The Synthesis of Precursors towards a Pin1-activatable Gd(III) MRI Contrast Agent

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Molecular Imaging: An Introduction

As the disciplines of biology and chemistry continue to converge, the need for new methods of visualizing biological phenomenon at sub-cellular and molecular levels continues to increase. To this end, molecular imaging has emerged as a multidisciplinary field that incorporates technologies, techniques and basic science from an array of sources with the goal of visualizing, characterizing and quantifying biological process \textit{in vivo} at the cellular and sub-cellular levels.\textsuperscript{1} Spearheading the development of molecular imaging is modern medicine which has much to gain from these new insights. Molecular imaging has already played a key role in the diagnosis, prognosis, and treatment of various cancers.\textsuperscript{2} \textit{In vivo} molecular imaging will continue to influence developments in medicine as new methods of imaging further expand the scope of biological observation and understanding.

Advances in biomedical engineering, synthetic chemistry and molecular biology have all contributed to the emergence of molecular imaging. From the landmark discovery of x-ray imaging in 1895, the development of medical imaging instrumentation has now burgeoned into a $6.6$ billion a year industry.\textsuperscript{3,4} Biomedical imaging modalities such as magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), ultrasound, and optical imaging continue to improve and evolve creating the hardware basis for molecular imaging.

Progress in the development of molecular imaging has also come from synthetic chemistry. Using injectable synthetic molecules with unique labels
(radioisotopes, magnetic and/or fluorescent tags) as probes has leveraged the resolving power of imaging instruments producing enhanced image resolution, sensitivity and specificity. Probes have evolved from radiolabeled molecules such as 2-deoxy-2-[F18]fluoro-D-glucose (\(^{18}\)FDG), a PET-based regional tracer for glucose metabolism, to very sophisticated ‘smart’ probes that specifically target and/or interact with an intended substrate. Specific examples of ‘smart’ molecular probes will be discussed in more detail later.

The identification of biomarkers for specific pathological processes has also contributed to advances in molecular imaging. By targeting specific biomarkers with high affinity and specificity, molecular probes elucidate details of sub-cellular processes with unparalleled clarity. The \textit{in vivo} monitoring of matrix metalloproteinase-2 (MMP-2) expression and inhibition is one of many such examples. As the discovery of novel biomarkers continues, the corollary development of new imaging strategies at the molecular level should be anticipated.

**Activatable Molecular Probes**

At the forefront of \textit{in vivo} molecular imaging is the development of injectable molecular probes that are both targeted and activatable. These ‘smart’ probes are relatively undetectable until they have interacted with their target at which point the probe undergoes a physiochemical change and ‘turns on’. The upshot is a significant increase in signal-to-noise ratio that quantifiably measures biological activity at target sites. Measurable increases in signal intensity of 12-
to 18-fold have been reported utilizing ‘smart’ probes.\textsuperscript{8,9} Currently most ‘smart’ probes have been designed for optical imaging and MRI modalities.

\textit{In vivo Optical Imaging}

Optical imaging consists of a variety of \textit{in vivo} molecular imaging techniques including two that employ ‘smart’ molecular probes: bioluminescence and fluorescence imaging. At the core of bioluminescence imaging is the incorporation of a reporter gene that encodes a light-producing enzyme (luciferase) in cells or genes of interest.\textsuperscript{10} Specific substrates (luciferins) act as ‘smart’ probes by reacting with \(O_2\) in the presence of catalyzing luciferase and ATP, thereby producing light that is detected as bioluminescence. However, the genetically altering nature of bioluminescence imaging confines this technique to basic science research and its imminent clinical utility is unlikely.\textsuperscript{11}

Fluorescence imaging on the other hand is quickly making inroads into the clinical arena. The underlying principle of fluorescence imaging differs significantly from bioluminescence in that fluorescence measures the emitted light following a photo-excitation and therefore requires an external light source. External light sources usually range in the near-infrared region (600 - 900 nm) to reduce absorbance from hemoglobin and lipids, absorbers in the visible and infrared region respectively.\textsuperscript{12} \textit{In vivo} fluorescence imaging utilizes ‘smart’ probes that fluoresce after interacting with their targets by exploiting the quantum-mechanical phenomenon of fluorescence resonance energy transfer (FRET).\textsuperscript{13} The general design of a FRET-based fluorescent probe involves two
different but closely situated fluorochromes that change proximity to one another via protein-protein interactions, proteolysis, or conformational change. Increasing the proximity of the two fluorochromes allows the fluorochromes to act independent of one another thus changing the ratio of observed fluorescence from each fluorochrome. Signal amplification of up to 600% has been reported using ‘smart’ FRET-based fluorescent probes.¹⁴

One of the major impediments to the further development of in vivo fluorescence imaging for clinical use has been the limited depth in tissue penetration. Because fluorescence-based optical imaging utilized radiation in the visible or near-infrared region, penetration greater than 1 cm poses a significant challenge.¹ To date, fluorescence-based in vivo optical imaging has been relegated to superficial, endoscopic or intraoperative procedures and do not involve non-invasive deep tissue penetration. Fluorescence molecular tomography (FMT) is the latest advancement in fluorescence-based imaging that aims to combine the 3-dimensional tomographic imaging methods of CT or MRI with optical imaging technology. Theoretical predictions based on tissue-like materials give estimates of up to 10 cm of tissue penetration using FMT.¹⁵ Until this becomes reality, in vivo optical imaging will remain relegated to superficial imaging procedures.

In vivo Magnetic Resonance Imaging

MRI-based molecular imaging has several advantages over optical imaging including higher spatial resolution, better depth penetration, and clinical
availability.¹ The resolution of an *in vivo* MRI is on the micrometer scale versus the millimeter scale resolution of optical imaging, a difference of three orders of magnitude.¹⁶,⁵ For molecular imaging purposes, this advantage in spatial resolution has allowed the *in vivo* tracking of at the single cell level using MRI.¹⁷ Because MRI employs radio-wave frequencies to gather imaging data, the non-invasive depth of penetration and imaging is virtually unlimited.

While fluorescence-based optical imaging is making inroads into the clinic, the use of MRI has fully been established. From 2004 to 2005, 816,512 MRI procedures were conducted in Canada amounting to 25.5 MRI exams per 1,000 people.¹⁸ For international comparison, consider the United States during the same time where 83.2 MRI exams per 1,000 people took place or England where 19.0 MRI exams per 1,000 people were conducted.¹⁸ These statistics reflect the frequent and common use of MRI as a clinical procedure making it an ideal modality vehicle for *in vivo* molecular imaging.

The major downside of MRI is a relatively low signal-to-noise ratio when compared with other imaging modalities. To overcome this challenge, MRI exams are often complemented with the administration of a contrast agent. As of 1999, 30% of MRI exams were conducted using a contrast agent.¹⁹ To fully appreciate the science of contrast agents and their enhancement of MR imaging quality, it is important to understand the fundamentals of MRI.

**MRI Basics**

The basis for magnetic resonance imaging (MRI) is in measuring the relaxation rates from the protons of water under a magnetic field after a radio
frequency pulse, similar to NMR spectroscopy.\textsuperscript{20} Application of an external magnetic field creates two possible nuclear spin states for protons: parallel or anti-parallel with respect to the external magnetic field. The parallel orientation corresponds to a lower energy level and a slightly greater number of protons take on this orientation. The ratio of proton spins in the anti-parallel or upper energy level to those in the lower energy level is given by a Boltzmann distribution and is exponentially related to the energy gap between the two spin states. On a macroscopic scale the more heavily populated lower spin state results in a net magnetic moment parallel to the external magnetic field also known as the longitudinal axis. A polarized radio frequency (RF) orthogonal to the longitudinal axis with an energy equal to the difference between the two spin states stimulates transitions between the lower energy spin state and the higher energy spin state.\textsuperscript{21} The net absorption of energy corresponds to the difference in populations of the two spin states under the external magnetic field prior to the RF. In effect, the RF diminishes the longitudinal net magnetic moment by creating equal populations of the two spin states. This also establishes a net magnetic moment orthogonal to the longitudinal axis along the transverse vector. The utilization of pulsed RF allows the net transverse magnetic moment to decay and the net longitudinal magnetic moment to reestablish itself under the external magnetic field. These two relaxation events are defined as, $T_1$ and $T_2$, corresponding to longitudinal and transverse relaxation time respectively. The reciprocal of the two relaxation times, $1/T_1$ and $1/T_2$, are known as the relaxation rates or relaxivities and denoted as $R_1$ and $R_2$ respectively.
The longitudinal and transverse relaxation processes describe two different pathways for restoring equilibrium magnetization from a non-Boltzmann distribution of spin states. The $T_1$ relaxation time is known as spin-lattice relaxation because as protons in the higher energy spin state relax back to the lower energy spin state, energy is released from the nuclei to its surroundings or lattice. Conversely, $T_2$ relaxation time is known as spin-spin relaxation because it involves the exchange of energy between the two spin states as spin coherence in the transverse vector is lost.

The $T_1$ and $T_2$ relaxation times of water protons are detected as induced voltages and translated via Fourier transform into images rendered by MRI. Differences in $T_1$ and $T_2$ relaxation times ultimately translate into the light vs. dark contrasts produced for each image pixel. Two key factors contribute to the $T_1$ and $T_2$ relaxation times of water protons in biological systems: the molecules interacting with water and the temperature. For mammalian tissue where temperature remains constant, the different tissue environments are responsible for causing water protons to relax at different rates producing the high-resolution anatomical images of MRI.

Towards ‘Smart’ MRI Contrast Agents

In addition to endogenous factors influencing water proton relaxation times, contrast agents are often utilized to manipulate $T_1$ and $T_2$ and enhance image quality. A contrast agent’s ability to decrease $T_1$ and $T_2$ (or increase $R_1$ and $R_2$) is a function of its electronic spins ($S$), its electronic spin relaxation time
(T₁ₑ), its proximity to the proton (r), the duration of contact with the proton (τ_m),
the number of interacting protons (q), and the rotational correlation time (τ_r).

