, ethanol was added dropwise to quench the reaction. After concentration under vacuum, the residue was dissolved in methylene chloride, washed with water, brine and dried over anhydrous sodium sulfate. The solution was concentrated by evaporation. The product was purified by column chromatography to give pure compound (7), 2.92 g (61% in yield). $^1$H-NMR (400 MHz acetone-d$_6$) $\delta$: 8.27-8.24 (m, 4H), 7.87-7.78 (m, 6H), 7.60-7.49 (m, 2H), 4.83-4.80 (t, $J$ = 5.6 Hz, 1H), 4.71-4.68 (t, $J$ = 6.0 Hz, 1H), 4.31-4.28 (t, $J$ = 6.4 Hz, 2H), 3.99 (s, 3H), and 2.35-2.32(m, $J$ = 6.0 Hz, 2H), 2.28-2.25(m, $J$ = 4.2 Hz, 2H).

4, 4’-((1E, 1’E)-(2-(3-Fluoroproyl)-5-methoxy-1,4-phenylene)bis(ethane-2,1-diyl))dianiline (8)

Under argon, to a solution of compound (7) (374 mg, 0.80 mmol) in absolute ethanol (60 mL), anhydrous tin(II) chloride (596 mg, 3.20 mmol) in hydrochloride acid (37%, 2 mL) was added in portions. Then the reaction mixture was refluxed for 12 h. After it was cooled to room temperature, the mixture was then concentrated under vacuum. Ethyl acetate (200 mL) was then used to dissolve the residue. The solution was adjusted to pH = 8-9 with 1 N sodium hydroxide solution. Then the organic portions were combined and washed with water, brine and dried over anhydrous sodium sulfate. After concentration, the residue was purified with a silica gel chromatography column (ethyl acetate: hexanes = from 1:1 to 2:1; v:v) to give compound (8), 77 mg. (yield: 23%) $^1$H-NMR (400 MHz, DMSO-d$_6$) 7.31-7.29 (m, 4H), 7.25-7.15 (m, 4H), 7.08-7.01 (m, 2H), 6.71-6.69 (m, 4H), 4.78-5.75 (t, $J$ = 6.0 Hz, 2H), 1H), 4.66-4.63 (t, $J$ = 6.0
Hz, 1H), 3.89 (s, 3H), 2.27-2.17 (m, \( J_1 = 6.0 \) Hz, \( J_2 = 25.6 \) Hz, 2H), and 1.35-1.22 (m, \( J_1 = 2.0 \) Hz, \( J_2 = 12.8 \) Hz, 2H).

3-(3-Fluoropropoxy)-4-nitrobenzaldehyde (9)

Potassium carbonate (13.80 g, 100 mmol) was added to a solution of 3-hydroxy-4-nitrobenzaldehyde (1.67 g, 10 mmol) in dry DMF (80 mL). The suspension was stirred for 1 h at 80 °C, 3-1-fluoro-3-iodopropane (2.26 g, 12 mmol) was added dropwise, then the whole mixture was stirred for another 3 h until the 3-hydroxy-4-nitrobenzaldehyde disappeared according to TLC. Then, the reaction was cooled to room temperature, and the whole mixture was added with water (300 mL) followed by extraction of ethyl acetate (3 × 150 mL) The combined organic layers were washed with water, brine, and dried over anhydrous sodium sulfate. The product was further purified by silica gel column chromatography using hexanes:ethyl acetate = from 9:1 to 4: (v:v) as eluents. The yield was 1.77g, 78%. 1H-NMR (400 MHz, DMSO-\( d_6 \)) δ: 7.89-7.87 (d, \( J = 8.8 \) Hz, 1H), 6.97 (s, 1H), 6.93-6.90 (m, 1H), 4.86-4.83 (t, \( J = 6.0 \) Hz, 1H), 4.74-4.71 (t, \( J = 5.6 \) Hz, 1H), 4.33-4.30 (m, \( J = 5.6 \) Hz, 2H), and 2.37-2.25 (m, \( J_1 = 5.6 \) Hz, \( J_2 = 56.8 \) Hz, 2H).

(E)-2-(3-Fluoropropoxy)-1-nitro-4-(4-nitrostyryl)benzene (10)

To a solution of diethyl 4-nitrobensylphosphonate (820 mg, 3 mmol) and compound (9) (681 mg, 3 mmol) in ethanol (5 mL) and DMF (5 mL), NaOMe in methanol solution (25%, 2.6 mL, 12 mmol) was added dropwise. The mixture was stirred at room temperature for 3 h. The precipitate was filtered and washed with a mixture of hexanes:ethyl acetate. The crude compound (10) was 789 mg and used without purification.
(E)-4-(4-Aminostyryl)-2-(3-fluoropropoxy) aniline (11)