Commonly administered contrast agents for MRI that take advantage of the
above listed properties include paramagnetic metal ions and superparamagnetic
particles. Both types of contrast agents contribute to image enhancement by
decreasing both T₁ and T₂ relaxation times of water protons and have
successfully been used for in vivo molecular imaging. However, paramagnetic
metal ions tend to decrease T₁ much more than T₂ and hence their use is favored
for T₁-weighted scans which result in positive contrast images.¹⁹

Superparamagnetic particles on the other hand tend to decrease T₂ much than
T₁ and are favored for T₂-weighted scans, which produce negative contrast
images.

The utility of superparamagnetic iron oxide particles has been
demonstrated in several capacities. Weissleder and coworkers observed the
detection of specific DNA sequences using complimentary oligonucleotides
cross-linked with a superparamagnetic iron oxide moiety.²³ When two cross-
linked oligonucleotides hybridize with a single-stranded target DNA sequence,
the T₂ relaxation time of the surrounding water protons decreases and contrast in
the region is enhanced. See Figure 1. In a different study the in vivo expression
of an engineered human transferrin receptor (ETR) was monitored using a
monocrystalline iron oxide nanoparticle (MION)-tagged transferrin.²⁴ Transgenic
mice expressing ETR on the surface of tumor cells were injected with MION-
tagged transferrin. Under a T₂-weighted MRI scan, significant increase in
Figure 1. P1 and P2 are DNA cross-linked iron oxide (CLIO) nanoparticles.\textsuperscript{23} Figure used without permission.

Contrast resulted from the 400% increase in $R_2$ relaxation rate of the water protons in tumor cells internalizing the MION-tagged transferrin. Studies such as these establish the potential utility of superparamagnetic particles as contrast agents for $T_2$-weighted \textit{in vivo} MR imaging.

Advances in MRI technology have favored $T_1$-weighted scanning and as a result paramagnetic metal ions have been exploited as MRI contrast agents far more than superparamagnetic particles.\textsuperscript{19} The most popular paramagnetic metal ion utilized as an MRI contrast agent is gadolinium(III). Several intrinsic properties make Gd$^{3+}$ a particularly attractive for this purpose. Gadolinium(III) is a lanthanide metal ion with an electronic configuration of $4f^7$ and seven unpaired electrons contributing to its high electron spin (S) and favorable electronic spin relaxation time ($T_{1e}$). With an appropriate multi-dentate ligand, gadolinium(III) forms relatively stable eight or nine coordinate complexes.\textsuperscript{25,26} This is particularly important because in aqueous environments free gadolinium(III) ions form
[Gd(H$_2$O)$_8$]$^{3+}$, a toxic compound that irreversibly binds to skeletal tissue.$^{27}$ Conversely, gadolinium(III) ligand complexes excrete efficiently from the body.$^{19}$

It should be mentioned that as with any prescribed drug, adverse side effects have been reported and precautions when using gadolinium-based contrast agents are necessary. Recent studies linking gadolinium-based MRI contrast agents and nephrogenic systemic fibrosis (NFS) are under investigated.$^{28}$ An overwhelming majority of NFS cases have resulted after the administration of a gadolinium-based contrast agent ($\sim$90%). Currently all FDA-approved gadolinium(III)-based MRI contrast agents come with strong warning of side effects but remain on the market.

Current FDA-approved Gd$^{3+}$ contrast agents are non-specific, nonselective and extracellular in their biodistribution.$^{19}$ The design of all FDA-approved Gd$^{3+}$ contrast agents is a nine coordinate ligand-metal complex. The ligand is a polyaminopolycarboxylate multi-dentate chelate that coordinates to eight of the nine coordination sites of the gadolinium(III) metal ion. See Figure 2.

![Figure 2. Currently FDA-approved Gd3+ contrast agents.$^{19}$](image-url)
This allows water molecules to rapidly exchange ($\tau_m$) at the ninth coordination site ($q = 1$). Endeavors to increase water exchange ($\tau_m$) and/or increase the number of coordination sites available for exchange ($q > 1$) have been considered using different ligand groups, however safety and stability concerns limit the viability of such pursuits.\(^{29}\)

An alternative route towards enhancing MR image contrast that does not decrease the denticity of the chelate involves manipulating the rotational correlation time ($\tau_r$) of the contrast agent. Adjusting rotational correlation time ($\tau_r$) is accomplished by creating Gd\(^{3+}\)-based contrast agent that bind a macromolecule. The effective size of the contrast agent changes which in turn changes its rotational speed. The most commonly targeted macromolecule for binding is human serum albumin, HSA. Aime and coworkers used a gadolinium(III)-DOTA (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) complex with three hydrophobic benzyloxymethyl (BOM) groups that bind HSA to demonstrate a five-fold decrease in relaxation time in the presence of HSA.\(^{30}\) Furthermore, decreasing in the number of BOM groups results in an increase in relaxation time. Both findings confirm that the increased image contrast is a result of the enhanced hydrophobicity of the probe and greater HSA interactions leading to increased rotational correlation time ($\tau_r$).

The use of gadolinium(III)-based pro-contrast agents ($q = 0$) that transform into contrast agents ($q = 1$) in the presence of biological or chemical stimuli has
been explored. Current approaches have used endogenous enzymes, changes in pH, and the concentration of specific biologically relevant cations to trigger alterations in the pro-contrast agent that would result in signal generation. These pro-contrast agents are also referred to as “activatable” agents. Several potential benefits for developing gadolinium(III)-based pro-contrast agents include: an increase in signal-to-noise ratio, lower dosages of contrast agent per MRI, and the acquisition of real-time biological activity. The image signal-to-noise ratio increases because a pro-contrast agent is essentially silent and only turns on in the presence of the specific targeted stimuli. Therefore, non-specific uptake of pro-contrast agent does not contribute to the background signal and lower dosage requirements can be expected. This is especially favorable given the high FDA standard dosages of 0.1 mmol Gd$^{3+}$ contrast agent per kilogram body mass of patient, which translate into gram quantities of Gd$^{3+}$. Ultimately, the most significant benefit of activatable MR contrast agents is the ability to monitor specific biological activity with high resolution. This makes activatable MR imaging a highly promising method for non-invasive in vivo molecular imaging.

Proof of concept for an enzymatically-triggered contrast agent was first established by Meade and coworkers with in vitro studies of their gadolinium(III)-DOTA complex referred to as EGad.$^{32}$ Unlike other gadolinium(III)-based contrast agents, EGad features a galactopyranose residue at the ninth coordination site of the Gd$^{3+}$ ion which effectively blocks water from the inner coordination sphere of Gd$^{3+}$. In the presence of β-galactosidase, the galactopyranose ring is cleaved off the complex, opening the ninth coordination
This irreversible cleavage step transforms the pro-contrast agent into the contrast agent and is directly related to the activity of β-galactosidase. Subsequent in vivo studies of the EGad predecessor, EGadMe, demonstrated the ability to monitor β-galactosidase gene expression in Xenopus laevis by MR imaging. While the success of EGad and EGadMe provides proof of concept for enzyme activated contrast agents, in vivo efficacy falls short of in vitro predictions. This phenomenon is likely due to interactions with endogenous ions that chelate to the paramagnetic contrast agent upon cleavage of the galactopyranose moiety. To account for the effect of physiological conditions, a new activatable Gd\(^{3+}\)-DO3A (DO3A = 1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane) complex for the visualization of β-glucuronidase was recently explored. Similar to the β-galactosidase activated EGad and EGadMe pro-contrast agents, the Gd\(^{3+}\)-DO3A complex bears a β-glucuronic acid moiety that is enzymatically cleaved off. However, the β-glucuronic acid moiety is not positioned to block water access to the gadolinium(III) ion. Instead the β-glucuronic acid moiety allows the preferential coordination of bidentate anions, such as carbonate, more effectively when it is attached to the complex than when it is enzymatically cleaved off. In the absence of the β-glucuronic acid moiety, water competes carbonate for gadolinium(III) coordination. In varying concentrations of bicarbonate anion, the Gd\(^{3+}\)-DO3A with attached β-glucuronic acid moiety exhibited a 1.1% increase in \(T_1\) per mM bicarbonate while the β-glucuronidase cleaved Gd\(^{3+}\)-DO3A complex displayed only a 0.14% increase in \(T_1\) per mM bicarbonate.
$T_1$ per mM bicarbonate. These results translate to a significant increase in image contrast at physiologically relevant \textit{in vitro} carbonate concentrations. However, when tested in human serum, the non-cleaved Gd$^{3+}$-DO3A complex exhibited higher contrast than its cleaved counterpart implying that anionic chelation is only one piece of the puzzle.

Another type of enzyme-activated gadolinium(III) contrast agent that deserves mention is one developed by McMurry and coworkers.\textsuperscript{35} They reported a pro-contrast agent that interacts with the human carboxypeptidase B, thrombin-activatable fibronolysis inhibitor (TAFI). TAFI are known for cleaving C-terminal lysine residues in the process of inhibiting clot degradation. The pro-contrast agent designed by McMurry and coworkers exploited the TAFI enzyme by masking a HSA binding group linked to Gd$^{3+}$-DTPA (DTPA = diethylenetriaminepentaacetic acid) with three lysine residues. As expected, the TAFI cleaves the lysine residues and allows for a 18-fold increase in binding to HSA. As mentioned earlier, manipulating the rotational correlation time ($\tau_r$) by binding gadolinium(III) complexes with HSA shows increase signal intensity.

The \textit{in vivo} monitoring of tissue pH via pH-sensitive MRI contrast agents could provide insight into physiological and pathological conditions. Several Gd$^{3+}$-based complexes responsive to varying H$^+$ concentrations have been investigated.\textsuperscript{36,37} Three gadolinium(III)-DO3A complexes featuring \textit{para}-substituted (-CF$_3$, -Me, -OMe) arylsulfonamide moieties demonstrated pH activation via on and off binding of the sulfonamide nitrogen to the Gd$^{3+}$ ion.\textsuperscript{37} The protonation constants (log $K_{\text{MLH}}$) for the -CF$_3$, -Me, and -OMe \textit{para}-
substituted arylsulfonamide nitrogen were determined to be 5.7, 6.4 and 6.7 respectively. Over a pH range of 7.4 to 6.8 the relaxivity, $R_1$, of the complex with a -CF$_3$ para-substituted arylsulfonamide group showed an increase of 48% in a human serum solution. While *in vivo* studies have not yet been conducted, these results are particularly promising for the detection hypoxic tumor environments (pH 6.8-6.9) where the arylsulfonamide nitrogen would remain protonated and unable to chelate to Gd$^{3+}$ metal ion. This would allow water to exchange with the paramagnetic ion, creating stronger image contrast. On the other hand, under the pH conditions of healthy tissue (pH 7.4), the arylsulfonamide nitrogen would act as an acid and be deprotonated. The coordinated arylsulfonamide nitrogen would impede water exchange at the Gd$^{3+}$ ion and ultimately yield lower image contrast.

Metal-responsive gadolinium(III)-based contrast agents for MRI have also been investigated. Using the concentration of metal ions as an activation parameter, pathologies and physiological processes related to specific metal ions are probed. The concentration of calcium (Ca$^{2+}$), which plays a pivotal role in cell communication and regulation, has been monitored using the gadolinium(III)-based MRI contrast agent, DOPTA-Gd (DOPTA = a derivative of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid linked to 1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (DO3A)).$^{38,39}$ See Figure 3. The MRI contrast agent consists of a Gd$^{3+}$-DOTA-like dimer that fluctuates between two conformational states depending on Ca$^{2+}$ concentration. The conformation in the absence of Ca$^{2+}$ prohibits water from the inner sphere of the
Gd$^{3+}$ ion while the conformation in the presence of Ca$^{2+}$ allows water to freely coordinate to the Gd$^{3+}$ ion. Change in the number of interacting protons (q) with the paramagnetic ion results in a significant increase in image contrast (~80% increase in relaxation rate, R$_1$, for each Gd$^{3+}$ unit). Furthermore, DOPTA-Gd is selective for Ca$^{2+}$ over Mg$^{2+}$ some 10$^5$-fold and not effected by changes in H$^+$ concentration changes between pH 6.8 – 7.4.

Zinc-responsive Gd$^{3+}$-based MRI contrast agents have also been reported. Similar in concept to DOPTA-Gd, the Gd$^{3+}$ DTPA-bisamide complex reported by Nagano and coworkers forms different conformational states that vary with Zn$^{2+}$ concentration. The Zn$^{2+}$-sensing gadolinium(III)-DTPA-bisamide complex differs from DOPTA-Gd in three significant ways: it is not dimeric like DOTPA-Gd, there are three conformational states instead of two, and T$_1$ increases with Zn$^{2+}$ concentration. The three conformational states correspond to different concentration ratios of Zn$^{2+}$:Gd$^{3+}$ DTPA-bisamide complex yielding
quantifiable data. However the increase in relaxation time $T_1$ with increased Zn$^{2+}$ concentration results in an inconclusive bright-to-dark signal. More recently Major et al. reported an improved zinc-sensing probe, Gd-daa3, (daa3 = an aryl diaminoacetate with three methylenes linked to DO3A) that reports with dark-to-bright signal output in the presence of Zn$^{2+}$ ions.$^{41}$

Gadolinium(III)-based MRI contrast responsive to Cu$^{2+}$ have been described by Que & Chang.$^{42}$ Copper-Gad-1 (CG1) is nearly identical in structure to Gd-daa3, minus a methyl group on the aryl ring of the capping (ninth coordinate) moiety. This structural difference lends CG1 a significant selectivity for Cu$^{2+}$ over Zn$^{2+}$, as well as limited interactions with other biologically relevant cations such as Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$. Selectively monitoring the concentration of Cu$^{2+}$ allows deeper insight into the physiological and pathological processes associated with it.

Intracellular deliver of activatable MRI contrast agents remains a challenge. Currently all FDA-approved gadolinium(III) MRI contrast agents are classified for extracellular imaging.$^{19}$ Fully realizing the goals of in vivo molecular imaging requires crossing the cellular membrane into the cytoplasm. Tung and coworkers originally pioneered this thought with the synthesis of a Gd$^{3+}$-DOTA complex labeled with a 13-mer HIV-tat derived membrane translocation peptide (tat-DOTA-Gd).$^{43}$ Comparing MR imaging of murine lymphocytes treated with tat-DOTA-Gd with untreated lymphocytes confirmed cellular uptake of the probe. Several other membrane-penetrable Gd$^{3+}$-DOTA complexes have followed suite featuring variations of polyarginine peptide tags.$^{44,45}$
Alternative methods for intracellular delivery of gadolinium(III)-based contrast agents include the use of micelles as carriers. *In vivo* delivery of Gd\(^{3+}\)-DPTA complexes conjugated to phosphatidyl ethanolamine (PE) and incorporated into polyoxyethylene (PEO) micelles has been demonstrated in rabbits.\(^{46}\) The PEO micelles loaded with Gd(III)-DPTA-PE were administered via subcutaneous injection to the rabbit's paw and the local lymphatic system, including vessels and nodes, were visualized by T\(_{1}\)-weighted MRI. Similarly, the development of micelles tagged with antibodies hold great promise for targeted intracellular delivery.\(^ {47}\)

As the frontier of *in vivo* molecular imaging continues to expand, intracellular activatable gadolinium(III)-based MRI contrast agents are poised to play a significant role. Developing these 'smart' contrast agents requires meeting several challenges. These include overcoming biological barriers in targeted cellular delivery, maintaining high specificity and selectivity for the targeted biomarker, amplifying the signal per biomarker, and finding suitable biomarkers that report on physiological and pathological processes. Overcoming these challenges will require the cross-disciplinary cooperation and collaboration.

**Pin1: An Introduction**

Pin1 is a peptidyl-prolyl *cis/trans* isomerase (PPIase) implicated in various cancers and neurodegenerative tauopathies.\(^ {48,49}\) While its enzymatic activity remains the same between the different pathologies, Pin1 is overexpressed in oncogenic cells and depleted in the nuclei of neurons associated with
taupathies.\textsuperscript{48,49} In both cases Pin1 expression is deregulated with detrimental effects implying the importance of its regulated expression. Monitoring Pin1 expression \textit{in vivo} could yield valuable information in the diagnosis, prognosis and treatment of various cancers and neurodegenerative taupathies.

As a peptidyl-prolyl \textit{cis/trans} isomerase, Pin1 catalyzes the isomerization of specific peptidyl-prolyl bonds of its substrates.\textsuperscript{50} The peptidyl-prolyl bond is unique because it exists in two distinct isomers: \textit{cis} or \textit{trans}. The two different conformations of a proline residue provide a backbone switch for rotation about the peptidyl-prolyl bond.\textsuperscript{51} Prolyl isomerization is considered slow and hence requires the assistance of an isomerase for a timely interchange.

Pin1 and its homologues are highly conserved across species and consequently serve similar functions.\textsuperscript{52} The role of Pin1 as a mitotic regulator has been well documented since the time of its discovery in a yeast two-hybrid screen for proteins interacting with NIMA.\textsuperscript{53} When Pin1 is depleted in yeast and HeLa cells mitotic arrest and subsequent apoptosis occurs. An overexpression of Pin1 in HeLa cells also inhibits mitosis interfering with the G2/M transition. Further evidence of being a mitotic regulator is demonstrated by the substrates that Pin1 binds and isomerizes. Some notable Pin1 targets are essential mitotic regulators such as NIMA, Rab4, and Cdc25C.\textsuperscript{54} Furthermore, Pin1 binds a host of MPM-2 antigens which have been identified to be involved in mitosis by the antibody Mitotic Phosphoprotein Monoclonal-2 (MPM-2).\textsuperscript{55}

The overlap in Pin1 and MPM-2 antibody substrates stems from both binding phosphorylated Ser/Thr-Pro motifs with very high selectivity.\textsuperscript{54} Kinetic
studies showed up to 1300-fold increase in $k_{\text{cat}}/K_m$ for Pin1 isomerase activity towards pSer/Thr-Pro compared with Ser/Thr-Pro demonstrating the importance of phosphorylation in substrate recognition. Flanking the pSer/Thr-Pro motif with various amino acids increases Pin1 substrate selectivity. Assaying different peptides sequences containing pSer-Pro revealed an optimal sequence for Pin1 binding dubbed “Pintide” (WFYpSPRLKK). Sequences such as Pintide reveal clues about the binding properties of Pin1 and the residues in the binding pocket that may be involved.