Anhydrous tin (II) chloride (569 mg, 3 mmol) in hydrochloride acid (37%, 2 mL) was added in portions to a solution of compound (10) (346 mg, 1 mmol) in absolute ethanol (30 mL). The reaction mixture was refluxed for 10 h. Then the solution was neutralized with 1N sodium hydroxide solution to pH = 8-9, followed by extraction with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water, brine and dried over anhydrous sodium sulfate. After concentration, the crude product was further purified by silica column chromatography using ethyl acetate:hexanes (from 1:2 to 1:1) as eluents. The yield was 70%, 206 mg. $^1$H-NMR (400 MHz, acetone-d$_6$) $\delta$: 7.27-7.22 (d, $J$ = 8.4 Hz, 2H), 7.07 (s, 1H), 6.88-6.83 (m, 3H), 6.67-6.63 (m, 3H), 4.77-4.74 (t, $J$ = 6.0 Hz, 1H), 4.65-4.62 (t, 1H), 4.19-4.16 (t, $J$ = 6.0 Hz, 2H), and 2.27-2.14 (m, $J_1$ = 6.0 Hz, $J_2$ = 23.2 Hz, 2H); $^{13}$C-NMR (100 MHz, acetone-d$_6$) $\delta$: 207.33, 149.45, 148.45, 138.96, 128.78, 126.53, 126.43, 121.78, 116.25, 115.91, 110.61, 83.55, 81.93, 65.72, 81.93, 65.72, 65.66, 32.33 and 32.13.

(E)-Tert-butyl (4-(4-nitrostyryl)phenyl)carbamate (12),

Under argon, NaOMe in methanol solution (25%, 13 mL, 60 mmol) was added dropwise to a solution of tert-butyl (4-(4-nitrostyryl)phenyl) carbamate (6.64 g, 30 mmol) and diethyl 4-nitrobenzylphosphonate (8.20 g, 30 mmol) in ethanol (50 mL) and DMF (50 mL). The mixture was stirred at room temperature for 7 h. The precipitate was filtered and washed with a mixture of cold ethyl acetate and hexanes( 1:1; v:v). The red solid was pure enough for further use. The yield was 61%, 6.23 g. $^1$H-NMR (400 MHz, acetone d$_6$) 8.23-8.20 (m, 2H), 7.84-7.82(m, 2H),
7.62-7.61 (m, 4H), 7.50-7.45 (m, 1H), 7.31-7.27 (m, 1H), and 1.49 (s, 9H); $^{13}$C-NMR (100 MHz, acetone d$_6$) δ: 207.28, 146.52, 142.26, 134.92, 132.50, 129.63, 128.69, 126.24, 125.77, 120.06, 119.83, 81.24, and 29.46.

(E)-Tert-butyl (6-hydroxyhexyl)(4-(4-nitrostyryl)phenyl)carbamate (13)

Under argon, a round bottom flask with (E)-tert-butyl (4-(4-nitrostyryl)phenyl)carbamate (12) (5.10 g, 15 mmol) in anhydrous THF (150 mL) and anhydrous DMF (150 mL) was cooled to 0 °C by an ice-water bath. Sodium hydride (0.72 g, 30 mmol) was added in portions. The suspension was stirred for 2 h at 0 °C, then 6-bromohexan-1-ol (4.62 mL, 33 mmol) was added dropwise over 30 min. The mixture was stirred for another 20 h at room temperature. The reaction was then quenched with ethanol. Then the mixture was concentrated under reduced pressure to remove THF, ethanol, and excess 6-bromohexan-1-ol. The residue was dissolved in DCM (300 mL). Then, the solution was washed with water (3 × 200 mL) and brine. After drying over anhydrous sodium sulfate, the clear solution was concentrated by rotary evaporation. The crude compound was purified by flash silica gel column chromatography using hexanes : ethyl acetate = 1: 1 to 1: 2 (v : v) as eluents to give pure compound (13), 3.77 g, (57%) $^1$H-NMR (400 MHz, acetone-d$_6$): δ: 8.25-8.22 (m, 2H), 7.87-7.85 (m, 2H), 7.67-7.65 (m, 2H), 7.54-7.50 (m, 1H), 7.40-7.35 (m, 1H), 7.33-7.30 (m, 2H), 3.71-3.67 (t, 2H), 3.54-3.49 (m, 2H), 3.46-3.43 (m, 1H), 3.36-3.20 (m, 1H), 2.90 (s, 1H), 1.43 (s, 9H), and 1.36-1.32 (m, 6H); $^{13}$C-NMR (100 MHz, acetone-d$_6$) δ: 150.29, 146.19, 145.05, 134.52, 129.19, 128.99, 128.00, 125.80, 111.84, 103.92, 81.28, 72.10, 63.28, 51.14, 34.64, 29.44, 28.20, 27.30.