The substrate specificity of Pin1 can be explained by examination of its crystal structure and analysis of site-directed mutagenesis data. Pin1 is a 163 amino acid polypeptide composed of an N-terminal WW domain and a C-terminal peptidyl-prolyl isomerase (PPIase) domain. As its name implies, the C-terminal PPIase domain isomerize pSer/Thr-Pro bonds between the cis and trans conformation. While the exact residues involve have not been elucidated, it is known that the C-terminal PPIase domain is solely responsible for the isomerase activity. Truncated Pin1 with no N-terminal WW domain are still able to isomerizes prolyl bonds. The N-terminal WW domain is involved in substrate recognition and binding by specifically targeting pSer and pThr residues. While mutating specific points in the WW domain does not affect PPIase activity, binding is significantly affected. Substituting the Tyr23 or Trp34 positions in the WW domain with Ala nullifies all binding activity. Similarly substituting the Arg68 and Arg69 positions with Ala reduced the $k_{\text{cat}}/K_m$ for phosphorylated Ser/Thr-Pro substrates 500-fold.
The importance of the WW domain is illustrated in the fact that it is found on all Pin1 homologues with the exception of plant enzymes.\textsuperscript{58} In addition to substrate recognition and binding the WW domain allows for activity regulation of Pin1 activity via phosphorylation of the Ser16 residue.\textsuperscript{60} Phosphorylation of Ser16 blocks the substrate binding site of the WW domain and hence impedes the recognition and binding of Pin1 substrates. Thus the inactive state of Pin1 is when Ser16 is phosphorylated and the active state is when Ser16 is dephosphorylated. The kinase and phosphatase responsible for phosphorylation and dephosphorylation of Pin1 are yet to be identified. Fluctuation in level of Pin1 phosphorylation during the cell cycle corroborates the regulation of Pin1 activity \textit{in vivo}.\textsuperscript{55} Using a combination of \textsuperscript{32}P labeling, immunoprecipitation followed by SDS-PAGE, and autoradiography it was shown that Pin1 is phosphorylated in growing HeLa cells. However, upon mitotic arrest only dephosphorylated Pin1 is observed. Kinetic studies of Pin1 dephosphorylation revealed that while the levels of Pin1 remained stable during the cell cycle, the two forms of Pin1 fluctuated. Thus it is understood that phosphorylation of Ser16 provides an “on” and “off” switch for Pin1.

The Ser16 residue of the WW domain is only one of several ways that Pin1 is regulated. One mechanism that has already been mentioned but deserves repeating is the high selectivity for phosphorylated Ser/Thr-Pro motifs of its substrates.\textsuperscript{54} Because Pin1 is so selective for pSer/Thr-Pro over Ser/Thr-Pro, Pin1 activity is essentially dependent on certain kinases and phosphatases that phosphorylate and dephosphorylate Pin1 substrates. In most cases Pin1
works in conjunction with both kinases and phosphatases. Another regulatory mechanism is Pin1’s substrate driven subcellular localization. While predominately a nuclear protein, Pin1 is often expressed in the cytoplasm depending on substrate interaction with the WW domain. This trait stems from Pin1’s small size (18kDa), its lack of functional nuclear localization or export signal, and strong substrate interactions. The expression of Pin1 is also regulated by growth signals through E2F transcription factors, which cause levels to fluctuate in normal cells but remain constant in cancer cells.

Understanding the role of Pin1 and its binding specificity to pSer/Thr-Pro motifs require a clear understanding of phosphorylation as a signaling mechanism. Generally speaking, phosphorylation and dephosphorylation regulate cellular signaling through activating/deactivating enzymes, amplifying signals, and creating binding motifs. For many cellular mechanisms, including cell cycle regulation, transcription, differentiation, and proliferation, the phosphorylation of serine and threonine preceding proline, also called “Pro-directed phosphorylation”, is an important mechanism in the signaling pathway. The Ser/Thr-Pro motifs are phosphorylated by a number of different proline-directed kinase families including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs) and glycogen synthatase kinase 3β (GSK-3β). These Pro-directed kinases are specific for proline residues following serine of threonine. Any point mutations of proline completely abolish phosphorylation and usually disrupt the signaling pathway. Moreover, Pro-directed phosphorylation renders these bonds resistant to isomerization by
other PPIases such as cyclophilins and FKPBs.\textsuperscript{52} Dephosphorylation of pSer/Thr-Pro motifs plays an equally important role in signaling and is carried out by phosphatases such as Ser/Thr phosphatase PP2A, phosphatase FCP1, RNA polymerase II C-terminal domain (RNA Pol II CTD) and calcineurin.\textsuperscript{50}

In the case of Pin1 and at its substrates, the phosphorylation a Ser/Thr-Pro creates a binding motif for Pin1. Pin1 then catalyzes the isomerization of the peptidyl-prolyl bond. This conformational change is believed to regulate the function of the substrate proteins.\textsuperscript{52,54} However, the exact nature or purpose of conformational changes about the Ser/Thr-Pro bond of Pin1 substrates is not fully understood.

In the absence of PPIases, the isomerization about a peptidyl-prolyl bond is considered a slow conformational interchange. \textit{In vitro} studies of peptide derivatives with Ser/Thr-Pro motifs gave catalytic parameters $k_{\text{cis} \rightarrow \text{trans}}$ of $5.1 \times 10^3$ – $13.1 \times 10^3$ sec\textsuperscript{-1}.\textsuperscript{67} Upon phosphorylation, Ser/Thr-Pro bonds isomerizes about 8 times slower. However, when Pin1 is added to the picture the phosphorylated Ser/Thr-Pro residues isomerize with $k_{\text{cat}}/K_M$ values of up to 1300-times that of the nonphosphorylated Ser/Thr-Pro derivatives.\textsuperscript{54} This high specificity for phosphorylated Ser/Thr-Pro motifs over their non-phosphorylated counterparts could provide an ideal target for measuring Pin1 activity.

The Pin1 \textit{cis/trans} isomerization of Ser/Thr-Pro motifs occurs in conformation specific manners. The phosphorylated Ser/Thr-Pro motifs of Pin1 substrates are always observed binding to the WW domain in the \textit{trans} conformation.\textsuperscript{54} Phosphorylation of Ser/Thr via kinases MAPK, ER2K, and CDK2
is also preferential for to the \textit{trans} conformation.\textsuperscript{68,69} Moreover, dephosphorylation by phosphatases such as PP2A only occur when the pSer/Thr-Pro motif is in the \textit{trans} conformation.\textsuperscript{70} Although \textit{cis} specific phosphatases and kinases are assumed to exist as well, they are yet unknown.

Both isomers of pSer/Thr-Pro bonds are likely to be found in folded phosphoproteins but as one might deduce from the preferential and specific \textit{trans} isomer events, \textit{trans} is strong favored. From approximately 100,000 Ser/Thr-Pro motifs found in sequence databases only about 1000 Ser/Thr-Pro bonds are found in the protein structure database with a propensity for the \textit{cis} conformation in the range of 10\% - 25\%.\textsuperscript{70} These estimates are further confirmed by considering the percentage of \textit{cis} Ser/Thr-Pro bonds in different peptide derivatives at a pH of 7.8 – a range from 5.7 \% to 30.9\%.\textsuperscript{67} Pro-directed phosphorylation of Ser/Thr-Pro motifs does not dramatically shift the \textit{cis}/\textit{trans} equilibrium and it is thus expected that pSer/Thr-Pro will be found in similar ratios to those listed above.\textsuperscript{67,70} The equilibrium ratio of \textit{cis} to \textit{trans} motifs does however change under various pH conditions.\textsuperscript{67} These strong conformational preferences and \textit{cis}/\textit{trans} ratios give indications of how Pin1 might be exploited using conformation specific Ser/Thr-Pro motifs.

**Pin1 and Cancer**

Given Pin1’s role as a mitotic regulator, associating Pin1 deregulation with cancer was never a distant thought.\textsuperscript{53} Observing the overexpression of Pin1 in human breast cancer cells and the corresponding elevation in cyclin D1
expression established the initial correlation.\textsuperscript{48} Subsequent studies revealed an elevated Pin1 expression for other cancers including colon cancer, lymphomas, melanomas, prostate and brain tumors. Furthermore, monoclonal antibody staining against Pin1, positively correlated levels of Pin1 with tumor grade. In addition to elevated Pin1 expression in cancerous tissue over normal tissue, the phosphorylation status of Pin1 is also different.\textsuperscript{48} Dephosphorylated Pin1 are predominant in cancerous cells whereas phosphorylated Pin1 are more abundant in normal tissue. This suggests that not only are the levels of Pin1 elevated in cancerous cell but also the activity.

Evidence linking an over expression of cyclin D1 with cell transformation has been previously established.\textsuperscript{71,72} In addition, inhibiting cyclin D1 expression has been linked with suppression of tumorigenicity.\textsuperscript{73} Though positive correlation between Pin1 and cyclin D1 was observed in various oncogenic cells, to confirm a direct relationship transfection studies were conducted.\textsuperscript{48} Breast cancer-derived cells transfected with a Pin1 expressing construct showed a 2-3 fold increase in cyclin D1 expression over non-transfected cells. Conversely breast cancer-derived cells transfected with an antisense Pin1 construct showed a significant reduction in cyclin D1.

The positive correlation between Pin1 and cyclin D1 is due to Pin1 activating the cyclin D1 promoter. The cyclin D1 promoter activation is achieved by interacting with the AP-1 binding site.\textsuperscript{48} However instead of directly interacting with the AP-1 binding site, Pin1 binds and isomerizes the pSer63-Pro and pSer73-Pro motifs of c-Jun.\textsuperscript{48} The c-Jun protein is part of the AP-1 complex.
responsible for transcriptional activation. The phosphorylation of c-Jun mediated by the c-Jun N-terminal kinase (JNK) or oncogenic Ras is followed by Pin1 isomerization. These events increase transcriptional activity of c-Jun towards the cyclin D1 promoter thus increasing cyclin D1 expression.

The upregulation of Pin1 observed in breast cancer also correlates to an increase in β-catenin. Increased levels of β-catenin in the nucleus enhance the transcription of certain genes including cyclin D1 and Myc with can lead to oncogenesis. The accumulation of nuclear β-catenin is facilitated by Pin1 catalyzed isomerization of the pSer246-Pro bond that inhibits binding to APC. Under non-isomerized conditions APC, a shuttling protein, binds and exports β-catenin to the cytoplasm for degradation. Thus the overexpression of Pin1 stabilizes β-catenin in the nucleus and gives rise to another oncogenic pathway.

The cytokine-NF-κB signaling pathway is deregulated in many cancers and has also been shown to be dependent on Pin1 prolyl isomerization. In non-transformed cells, IκBα shuttles NF-κB to the cytoplasm and inhibits its activity. Surprisingly, in many cancers NF-κB is activated and IκBα levels are elevated suggesting some other regulatory factor. This other factor turns out to be Pin1 which binds and isomerizes p65 on its pThr254-Pro residue and thus inhibits binding to IκBα. The upshot is deregulated transcription through an increase of nuclear p65 and enhanced NF-κB activity.