(E)-Tert-butyl (6-fluorohexyl)(4-(4-nitrostyryl)phenyl)carbamate (14)
Under argon, (E)-tert-butyl (6-hydroxyhexyl)(4-(4-nitrostyryl)phenyl)carbamate (13) (3.10 g, 7 mmol) was dissolved in anhydrous DCM (150 mL). Then the solution was cooled to -76 °C. One hour later, DAST (4.65 mL, 25 mmol) was added dropwise at -76 °C. The reaction mixture was stirred for another 16 h. The reaction mixture was warmed to room temperature gradually. The reaction was then quenched with saturated sodium bicarbonate solution until no bubbles came out. Then the mixture was diluted with DCM (200 mL) and washed with water (3 × 200 mL) and brine. After it was dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was purified by flash silica gel column chromatography using ethyl acetate: hexanes from 1:4 to 1:2 as eluents to give 2.36 g of pure compound (14). (77%) 1H NMR (400 MHz, acetone d6): δ 8.26-8.24 (d, 2H), 7.89-7.87 (d, 2H), 7.68-7.66 (m, J = 8, 2H), 7.56-7.37 (m, 2H), 7.41-7.37 (m, 2H), 3.71-3.67 (t, 2H) 3.53-3.34 (m, 4H), 1.58-1.46(m, 6H) 1.43 (s, 9H). 13C NMR (100 MHz, acetone d6): 150.29, 146.19, 145.05, 134.50, 129.19, 128.99, 128.00, 125.80, 111.84, 92.81, 81.28, 72.10, 63.28, 51.05, 32.13, 21.94, 30.02, 29.43, 27.94, 26.60, 26.55.

(E)-4-(4-Aminostyryl)-N-(6-fluorohexyl)aniline (15)

Under argon, tin (II) chloride (2.28 g, 12 mmol) in hydrochloride acid (37%, 5 mL) was added in portions to a solution of (E)-tert-butyl(6-fluorohexyl)(4-(4-nitrostyryl)phenyl)carbamate (14). The reaction mixture was refluxed for 7 h. Then the solution was basified with 1N sodium hydroxide until pH was around 8-9. After extraction with ethyl acetate (3 × 200 mL), the combined organic layers were washed with water, brine and dried over anhydrous sodium sulfate. After it was concentrated over anhydrous sodium sulfate, the crude product was further
purified by silica gel column chromatography using hexanes: ethyl acetate (from 2:1 to 1:1) as eluents. The yield was 43%, 537 mg. $^1$H-NMR (400 MHz, acetone-d$_6$) δ: 7.46-7.32 (m, 1H), 7.28-7.22 (m, 3H), 7.04-6.81 (m, 2H), 6.68-6.58 (m, $J = 5.6$ Hz, 4H), 4.49-4.45 (t, $J = 6.0$ Hz, 7.2 Hz 1H), 4.37-4.34 (t, $J = 6.0$ Hz, 1H), 3.11-3.08 (t, $J = 6.0$ Hz, 2H), 1.70-1.65 (m, $J = 4.2$, Hz 4H), and 1.43-1.38 (m, $J = 3.6$ Hz, 4H); $^{13}$C-NMR (100 MHz, acetone-d$_6$): δ: 207.33, 150.11, 149.24, 128.76, 128.67, 116.24, 114.11, 86.26, 84.63, 72.15, 72.05, 62.55, 32.15, 31.95, 28.69, 28.45, 27.80, 27.58, 26.75, and 26.70.

5.3 *Ex Vivo* brain tissue staining

The binding affinity and selectivity of fluorinated CIC and MeDAS probes to myelinated white matter was further evaluated *ex vivo* according to a protocol developed by our lab.[74, 75] A dose of 1.0 mg of fluorinated analog (i.e. 50 mg/kg bodyweight) was administered via tail vein injection to either wild type or lysolecithin-treated rats. Two hours after injection, rats were perfused with saline to clear the blood vessels in the brain, then perfused transcardially with 4% paraformaldehyde in PBS (pH: 7.3). After the brain was removed and dehydrated with 10%, 20% and 30% sucrose sequentially, the embedded brains in freezing compounds (OCT; Fisher Scientific, Suwanee, Ga) were sliced. The staining was then directly examined by fluorescent microscopy (Leica DRMB microscope).

5.4 LPC rat model of focal demyelination

Sprague Dawley female rats, 6–8 weeks of age, were used for this study. Animal surgery and care was performed in accordance with the Institutional Animal Care and Use Committee of
Case Western Reserve University. Animals were anesthetized using a mixture of ketamine hydrochloride (64.20 mg/kg), xylazine hydrochloride (12.09 mg/kg), and acepromazine (12.90 mg/kg) and positioned in a stereotaxic frame (Stoelting). A small incision was made in the scalp, and the corpus callosum was targeted using the following stereotaxic coordinates, relative to bregma: anterior–posterior, 0.00 mm; medial–lateral, 2.00; and dorsal–ventral, 3.40. A small hole was drilled in the skull, and a 26S gauge needle attached to a 10 µL Hamilton syringe was lowered into the corpus callosum according to the dorsal–ventral coordinate. A micro-injector pump (Stoelting) controlled the infusion of 6 µL of lysolthicin (lysophosphatidyl chorine) at a rate of 0.25 µL/min, after which the needle was left in place for 2 min to prevent liquid reflux out of the brain parenchyma. The incision was then closed using 5-0 Ethicon sutures, and the animals are allowed to recover on a heating pad. MRI imaging of demyelination of the rats was performed nine days after surgery. The rats were kept alive after MRI studies for ex vivo braining tissue staining.

5.5 MRI imaging

The demyelination of the LPC rat model was characterized by MR imaging technique. MR image acquisition was conducted on a Bruker Biospin horizontal magnet (7T; 30 cm bore) with a transmit/receive rat volume coil (73 mm volume ID cylinder transceiver coil). LPC treated rat was mounted on the holder for each scan under anesthesia consisting of 2% isofluorane and 98% oxygen. The sequence was T2-weighted turbo spin echo (TR/TE = 3000/60 ms, resolution = 1 mm × 200 µm × 200 µm, field of view = 50 mm × 50 mm). During acquisition, the respiration rates of the rats were monitored and regulated within the range of 50-70 breath/min by adjusting the percentage of isofluorane, while the animals’ body temperature was kept around 37 ± 2°C by thermal control of warm air directly from the magnet core. Multi-slices of the region
of interest were selected in axial directions to focus on the region of the corpus callosum in rat brain.

5.6 Results and discussion

Three proposed fluorinated imaging probes were successfully synthesized with reasonable yields. In order to know if they can cross the blood brain barrier (BBB) and selectively bind to the myelin sheaths, ex vivo fluorescent tissue staining was conducted via i.v. injection. The results showed that all of them can cross the BBB of rat brain and selectively bind to myelinated areas in CNS, such as corpus callosum and striatum. Both FC₃-BDB and FC₃-DAS have binding affinity and selectivity for myelinated white matter in CNS similar to that of BDB and MeDAS respectively.[75, 78] FC₆-DAS has stronger fluorescence than FC₃-DAS according to the results of ex vivo brain tissue staining as shown in Fig.5.1.

Figure 5.1 (A) Ex vivo FC₃-BDB staining of myelin sheaths in the region of corpus callosum (CC) and myelinated bundles within the striatum (St) with stronger fluorescence in wild type rat brain. (B) Ex vivo FC₃-DAS staining of wild type rat brain with the strong fluorescence in myelinated areas of CC and St. (C) FC₆-DAS ex vivo staining in CC and St regions of LPC treated rat brain (treatment directly into the left hemisphere of the corpus callosum).
The focal demyelinated lesions induced by LPC were clearly detected by ex vivo staining and in vivo MRI.[77, 93] From the ex vivo results, FC₆-DAS shows sharp contrast between myelinated and non-myelinated areas in the rat brains. When part of the corpus callosum was demyelinated by LPC, the ex vivo staining clearly detected the lesion areas due to the lack of fluorescence. When the LPC agent locally damaged the myelin sheaths of the corpus callosum in a small area as detected by MRI ((B) in Fig. 5.2).

**Figure 5.2** (A) FC₆-DAS ex vivo staining in corpus callosum(CC) and striatum(St) regions of LPC treated rat brain which has localized demyelination lesion in the left part of the CC (B) MRI image of LPC treated rat brain with localized demyelination in the left part of CC indicated by the arrow. (C) FC₃-DAS ex vivo staining in CC and St regions of the LPC treated rat brain with demyelination lesion of the left part of CC. (D) MRI image of the LPC treated rat brain with demyelination of the whole left part of CC indicated by the arrow.
It was clearly visible in the ex vivo staining by FC₆-DAS (Image (A) in Fig. 5.2). The results also demonstrated that FC₃-DAS’s high binding affinity and selectivity for myelin sheath when half of the corpus callosum was demyelinated by LPC (shown in images (C) in Fig. 5.2). This result was also confirmed by MRI imaging (image (D) in Fig. 5.2). Therefore, all three of these fluorinated molecules stain myelinated white matter in the rat brain. The retention of these agents was significantly decreased in demyelinated lesions.