The relationship between Pin1 and cancer is further strengthened through considering Pin1-knockout mice. Originally theses cloned mice were thought to develop normally. Their phenotype resembled normal mice and only differed
on a cellular level with the inability to restart proliferation after entering G\textsubscript{0}.

However upon closer study many cell proliferation abnormalities and fertility defects emerged among these Pin1-/- mice\textsuperscript{83,84} Some notable abnormalities included testicular atrophy, retinal degeneration and mammary gland impairment – all characteristic of cyclin D1-knockout mice. Moreover, cyclin D-1 levels were severely depressed in Pin1-deficient mice. The levels of β-catenin were also reduced in the tissues of Pin1-null mice\textsuperscript{75} These results confirm a relationships between Pin1, cyclin D1 and β-catenin \textit{in vivo}.

Further analysis of this relationship revealed that in addition to Pin1’s regulation of cyclin D1 transcription via Jun/AP-1 and β-catenin/APC pathways, Pin1 also isomerizes the pThr286-Pro site of cyclin D1\textsuperscript{75} This conformational change inhibits the binding of nuclear exporter, CRM1, which shuttles cyclin D1 to the cytoplasm for degradation. Thus Pin1 also stabilizes nuclear cyclin D1 in addition to regulating its transcription.

The paramount question of interest in studying Pin1 and cancer is how Pin1 transcription is upregulated. Though definitive evidence is lacking, the Rb/E2F pathway seem to be implicated. The promoter of the human PIN1 gene, located on chromosome 19p13, lacks a TATA and CAAT box\textsuperscript{85,62} However, the promoter does have two GC boxes and three E2F-binding sites. \textit{In vitro} and \textit{in vivo} studies show that binding EF2 enhances PIN1 promoter activity and subsequent Pin1 mRNA levels\textsuperscript{62} High levels of EF2 protein are observed in breast cancer and may indicate a deregulation of the Rb/E2F pathway\textsuperscript{86} Since deregulation of the Rb/E2F pathway is also observed in many other cancers it is
reasonable to infer that this may be a possible factor in overexpression of Pin1.\textsuperscript{87} While it may be safe to conclude that E2F is involved in Pin1 expression, further in vivo studies are necessary before determining all the factors in the upregulation of the PIN1 gene.

It is important to realize that Pin1 does not always exhibit a negative role in relation to oncogenesis. The regulation of p53 stability in response to DNA damage demonstrates a positive role Pin1 plays in normal cells.\textsuperscript{88} The p53 protein is a tumor suppressor that regulates cellular processes such as cell cycle checkpoints, genomic stability, transcriptional activation and apoptosis.\textsuperscript{89,90} In the event of DNA damage Pin1 binds and isomerizes p53 at the pSer33-Pro and pSer46-Pro motifs thus inhibiting p53 binding to its ubiquitin ligase, MDM2.\textsuperscript{88} This positive regulation of p53 prolongs its half-life and subsequently increases the transcriptional activity of p53 towards the \textit{p21} promoter. The resulting increase in cell cycle inhibitor p21 curtails the effects of DNA damage and potential oncogenesis by causing effected cells to undergo apoptosis. Thus Pin1 provides stability to tumor suppressors that in turn prohibit the start of transformed cell proliferation.

While the jury is still out on Pin1’s exact role in cancer, the deregulation of Pin1 in cancer has been established. Compelling evidence suggests it may act as an oncogenic catalyst.\textsuperscript{91} Support for this theory stems from the multiple signaling pathways that Pin1 is implicated in. Three of these pathways include the Ras/JNK signaling pathway, the Wnt-\(\beta\)-catenin signaling pathway, and the Rb/E2F signaling pathway.\textsuperscript{48,62,75} Pin1 collaborates with the Ras/JNK signaling
to increase the transcriptional activity of Jun towards cyclin D1. Pin1 can also activate β-catenin, which in turn induces the transcription of the cyclin D1 gene, Jun and Myc. The transcribed Myc induce activation of the E2F family of genes. The Pin1 gene itself is activated by E2F creating a positive feedback loop. While Pin1 itself may not be sufficient to transform cells or promote cell proliferation, it is likely to play the role of an indispensable translator or amplifier of oncogenic signal transduction.

**Pin1 and Taupathies**

A distinguishing feature of neurodegenerative diseases such as Alzheimer’s disease (AD) are the formation of prominent neuronal lesions also known as neurofibrillary tangles (NFTs). Pin1 was first implicated in Alzheimer’s after observing the localization of Pin1 in the cytoplasm and NFTs of AD neurons. Similar observations have linked Pin1 with other taupathies such as frontotemporal dementias and age-related neurodegeneration. The NFTs are composed of the microtubule-associated protein tau in the form of paired helical filaments (PHF). The tau proteins found in NFTs are hyperphosphorylated rendering them ineffective to binding microtubules and promoting microtubule assembly. Pin1 restores these abilities by binding the pThr231-Pro motif of tau or by promoting tau dephosphorylation via conformation specific PP2A activity. Recall that PP2A is specific for the trans conformation. By isomerizing the pThr231-Pro bond to the trans position PP2A can act upon this motif and dephosphorylate tau.
Further study using $^1$H NMR and CD spectroscopy confirm the existence of cis and trans forms of the Thr231-Pro motif of tau but no difference in the equilibrium distribution of the isomers following phosphorylation. This evidence implies that only one isomer form associates with microtubulin binding. A decrease in the specific isomer and a reduction in isomerization rate associated with phosphorylation could explain the loss of binding microtubulin. Furthermore, reduced microtubulin binding allows more phosphorylated tau to form PHFs. Incidentally this in turn causes a reduction of available Pin1 by binding it to PHFs. Consequently, Pin1 is sequestered in the neurofibrillary tangles.

Evidence that Pin1 gets trapped in the paired helical filaments of NFTs was confirmed by measuring Pin1 in the soluble and insoluble fraction of AD and normal brain extracts. The level of soluble Pin1 in AD neurons was on average about 5-fold less than the soluble Pin1 of normal neurons. Using immunoprecipitation analysis, it was found that the overall levels of Pin1 for AD and normal brains were not significantly different. However in normal brain extracts Pin1 was predominantly found in the soluble fractions where as in AD brains Pin1 resided in the insoluble fraction. These results along with the depletion and localization of Pin1 to the cytoplasm are consistent with Pin1 being trapped in the NFTs.

The fact that Pin1, a predominantly nuclear protein, is redistributed to the cytoplasm and NFTs of AD neurons suggests that its distribution is driven by target proteins. In this case, the target proteins are the phosphorylated tau proteins and more specifically their pThr231-Pro residues. However once
bound to these residues Pin1 becomes trapped in the neurofibrillary tangles made up of the phosphorylated tau proteins. The exact kinase responsible for phosphorylating the Thr231-Pro motif is unknown, yet evidence points to mitotic kinases which further supports an association with Pin1.\textsuperscript{49,54}

Physiological effects observed in taupathies such as Alzheimer’s disease may be explained by considering the depletion of Pin1 from the neurons. Pin1 is predominantly a nuclear protein involved in mitotic regulation.\textsuperscript{53} The depletion of Pin1 in HeLa cells resulted in mitotic arrest and apoptosis. This may correspond to neural death and a corresponding loss in mental capacity observed in neurological disorders. The hyperphosphorylation of tau proteins and their inability to bind microtubules is also the result of Pin1 depletion.\textsuperscript{97} The compromised integrity of microtubule formation impedes neuronal processing and transferring of signals to other neurons.\textsuperscript{99} Furthermore embryonic hippocampal cultures from tau deficient mice revealed delays in the maturation of axonal and dendritic extensions.\textsuperscript{100} The tau proteins of normal brains are usually found in the neuronal axon where they function to regulate cell structure, maintain cell polarity and facilitate axonal transport.\textsuperscript{101} These normally functioning tau proteins are replace by NFTs in AD neurons causing progressive disruptions. Thus decreases in mental function and memory loss are both understood by considering the loss in neuronal networks due to increasing neurofibrillary tangles which can ultimately be traced back to depletion of Pin1.

Murine models of the Pin1-/- genotype exhibited age-dependent tau hyperphosphorylation and NFT-specific conformations illustrating the pivotal role
of Pin1 in neurodegenerative disease. Clinical manifestations of Pin1-/- mice involved motor and behavioral deficits characteristic of neuropathy. Other physiological effects observed include tau filament formation and neuronal degeneration of central nervous system. Though this data may appear to implicate reduction of Pin1 as the cause for neurological disorders other studies reveal a more complex relationship.

In a separate study Pin1-null mice demonstrated a reduction in amyloid β production. The aggregation of the extracellular plaque mainly composed of amyloid β is also a physiological hallmark of AD and central to its pathogenesis. By comparing the Pin1-null mice with their wild-type counterparts, an in vivo correlation could be drawn linking Pin1 to an amyloid β generating pathway accounting for approximately 23% of the total amyloid β in the brain. The mechanism by which Pin1 facilitates amyloid β production is thought to occur via the Thr668-Pro motif of the amyloid precursor protein. When this motif is phosphorylated by a number of possible kinases Pin1 binds pThr668-Pro affecting amyloid precursor protein metabolism and the subsequent production of amyloid β. These results seem to suggest Pin1’s direct involvement in AD pathology.

The association of mutations at specific positions of the PIN1 promoter and Alzheimer’s patients revealed two significant single nucleotide polymorphisms (SNPs) at positions -842 (G→C) and -667 (C→T). The frequency of the -842C allele in Alzheimer’s patients was roughly 3 times that of their healthy counterparts. Furthermore the presence of the -842C allele raised
the risk of developing Alzheimer’s disease significantly (odds ratio = 3.044, 95% confidence interval, 1.42-6.52). The -842 and -667 SNPs are in linkage disequilibrium and characterized by three different haplotypes. The CC haplotype was observed in about 16% of the AD patients studied while only 6% of the healthy controls. The Pin1 expression of among both -842C allele carriers and CC haplotypes was lower than non-SNP genotypes in peripheral mononuclear cells. The polymorphisms observed in the PIN1 promoter indicate genetic mechanisms via gene expression and predispositions associated with AD.