Encouraged by the imaging studies, in vivo imaging studies of myelination in rat brain by [¹⁸F]FC₃-DAS were conducted. This compound was chosen because its chemical synthesis is more straightforward than that of FC₃-BDB (see in Schemes 5.2 and 5.3). Thus, [¹⁸F] FC₃-DAS was used for in vivo myelin imaging by PET/MRI.
Chapter Six
Radiosynthesis of $^{18}\text{F}]\text{FC}_3$-DAS and in vivo PET/MRI Studies

6.1. Introduction

Encouraged by the ex vivo brain tissue staining results with the fluorinated myelin imaging probes, FC$_3$-DAS was selected for $^{18}\text{F}$-labeling and in vivo PET imaging. The radiosynthesis was realized via nucleophilic $^{18}\text{F}$- substitution reactions which are the most efficient procedures for the $^{18}\text{F}$-labeled PET tracer production.[94, 95] The first step was to synthesize the requisite radiolabeling precursor. Usually the radiolabeling precursor for $^{18}\text{F}$ direct nucleophilic substitution consists of a highly reactive leaving group, such as mesylate, tosylate, triflate, or iodide, etc. For this reason, the potentially competing reactive sites for nucleophilic attack were protected. During the radiosynthesis of $^{18}\text{F}]\text{FC}_3$-DAS, the nitro groups were reduced to amino groups after the $^{18}\text{F}$- nucleophilic substitution, therefore, it took two steps to obtain the final product. This two-step synthesis is the routine procedure for the radio-labeling of molecules which involve deprotection.[96] $^{18}\text{F}$ was made with an on-site cyclotron and used to displace the tosylate group under basic conditions. Then the nitro groups were reduced to give $^{18}\text{F}]\text{FC}_3$-DAS. After that, the $^{18}\text{F}]\text{FC}_3$-DAS fraction was collected by HPLC. PET/MRI imaging was done in vivo with $^{18}\text{F}]\text{FC}_3$-DAS as the radio tracer for myelin imaging.

6.2. Chemical synthesis and radiolabeling of $^{18}\text{F}]\text{FC}_3$-DAS

The synthesis of the $^{18}\text{F}$ labeling precursor is similar to that of FC$_3$-DAS, using the same material. The side chain with hydroxyl group was introduced via $\text{S}_\text{N}2$ substitution. Then, the hydroxyl group was protected with a tetrahydropyranyl group (THP). The protective group
was removed after HWE coupling reaction. The free hydroxyl group was tosylated to yield \(^{18}\text{F}\)F labeling precursor. For the radiosynthesis, the first step was to substitute the tosylate group with \(^{18}\text{F}\)F. The second step was to reduce the nitro groups to amino groups by tin (II) chloride under acid conditions to obtain \(^{18}\text{F}\)FC\(_3\)-DAS. The synthetic route is shown in Scheme 6.1.

Scheme 6.1 Chemical synthesis and radiosynthesis of \(^{18}\text{F}\)FC\(_3\)-DAS

3-(3-Hydroxylpropoxy)-4-nitrobenzaldehyde (1)
Potassium carbonate (27.6 g, 200 mmol) was added to the solution of 3-hydroxy-4-nitrobenzaldehyde (3.34 g, 20 mmol) in dry DMF (80 mL). The suspension was stirred for 2 h at 80 °C. 3-Bromopropan-1-ol (3.34 g, 24 mmol) was added dropwise, then the mixture was stirred for another 3 h, until the 3-hydroxy-4-nitrobenzaldehyde disappeared as assessed by TLC. Then the reaction mixture was cooled to room temperature, and the entire mixture was added to water (500 mL), followed by extraction with ethyl acetate (3 × 300 mL). The combined organic layers were washed with water, brine, and dried over anhydrous sodium sulfate. The product was further purified by silica gel column chromatography, using hexanes:ethyl acetate = 9:1 to 4:1 as eluents. The yield was 71%, 3.84 g.

4-Nitro-3-((tetrahydro-2H-pyran-2-yl)oxy)propoxy)benzaldehyde (2)

A solution of compound 1 (3.38 g, 15 mmol) in dry DCM (100 mL) with pyridinium p-toluenesulfonate (PPTS) (0.25 g, 1 mmol) was stirred for 17 h at room temperature. The solution was diluted with diethyl ether and washed by half-saturated brine (50 mL) to remove the excessive PPTS. After drying over anhydrous sodium sulfate, the clear solution was concentrated under reduced pressure to produce crude compound (2). It was further purified by flash silica gel column chromatography using hexanes:ethyl acetate = 7:1 (v:v) as eluents to produce pure compound (2), 3.41 g. (73%)

(E)-2-(3-(2-Nitro-5-(4-nitrostyryl)phenoxy)propoxy)tetrahydro-2H-pyran (3)

Under argon, NaOMe in methanol solution (25 %, 6.5 mL, 30 mmol) was added dropwise to a solution of diethyl 4-nitrobenzylphosphonate (1.92 g, 7 mmol) and compound (2)
(2.17 g, 7 mmol) in ethanol (15 mL) and DMF (15 mL). The mixture was then stirred at room temperature for 7 h. The precipitate was filtered and washed with the mixture of hexanes : ethyl acetate = 1:1 (v:v). The yield was 71%, 2.13 g. $^1$H-NMR (400 MHz, CDCl$_3$) δ: 8.30-8.27 (m, 2H), 7.95-7.89 (m, 3H), 7.70-7.58 (m, 3H), 7.42-7.39 (m, 1H), 4.58-4.56 (t, 1H), 4.33-4.30 (t, 1H), 3.85-3.79 (m, 1H), 3.73-3.68 (m, 1H), 3.56-3.50 (1H), 3.43-3.38 (m, 1H), 2.05-1.99 (m, 2H), 1.71-1.58 (m 2H), 1.48-1.37 (m, 4H).

(E)-3-(2-Nitro-5-(4-nitrostyryl)phenoxy)propan-1-ol (4)

Under argon, a solution of compound 3 (1.71 g, 4 mmol) and PPTS (101 mg, 0.4 mmol) in DMF (20 mL) and ethanol (50 mL) was stirred at 55 °C using silicon oil bath for 19 h. The solvent was then evaporated under vacuum. The residue was dissolved in ethyl acetate (200 mL). The solution was washed with water and half-saturated brine. After drying over anhydrous sodium sulfate, the solution was concentrated. The crude product was purified by flash silica gel column chromatography using ethyl acetate : hexanes = 1:3 (v : v) as an eluent to give 882 mg pure compound (4). (64%) $^1$H NMR (400 MHz, acetone-d$_6$) δ: 8.30-8.28 (m, 2H), 7.95-7.90 (m, 3H), 7.71-7.59 (m, 3H), 7.41-7.38 (m, 1H), 4.63-4.60 (m, 1H), 4.32-4.29 (m, 2H), and 1.94-1.88 (m, 2H); $^{13}$C-NMR (100 MHz, acetone-d$_6$) δ:151.37, 150.67, 124.60, 124.12, 115.00, 98.89, 65.78, 64.17, 62.23, 56.01, 30.677, 29.97, 25.44, 19.56, 16.08, and 16.04.

(E)-3-(2-Nitro-5-(4-nitrostyryl)phenoxy)propyl 4-methylbenzenesulfonate (5)

Compound 4 (689 mg, 2 mmol) was dissolved in anhydrous pyridine (50 mL) at 0 °C. After stirring for 2 h, $p$-toluenesulfonyl chloride (133 mg, 6.9 mmol) was added and then the ice
bath was removed. After the reaction was stirred for another 8 h at room temperature, the mixture was transferred to a separatory funnel (250 mL) and was extracted with diethyl ether (3 × 75 mL). The organic extracts were combined and washed sequentially with cold 1N HCl (100 mL), water (100 mL), and brine (100 mL). The solution was dried over anhydrous sodium sulfate. After concentration under reduced pressure, the tosylate product was purified by flash silica gel column chromatography with the eluent of hexanes : ethyl acetate = 3:1 to get compound (5), 409 mg. (41%) 1H NMR (400 MHz, acetone-d$_6$) δ: 8.31-8.29 (m, 2H), 7.96-7.91 (m, 3H), 7.73-7.58 (m, 4H), 7.51-7.39 (m, 2H), 7.28-7.26 (m, 2H), 4.24-4.22 (m, J= 4.2 Hz, 2H), 4.17-4.15 (m, J= 3.2 Hz, 2H), 2.28 (s, 3H), and 2.12-2.06 (m, J= 3.2 Hz, 2H).

(E)-4-(4-Aminostyryl)-2-(3-[18F]fluoropropoxy) aniline (7)

[18F]Fluoride was generated by an on-site Scanditronix MC-17 cyclotron. After [18F]F$^-$ (161 mCi) was trapped on an anion Sep-pak column, it was rinsed into a vial by a mixture of K222 (15 mg) in acetonitrile (0.8 mL) and K$_2$CO$_3$ (5 mg) in H$_2$O (0.2 ml). After the azotropic distillation with acetonitrile (3 × 1 mL), compound (5) (5 mg) in anhydrous DMF (0.3 mL) was added to a vial containing [18F]F$/\text{K222}$. The reaction mixture was then stirred for 10 min at 100 °C to produce (E)-2-(3-[18F]fluoropropoxy)-1-nitro-4-(4-nitrostyryl)benzene (6), which was confirmed by HPLC with the same retention time of unlabeled (E)-2-(3-fluoropropoxy)-1-nitro-4-(4-nitrostyryl)benzene. Then the concentrated compound (6) in absolute ethanol (0.3 mL) was reduced with tin (II) chloride (22 mg) in hydrochloride acid (37%, 20 µL). The mixture was refluxed at 100 °C for 15 min. Then the mixture was basified with 1 N sodium hydroxide to pH 8-9 and then extracted with ethyl acetate (2 × 2 mL). The extracts were combined and concentrated to 1.5 mL, then loaded onto HPLC to produce purified [18F]FC$_3$-DAS which was
confirmed by (E)-4-(4-aminostyrlyl)-2-(3-fluoropropoxy) aniline via running co-injection HPLC spectra. The radio synthesis took around 90 min. The final product was 3.72 mCi and the purity was 98%.