The relationship between Pin1 and taupathies is not simple to say the least. While much of the data points towards an inverse relationship between Pin1 expression and NFT formation, other relationships such as Pin1 and amyloid β plaque formation seems to exhibit a positive correlation. Further study is necessary before any conclusions can be drawn. The present knowledge does provide insight into the possible mechanisms these neurological degenerative conditions may utilize and the involvement of Pin1.

**Pin1 Deregulation as a Biomarker**

While our understanding of Pin1 in oncogenesis and taupathies has expanded greatly over the past few years, much more progress is needed before safely exploiting Pin1 expression as a therapeutic avenue. In the interim, using Pin1 expression as a biomarker for cancer and neurodegeneration could yield positive results in both diagnosis and prognosis. Indeed, studies correlating Pin1
expression and various cancers have already been conducted while others wait to be. The initial results indicate that Pin1 is an ideal biomarker candidate for cancer diagnosis and prognosis.

The use of Pin1 expression as a marker for cancerous cells seems ideal given the fact that Pin1 is highly overexpressed in many human cancers. Monitoring Pin1 expression may also serve as an important prognostic tool for patients managing cancer. A prognostic factor already in use for breast cancer is the measuring of $\beta$-catenin. Recall that in breast cancer the elevation of Pin1 strongly correlates with an upregulation of $\beta$-catenin. Hence monitoring Pin1 expression may also be of prognostic value in breast cancer as well as other cancers.

A study correlating Pin1 expression and the clinical outcome of 580 prostate cancer patients has validated the significant prognostic value of measuring Pin1 expression. The patients all underwent radical prostatectomy by the same surgeon and did not receive any preoperative adjuvant therapy. Using immunocytochemistry it was found that Pin1 expression is overexpressed both in the intensity of expression and the percentage of Pin1-positive cells in prostate cancer cells. Pin1 expression was also found to positively correlate with lymph node metastasis and clinical recurrence. The expression of Pin1 was semi-qualitatively assessed using automated imaging analysis to measure the Pin1 staining intensity. Summing the highest intensity of Pin1 staining yielded a sum index of Pin1 expression. This index number along with and the average percentage of Pin1-positive cells were used to predict the recurrence in the
prostate cancer patients following surgery. A recurrence was measured by the reappearance of prostate cancer specific antigens (PSA). The predictive value of Pin1 expression outperformed many currently used predictors including lymph node metastasis, Preoperative PSA levels, Gleason score, surgical margins, seminal vesicle status, and extracapsular extension. In comparing a patient with a high Pin1 sum index with a patient with low Pin1 index, the patient with a high score is more than 8 times more likely to have a recurrence. The predictive power of Pin1 expression was even evident for patients with Gleason scores of 6 or 7, where predicting a clinical outcome is near impossible. These results proved that Pin1 expression has significant prognostic value and could potentially be used as a tool to guide cancer treatment.

While progress for diagnosing neurodegenerative diseases using Pin1 has not reached preliminary trials like that of prostate cancer, several proposals have been made. Levels of tau phosphorylated at Thr231 in the cerebrospinal fluid have been shown to correlate with the progression of Alzheimer’s disease. Hence monitoring this phospho-epitope has been proposed as a diagnostic tool for AD. It is known that Pin1 binds tau proteins in a phosphorylation-dependent manner on the pThr231-Pro motif. Thus by measuring the binding of Pin1 to tau proteins at the pThr231-Pro site, a level of disease progression could be assessed. In addition to diagnosing and monitoring AD, a measure of susceptibility could be made by assaying levels of Pin1 found in the soluble faction of brain samples. Because Pin1 expression is inversely proportional to the vulnerability of forming neurofibrillary tangles, one could easily measure the
potential for developing Alzheimer’s disease or other taupathies. Although these potential Pin1-dependent diagnostic tools are not cures, they may offer opportunities for early detection, assistance in identifying suitable therapy, or progressive monitoring of disease.

**Current Research**

The current thesis research project involves the synthesis of novel enzyme-activatable Gd(III) contrast agents for MRI. Targeting Pin1 via its high specificity towards phosphorylated-Ser/Thr-Pro motifs, the goal of this project is to visualize the expression and activity of Pin1 in cancer cells or neurons. Non-invasive *in vivo* visualization of Pin1 expression and activity at the molecular level could provide information leading to earlier disease detection and better treatment protocols.

The current molecular probe has been designed as a pro-contrast agent (q = 0) that ‘turns on’ (q = 1) by exploiting Pin1’s high specificity for phosphorylated-Ser/Thr-Pro motifs and its isomerase activity (see Figure 1). As a pro-contrast agent, all nine coordination sites of the gadolinium(III) metal ion are blocked. Following the phosphorylation of Ser/Thr-Pro motif, Pin1 can recognize its substrate and isomerizes the peptidyl-prolyl bond, changing the conformation (*trans* → *cis* or *cis* → *trans*) of the phosphorylated-Ser/Thr-Pro motif. It is hoped that this conformation change will reversibly rearrange the phosphorylated-Ser/Thr-Pro motif away from the gadolinium(III) ion, freeing the
ninth coordination site to exchange water and ‘turning on’ the probe (see Figure 4).

The current thesis research project has been devoted to the solution phase synthesis of the prototype probe shown in Figure 4. This work has featured amide bond formation between an amino acid and DOTA ligand, synthesis of tBu-protected DOTA starting compound, solution phase peptide synthesis, and purification by liquid chromatography (LC). Each aspect of the current thesis research project will be disclosed in full detail.

![Figure 4](image-url) A prototype Gd\(^{3+}\) pro-contrast agent activated by phosphorylation and Pin1 mediated isomerization.

**Experimental Section**

**General Methods**

All reagents were purchased from commercial sources and used without further purification. The following starting products: 1,4,7,10-tetraazacyclododecane-1,4,7-tris(t-butyl acetate)-10-succinimidyl acetate,
1,4,7,10-tetraazacyclododecane-1,4,7-tris-tert-butyl acetate-10-acetic acid, and 1,4,7,10-tetraazacyclododecane were all purchase from Macrocyclics (Dallas, TX). The D-phenylalanine methyl ester hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO). The L-proline methyl ester hydrochloride was purchase from Bachem (Torrance, CA). The Fmoc-O-t-butyl-L-serine was procured from Advanced ChemTech (Louisville, KY). All other coupling reagents, solvent, and modifiers were purchased from Sigma-Aldrich, Mallinkrodt Baker, or Fisher Scientific. Water was purified using a Millipore Direct-Q3 filtration system. NMR data was gathered using a Varian Inova 300 MHz instrument. Analytical HPLC was conducted using an Hewlett-Packard 1090 HPLC system with a C-18 reverse phase column (25cm x 4.6mm). The pH was monitored using colorpHast (pH 2.0-9.0) indicator strips from EMD Chemicals (Gobbstown, N.J.). Flash chromatography was conducted using a CombiFlash Companion with a 4.3 g. RediSep C-18 reverse phase column. Mass spectrometry was performed using a Varian 500-MS system.

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-tert-butylester-1-yl-acetyl-D-Phe-OMe (DOTA-(tBu)₃-D-Phe-OMe): 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-tert-butyl acetate-10-acetic acid (100.9 mg, 0.1762 mmol) and HATU (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) (67.6 mg, 0.1762 mmol) were combined in 325 µL of DMF, followed by addition of DIEA (30.7 µL, 0.1762 mmol). The homogeneous solution was stirred at room temperature under
nitrogen atmosphere for 30 min. In a separate vessel, a 0.155 M solution of D-
phenylalanine methyl ester hydrochloride in DMF was prepared and a 325 µL
aliquot was mixed with 61.4 µL of DIEA. The two solutions were then combined
with pH adjusted to ~ 8 and stirred under nitrogen atmosphere at room
temperature for 24 hrs. The reaction mixture was washed with EtOAc and a
saturated aqueous LiBr solution (5 times). The organic layer was dried in vacuo
resulting in a viscous orange gel. The gel was dissolved in acetonitrile (ACN)
and analyzed via HPLC at a flow rate of 0.4 mL/min using the following method:
(Solvent A = 0.1% TFA (vol/vol) in H₂O, Solvent B = 0.1% TFA (vol/vol) in ACN) 0
– 60% B over 40 min, 60 – 100% B over 20 min, hold for 10 min at 100% B, 100
– 0% B over 20 min, and 0% B for 10 min. Two absorption peaks with retention
times of 20 min and ~40 min were observed at 254 nm. Flash liquid
chromatography following the same method at a flow rate of 5 mL/min produced
two peaks when monitored at 212 nm with similar retention times. The peak at
~40 min from flash chromatography was dried under vacuum and analyzed by 1H
NMR (300 MHz, 25°C, [D₆]DMSO): δ 1.40 (s, 27 H, tBu), 2.7 – 3.2 (m, 24 H,
CH₂N), 3.63 (s, 3 H, OCH₃), 7.2 – 7.25 (m, 5 H, Phe). ESI-MS m/z: (M + H)⁺
734.2 (100%).
Figure 5. Synthesis of DOTA-(tBu)₃-D-Phe-OMe.