6.3 PET/MRI in vivo brain imaging

6.3.1 Micro PET imaging

MicroPET studies were conducted in a Concord R4 microPET scanner under anesthesia with 2.0% isoflurane carried by oxygen. The dynamic PET image acquisition was performed immediately after tail vein injection of [18F]FC7-DAS (~500 μCi for each rat) over 90 min in a list mode of emission acquisition. This was followed by 10 min transmission acquisition using 57Co which was used for attenuation correction. A heating lamp was used to maintain the animals’ body temperature around 37 ± 2 °C. Image reconstruction was made into a matrix of 128 × 128 with slices of 0.8 mm in thickness.

6.3.2 Micro MRI imaging

MRI scans were performed immediately after the microPET scans while the animals were still under anesthesia to keep the rats immobile. MR image acquisition was conducted on a Bruker Biospin horizontal magnet (9.4T; 30 cm bore) with a transmit/receive rat volume coil. Rats were mounted on the holder for each scan under the anesthesia consisting of 2% isoflurane and 98% oxygen. The sequence was T2-weighted turbo spin echo (TR/TE = 3000/60 ms, resolution = 1 mm × 200 μm × 200 μm). During acquisition, the respiration rates of the rats were monitored and regulated within the range of 50-70 breath/min by adjusting the percentage of isoflurane, while the animals’ body temperature was kept around 37 ± 2°C by thermal control of
warm air directly from the magnet core. Multi-slices of the region of interest were selected in three directions: axial, coronal and sagittal.

6.4 Results

In vivo PET/MRI images showed that the $[^{18}\text{F}]\text{FC}_3$-DAS can cross the BBB and enter the rat brain. A rat model was used because a rat brain is relatively large compared to the mouse brain. Fusion images clearly indicated that the uptake of the $[^{18}\text{F}]$ labeled radiotracer was located in the brain area as shown in Fig. 6.1.

![Figure 6.1](image)

**Figure 6.1.** In vivo images of MRI, PET ($[^{18}\text{F}]\text{FC}_3$-DAS as a probe), and fusion results in three directions of the rat brain. (A) PET image of axial view. (B) PET image of coronal view. (C) PET image of sagittal view. (D) MRI image of axial view. (E) MRI image of coronal view. (F) MRI image of sagittal view. (G) PET and MRI fusion image of axial view. (H) PET and MRI fusion image of coronal view. (I) PET and MRI fusion image of sagittal view.
The binding affinity and specificity of the $[^{18}\text{F}]\text{FC}_3\text{-DAS}$ probe to brain myelin was illustrated with assistance of the high special resolution of MRI images. The images of PET and MRI well overlapped in three directions: axial, coronal and sagittal. MRI images were acquired to localize the desired field of view, the myelinated white matter in the brain. The uptake of the imaging probes in the whole rat brain was evaluated through the fusion images (Fig. 6.1. G, H and I). The standardized uptake value (SUV) of the probe in white matter areas (allosum and cerebellum) and gray matter area (cerebral cortex) were quantitatively calculated. The trend of SUV in these areas over time was shown in Fig. 6.2.

**Figure 6.2.** (A) Average of the radioactivity concentration of $[^{18}\text{F}]\text{FC}_3\text{-DAS}$ in terms of SUV as a function of time in the rat brain white matter areas (corpus callosum and cerebellum) and a gray matter area (cerebral cortex) (B) 60 minute average SUV values for white matter areas and a gray matter area (cerebral cortex) in the rat brain.
These in vivo data showed that highly myelinated white areas had higher average SUV than that of the barely myelinated gray matter area, cerebral cortex, by 32%. The SUV was higher at early time points and decreased in value at later time points.

6.5 Discussion and conclusions

Both the in situ brain tissue staining and in vivo PET imaging showed that FC₃-DAS can cross the BBB and bind to the myelinated white matter with specificity. Part of the radioactivity uptake in Fig. 6.2 (A) might be due to nonspecific binding. After the nonspecific binding was cleared either by blood stream or other physiological metabolism in the body, the only specific binding contributes to the quantitative measurement of SUV uptake at later time points. This is why radionuclides with longer half-life like ¹⁸F are very beneficial in the clinical field. It gives a quantitative diagnosis with less background noise and provides reliable results.[74, 97] When incorporated with [¹⁸F]F radionuclide, [¹⁸F]FC₃-DAS can work as an image marker to quantitatively measure the level of myelination by in vivo MRI/PET imaging techniques. MRI possesses high sensitivity and great spatial resolution but lacks the specificity and ability to quantify myelin loss and remyelination. In vivo PET images with a myelin marker can facilitate the diagnosis of myelin disorders and myelination.[98-100]