1,4,7,10-Tetraazacyclododecane-1-carboxymethyl-benzylester (cyclen carboxymethyl-OBzl): 1,4,7,10-Tetraazacyclododecane (509.6 mg, 2.96 mmol), also known as cyclen, was sparingly dissolved in ~5 mL of CHCl₃. About 10 – 15 µL of triethylamine was added to further dissolve solid. A solution of benzyl-2-bromoacetate (234 µL, 1.48 mmol) in 25 mL of CHCl₃ was added drop wise to the 1,4,7,10-tetraazacyclododecane slurry for the duration of 3 hrs while stirring under nitrogen atmosphere at room temperature. The slurry was stirred for an additional hour following the complete addition of benzyl-2-bromoacetate. The reaction mixture was filtered and the filtrate dried under vacuum. The resulting viscous orange oil was separated via flash chromatography at a flow rate of 5 mL/min using the following method: (Solvent A = 0.1% TFA (vol/vol) in H₂O, Solvent B = 0.1% TFA (vol/vol) in ACN) 0 – 60% B over 40 min, 60 – 100% B over 20 min, hold for 10 min at 100% B, 100 – 0% B over 20 min, and 0% B for 10 min. Four peaks were observed using a 220 nm detection lamp. The third peak at retention time ~25 min was dried under vacuum and followed up with ¹H NMR analysis (300 MHz, 25°C, [D₆]DMSO): δ 2.8 – 3.1 (m, 16 H, CH₂N), 3.56 (s,
2 H, CH$_2$COO), 5.14 (s, 2 H, CH$_2$Phe), 7.3 -7.4 (m, 5 H, Phe). ESI-MS m/z: (M + H)$^+$ 321.3 (100%).

**Figure 6.** Synthetic scheme for Cyclen carboxymethyl-OBzl.

9-fluorenylmethoxycarbonyl-Ser-tert-butoxide-Pro methyl ester (Fmoc-Ser-(tBu)-Pro-OMe): Fmoc-Ser(tBu)-OH (105.9 mg, 0.276 mmol) was dissolved with 1-Hydroxybenzotriazole (HOBt, 74.6 mg, 0.552 mmol) in ~4 mL of ACN and stirred under nitrogen atmosphere at room temperature as non-homogeneous white solution. Diisopropyl carbodiimide (DIC, 70.1 mg, 0.552 mmol) in 0.5 mL of ACN was added to above solution turning it homogeneous followed by precipitate formation. Addition of DIEA (95.8 µL, 0.552 mmol) turned solution an orange color and precipitation continued. Continued to stir of 2.5 hrs. Precipitate was filtered off and filtrate was combined with a solution of proline methyl ester (50.3 mg, 0.304 mmol) and DIEA (95.8 µL, 0.552 mmol) in ~5 mL of ACN. The combined solution was stirred at room temperature under nitrogen atmosphere for 44 hrs. The solvent was mixed with celite and dried under vacuum. The resulting celite mixture was purified with reverse phase flash chromatography at a flow rate of 5 mL/min using two method: Method 1 - (Solvent A = 0.1% TFA (vol/vol) in H$_2$O, Solvent B = 0.1% TFA (vol/vol) in ACN) 0 – 60% B over 40 min,
60 - 100% B over 20 min, 100% B flush for 20 min, 100 - 0% B over 20 min, 0% B for 10 min. Four peaks were observed using a 220 nm. detector at retention times 23 min, 42 min, 45 min, and 48 min. Peaks at retention times 42, 45 and 48 min were not base line resolved. The characterization by $^1$H MNR spectroscopy and mass spectroscopy indicate a possible sodium adduct of Fmoc-Ser-(tBu)-Pro-OMe dipeptide, however at the present time conclusive evidence is yet to be established.

![Figure 7](image.png)

**Figure 7.** Synthesis of Fmoc-Ser-(tBu)-Pro-OMe.

**Synthesis**

The primary focus of the thesis research centered on synthesizing a ligand complex and peptide motif using solution phase methods as outlined in Figures 5, 6, & 7. The three general reaction methods were largely adapted from two prior published papers. All three synthetic schemes have entailed multiple approaches, yielded limited success, and opened many avenues for continued advancement.
Initially, the interest was on finding optimal conditions for creating an amide bond between the DOTA – type ligand and an amino acid. The two synthetic methods outlined in Figure 8 were attempted several times and finally resulted in the development of the synthesis outlined in Figure 5. The use of phenylalanine methyl ester as the amino acid was for several reasons: the methyl ester functionality prevents side product formation via further peptide coupling reactions, the phenyl group provides a UV-fluorophore utilized during chromatographic separation, and phenylalanine methyl ester was readily available. The use of glycine methyl ester was also explored however for
reasons unknown phenylalanine methyl ester couples more readily with DOTA in forming an amide bond.

As illustrated in Figure 8, two different DOTA – based starting compounds were explored. Both DOTA starting compounds are protected with tert-butyl groups on three of the carboxylic acid moieties, limiting reactivity to a single carboxylic acid group. The top synthetic scheme involves an \( N \)-hydroxysuccinimide ester activated DOTA compound (DOTA-(tBu)\(_3\)-OSu) and the bottom synthetic scheme is the free DOTA acid (DOTA-(tBu)\(_3\)-OH).

Surprisingly, the top synthetic scheme with the activated DOTA was less successful and actually prompted the reaction with free DOTA acid.

The reaction conditions were varied over time to find an optimal method. The ratio of starting materials DOTA to phenylalanine of 3.5 : 1 seemed to produce the best results. Even with a large excess of DOTA, analytical HPLC showed that some of the starting phenylalanine methyl ester does not react to completion. A slightly alkaline pH ~8 is optimal reaction conditions. While DMF and NMP were equally suitable solvents, the difficulty of removing NMP over DMF makes the later a better solvent for this reaction. Below are listed the reaction parameters for each of the reactions in detail.

<table>
<thead>
<tr>
<th>Rxn #</th>
<th>Date</th>
<th>DOTA Reagent / Eqv</th>
<th>Amino Acid / Eqv</th>
<th>Coupling Agent</th>
<th>Solvent</th>
<th>Eqv DIEA</th>
<th>Rxn Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/10/07</td>
<td>DOTA-(tBu)(_3)-OSu 1 eqv</td>
<td>Phe-OMe 1.1 eqv</td>
<td>None</td>
<td>DMF</td>
<td>5 eqv</td>
<td>overnight</td>
</tr>
<tr>
<td>2</td>
<td>6/06/07</td>
<td>DOTA-(tBu)(_3)-OSu 1.2 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HOBt 1.1 eqv</td>
<td>DMF</td>
<td>2.2 eqv</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>3</td>
<td>6/07/07</td>
<td>DOTA-(tBu)(_3)-OSu 1 eqv</td>
<td>Gly-OMe 4 eqv</td>
<td>HOBt 1 eqv</td>
<td>DMF</td>
<td>1.8 eqv</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>4</td>
<td>6/15/07</td>
<td>DOTA-(tBu)(_3)-OSu 1 eqv</td>
<td>Gly-OMe 1 eqv</td>
<td>HOBt 1 eqv</td>
<td>DMF</td>
<td>20 eqv</td>
<td>20 hrs.</td>
</tr>
<tr>
<td>5</td>
<td>6/26/07</td>
<td>DOTA-(tBu)(_3)-OSu 1 eqv</td>
<td>Gly-OMe 1 eqv</td>
<td>HOBt 1 eqv</td>
<td>DMF</td>
<td>20 eqv</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>Rxn #</td>
<td>Date</td>
<td>DOTA Reagent / Eqv</td>
<td>Amino Acid / Eqv</td>
<td>Coupling Agent</td>
<td>Solvent</td>
<td>Eqv</td>
<td>Rxn Time</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
<td>--------------------</td>
<td>------------------</td>
<td>----------------</td>
<td>-----------</td>
<td>-----</td>
<td>----------</td>
</tr>
<tr>
<td>6</td>
<td>7/09/07</td>
<td>DOTA-(tBu)$_3$-OSu 1 eqv</td>
<td>Gly-OMe 1 eqv</td>
<td>HOBt 1 eqv</td>
<td>DMF</td>
<td>20 eqv</td>
<td>28 hrs.</td>
</tr>
<tr>
<td>7</td>
<td>7/24/07</td>
<td>DOTA-(tBu)$_3$-OSu 1 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HOBt 1 eqv</td>
<td>DMF</td>
<td>20 eqv</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>8</td>
<td>8/07/07</td>
<td>DOTA-(tBu)$_3$-OSu 1 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HOBt 1 eqv &amp; DIC 2.6 eqv</td>
<td>DMF</td>
<td>3 eqv pH 7-8</td>
<td>20 hrs.</td>
</tr>
<tr>
<td>9</td>
<td>9/04/07</td>
<td>DOTA-(tBu)$_3$-OH 3.5 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HATU 3.5 eqv</td>
<td>NMP</td>
<td>7 eqv</td>
<td>4 hrs.</td>
</tr>
<tr>
<td>10</td>
<td>9/15/07</td>
<td>DOTA-(tBu)$_3$-OH 3.5 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HATU 3.5 eqv</td>
<td>NMP</td>
<td>7 eqv</td>
<td>4 hrs.</td>
</tr>
<tr>
<td>11</td>
<td>9/22/07</td>
<td>DOTA-(tBu)$_3$-OH 3.5 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HATU 3.5 eqv</td>
<td>DMF</td>
<td>23 eqv pH ~8</td>
<td>41 hrs.</td>
</tr>
<tr>
<td>12</td>
<td>11/09/07</td>
<td>DOTA-(tBu)$_3$-OH 3.5 eqv</td>
<td>Phe-OMe 1 eqv</td>
<td>HATU 3.5 eqv</td>
<td>DMF</td>
<td>10.5 eqv</td>
<td>24 hrs.</td>
</tr>
</tbody>
</table>

The outcomes of each of the reaction listed above varied and only three of the reactions were fully characterized by via $^1$H NMR, mass spectrometry, or both for the product of interest. Reaction number 12 also featured in Figure 5 yielded the best results. Percent yields were not obtained due to issues with product purity and separation. All of the reactions required subsequent separation steps including preparatory TLC, precipitate filtration, liquid-liquid extraction, normal phase liquid chromatography, and reverse phase liquid chromatography. The various chromatography methods will be explained in more detail later.