The mechanism of specific binding of mono stilbene or bi-stilbene molecules to myelin sheath remains under investigation. It has been hypothesized that the binding affinity is probably due to the hydrogen bounding between the amino groups of the myelin imaging probes and the electrophiles within the proteins in the brain.[101-103] A more convincing rationale is that the planar structure of the mono-stilbene and bi-stilbene has the ability to fit into the channels of the myelin basic protein (MBP)’s beta-sheets.[104] Previous research on the binding affinity and
specificity of the myelin imaging probes developed in our lab showed that BDB, CIC and MeDAS all specifically bind to myelin basic proteins. The myelin staining patterns of these probes were consistent with the results obtained with black gold or MBP antibody.[75, 78, 91]

Taken together, those myelin imaging probes specifically bind to MBP. Possessing a planner structure, they might also fit into the channels of the myelin beta-sheet strands.[105, 106]

In summary, \[^{18}\text{F}]\text{FC}_3\text{-DAS}\ has been successfully developed. It can be used as an in vivo myelin imaging probe for PET/MRI quantitative analysis of myelination and efficacy evaluation of myelin repair therapies which are currently under development. So far, one of the most promising treatments for demyelination disease such as MS is to promote myelin repair. Therefore, PET imaging probes that can provide quantification of myelination is desperately needed for the evaluation of drug efficacy.
Appendices

NMR, HR-ESIMS and HPLC Spectra
$\text{N-OCH}_2\text{CH}_2\text{OTs}$
N-OCH₂CH₂CH₂CH₂OH
$\text{N-OCCH}_2\text{CH}_2\text{CF}_3$
HCl • NH₂OCH₂CH₂F
HCl • NH₂OCH₂CH₂CH₂F
HCl • NH₂OCH₂CH₂CH₂F
HCl • NH₂OCH₂CH₂CH₂CH₂F
HCl • NH$_2$OCH$_2$CH$_2$CH$_2$CH$_2$F

ppm (f1)

1.0

5.0

108
HCl • NH₂OCH₂CF₃
HCl • NH₂OCH₂CF₃
HCl • NH₂OCH₂CH₂CF₃
HCl • NH$_2$OCH$_2$CH$_2$CF$_3$
HCl • NH$_2$OCH$_2$CH$_2$CH$_2$CF$_3$
NH$_2$OCH$_2$CH$_2$F

**Observed**

**Theoretical**

NL:
3.02E5
Wu-1C2F-20090805-R01#31-68 RT:0.08-0.40 AV:22 F:
FTMS + p ESI Full m/s
[50.00-500.00]

NL:
2.28E4
C$_2$H$_2$NOF:
C$_2$H$_2$N$_2$O$_2$F$_1$
p (gss, s /p:40) Chrg 1
R: 500000 Res .Pwr .@FWHM
MH⁺
C₂H₅NOF₃⁺
Error = 42 ppb

NH₂OCH₂CF₃
Observed

C₂H₅NOF₃:
C₂H₅N⁺O⁺F₃
p (gs, s/p/40) Chrg 1
R: 345000 Res .Pwr . @FWHM

NL:
3.05E5
Wu-4-20101025-R01#107-
192 RT: 0.69-1.10 AV: 26 F:
FTMS + p ESI SIM m/s
[111.03-121.03]
NH₂OCH₂CH₂CH₂F

Observed

Theoretical

C₃H₉NOF:
C₃H₃N₁O₁F₁
p (gss, s /p:40) Chrg 1
R: 500000 Res .Pwr . @FWHM
NH₂OCH₂CH₂CH₂CH₂F

Observed

Theoretical

NH₂OCH₂CH₂CH₂CH₂F

NL: 5.02E6
Wu-4C4F-20090805-R01#48-73 RT: 0.07-0.41 AV: 24 F:
FTMS + p ESI Full m s [100.00-250.00]

NL: 2.23E4

C₄H₁₁NOF:
C₄H₉N₂O₂F₆
p (gss, s ip:40) Chrg 1
R: 400000 Res .Pwr . @FWHM
**NH₂OCH₂CH₂CF₃**

**Observed**

**Theoretical**

NL: 1.89E5
Wu-SC3F3-20090805-R02#45-320 RT: 0.12-1.79 AV: 99 F:
FTMS + p ESI Full m.s [100.00-250.00]

NL: 2.26E4
C₃H₇NOF₃:
C₃H₇N₂O₂F₃ p (gss, s/p:40) Chrg 1
R: 300000 Res .Pwr . @FWHM

Theoretical and observed data for the molecule NH₂OCH₂CH₂CF₃.
NH$_2$OCH$_2$CH$_2$CH$_2$CF$_3$

**Observed**

**Theoretical**

NL: 1.24E7 Wu-6C4F3-20090805 - R02#112-199 RT: 0.24-1.17 AV: 63 F: FTMS + p ESI Full ms [100.00-250.00]

C$_4$H$_9$NOF$_3$:
C$_4$H$_9$N$_1$O$_1$F$_3$
p (gss, s /p:40) Chrg 1
R: 250000 Res .Pwr . @FWHM
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