The synthesis of 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester is of great interest because it serves as a precursor in making the starting reagent, DOTA-(tBu)$_3$-OH. Though DOTA-(tBu)$_3$-OH is commercially available, it is very expensive (US$420.00 per 500 mg) and impractical to purchase on a large scale. Furthermore, methods for synthesizing of DOTA-(tBu)$_3$-OH are readily available in the literature.$^{109,110}$

Synthesizing 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester employed a technique known as reverse addition. To a concentrated
1,4,7,10-tetraazacyclododecane (2 equivalents) solution in CHCl₃ is added a relatively dilute solution of benzyl-2-bromoacetate (1 equivalent) in CHCl₃ over a span of three to four hours. While this synthesis will also result in side products like the diacylated, triacylated and tetraacylated 1,4,7,10-tetraazacyclododecane products, the reverse addition technique under the concentration and time conditions listed above favor the monoacylated product, 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester.

As with all syntheses involved in the thesis project, purification steps were required. Liquid chromatography was explored extensively in separating out the product of interest. Normal phase liquid chromatography methods with silica-based stationary phases and alumina-based stationary phases were investigated. Reverse phase liquid chromatography with C18 column ultimately provided the best separation. The following table provides information regarding the reactions in synthesizing 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester:

<table>
<thead>
<tr>
<th>Rxn #</th>
<th>Date</th>
<th>1,4,7,10-Tetraazacyclododecane Eqv / Dilution in CHCl₃</th>
<th>Benzy1-2-bromoacetate Eqv / Dilution in CHCl₃</th>
<th>Rxn Time</th>
<th>Purification Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/15/07</td>
<td>2 eqv / 0.83 M</td>
<td>1 eqv / 2.1 M</td>
<td>1 hr.</td>
<td>Silica column – CHCl₃/EtOH/NH₃</td>
</tr>
<tr>
<td>2</td>
<td>3/28/07</td>
<td>2 eqv / 0.83 M</td>
<td>1 eqv / 0.073 M</td>
<td>4 hrs.</td>
<td>Silica column – CHCl₃/EtOH/NH₃</td>
</tr>
<tr>
<td>3</td>
<td>8/29/07</td>
<td>2 eqv / 0.83 M</td>
<td>1 eqv / 0.073 M</td>
<td>4 hrs.</td>
<td>Silica column – Hexane/EtOAc</td>
</tr>
<tr>
<td>4</td>
<td>1/02/08</td>
<td>2 eqv / 0.59 M + 10–15 µL TEA</td>
<td>1 eqv / 0.059 M</td>
<td>3 hrs.</td>
<td>C18 column – H₂O/ACN</td>
</tr>
<tr>
<td>5</td>
<td>1/31/08</td>
<td>2 eqv / 0.59 M</td>
<td>1 eqv / 0.059 M</td>
<td>5 hrs.</td>
<td>C18 column – H₂O/ACN</td>
</tr>
<tr>
<td>6</td>
<td>2/27/08</td>
<td>2 eqv / 0.57 M</td>
<td>1 eqv / 0.033 M</td>
<td>4 hrs.</td>
<td>none</td>
</tr>
</tbody>
</table>

The last reaction (#6) is yet to be purified. Reaction number 4 also featured as Figure 6 resulted in the best product formation. The purpose of adding
triethylamine was not to adjust pH but rather to help dissolve the 1,4,7,10-tetraazacyclododecane starting product. However the solution remained heterogenous. In reaction 5 the 1,4,7,10-tetraazacyclododecane was from a separate purchase and completely dissolved in the chloroform solvent. The liquid chromatography UV spectra (220 nm) of both reaction 4 and 5 seem similar indicating triethylamine and pH did not significantly effect reaction outcome.

The analogous synthesis of 1,4,7,10-tetraazacyclododecane-1-acetic acid-1,1-dimethylethyl ester was attempted according to prior procedure.\textsuperscript{111}The synthesis follows the same reverse addition parameters as the synthesis of 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester. The \textsuperscript{1}H NMR for 1,4,7,10-tetraazacyclododecane-1-acetic acid-1,1-dimethylethyl ester was consistent with literature values. However, t-butyl functional groups do not offer distinct fluorescence absorber as do benzyl groups during liquid chromatography monitored by UV, making the purification of 1,4,7,10-tetraazacyclododecane-1-acetic acid-1,1-dimethylethyl ester less favorable to that of its benzylester counterpart. Thus for the ultimate purpose of synthesizing DOTA(tri-protected)-OH, the initial synthesis of cyclen carboxymethyl-OBzl is a better alternative.

The solution phase synthesis of the dipeptide Ser-Pro motif was conducted twice according to the scheme illustrated in Figure 3. The Fmoc-serine(tBu)-OH was activated using two coupling agents, DIC and HOBt. The two reactions differed in the order in which the coupling reagents were added. For the first reaction HOBt was added to the Fmoc-serine(tBu)-OH followed by
DIC. The reverse order was followed for the second reaction. In both reactions the addition of the two coupling reagents was followed by addition of the base, DIEA, which turned the solutions into an orange color. In the first reaction precipitate formed immediately and was filtered off after 2.5 hours of stirring under nitrogen atmosphere at room temperature. In the second reaction the solution remained homogeneous and no precipitate formed immediately. Only after two days of unperturbed sitting did long needle shaped crystals form. In both the first and second reactions the filterate/mother liquor was combined with a basic solution of H-Pro-OMe and allowed to react for 44 and 6 hours respectively. Combining the activated serine was with the proline methyl ester yielded a mixture of products that were separated using reverse phase flash chromatography with H$_2$O and ACN as mobile phases. Subsequent identification via $^1$H NMR spectra for these products proved inconclusive.

**Chromatography**

The most challenging aspect of the current thesis research has involved the separation and purification of desired products. Chromatography in the form of thin-layer chromatography (TLC), liquid chromatography via manual column (LC), flash chromatography, and high performance liquid chromatography (HPLC) have played a major role in identifying compounds of interest and separating them from other side products.

Initially normal phase liquid chromatography was attempted for the separation of the following three compounds of interest: 1,4,7,10-
Tetraazacyclododecane-1-carboxymethyl-benzylester, DOTA-(tBu)$_3$-D-Phe-OMe, and DOTA-(tBu)$_3$-Gly-OMe. The mobile phases included mixtures of CHCl$_3$/EtOH/NH$_3$, CH$_2$Cl$_2$/MeOH, and Hexane/EtOAc predetermined by TLC separation. The stationary phases of both the column and TLC plate were either silica or alumina. Column chromatography using manual column techniques and flash chromatography were both explored. The following table summarizes the various attempts at normal phase liquid chromatography (LC) separation:

<table>
<thead>
<tr>
<th>Column #</th>
<th>Date</th>
<th>Product of Interest</th>
<th>Stationary Phase</th>
<th>Mobile Phase</th>
<th>Observed Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3/06/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica - Flash</td>
<td>CHCl$_3$/EtOH/NH$_3$ ratio = 16:9:4</td>
<td>2 peaks @ 254 nm</td>
</tr>
<tr>
<td>2</td>
<td>3/08/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica - Flash</td>
<td>CHCl$_3$/EtOH/NH$_3$ ratio = 4:5:2</td>
<td>1 peak @ 254 nm</td>
</tr>
<tr>
<td>3</td>
<td>3/08/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica - Flash</td>
<td>CHCl$_3$/EtOH/NH$_3$ ratio = 4:5:2</td>
<td>1 peak @ 254 nm</td>
</tr>
<tr>
<td>4</td>
<td>3/29/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica - Manual</td>
<td>CHCl$_3$/EtOH/NH$_3$ ratio = 4:5:2</td>
<td>3 peaks @ 254 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td># / vol of fractions: 35 / 1.5 mL</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3/30/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica - Manual</td>
<td>CHCl$_3$/EtOH/NH$_3$ ratio = 4:5:2</td>
<td>4 peaks @ 254 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td># / vol of fractions: 60 / 0.75 mL</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6/20/07</td>
<td>DOTA-(tBu)$_3$-Gly-OMe</td>
<td>Silica - Flash</td>
<td>CH$_2$Cl$_2$/MeOH ratio = 15:1</td>
<td>1 peak @ 254 nm</td>
</tr>
<tr>
<td>7</td>
<td>6/29/07</td>
<td>DOTA-(tBu)$_3$-Gly-OMe</td>
<td>Silica - Flash</td>
<td>CH$_2$Cl$_2$/MeOH ratio = 15:1</td>
<td>1 peak @ 220 nm</td>
</tr>
<tr>
<td>8</td>
<td>7/24/07</td>
<td>DOTA-(tBu)$_3$-D-Phe-OMe</td>
<td>Alumina – Manual</td>
<td>Hexane:EtOAc ratio = 2:9</td>
<td>1 peak @ 254 nm</td>
</tr>
<tr>
<td>9</td>
<td>9/18/07</td>
<td>Cyclen carboxymethyl-OBzl</td>
<td>Silica – Flash</td>
<td>Hexane:EtOAc ratio = 1:1</td>
<td>3 peaks @ 254 nm</td>
</tr>
</tbody>
</table>

The use of C18 reverse phase chromatography via flash chromatography and HPLC significantly improved separating resolution for desired compounds. The solvents A and B for all reverse phase chromatography corresponded to H$_2$O and ACN respectively. A mobile phase modifier of 0.1% triflouroacetic acid was added to both solvents. Eluting methods included time-dependent
concentration gradients as well as isocratic methods. Method development was based off of a prior established procedure with additional modification from trial and error.\textsuperscript{108} The C18 reverse phase chromatography purification attempts are listed below:

<table>
<thead>
<tr>
<th>Column #</th>
<th>Date</th>
<th>Product of Interest</th>
<th>Instrument / Flow rate</th>
<th>Method A = H\textsubscript{2}O B = ACN</th>
<th>Observed Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7/05/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-Gly-OMe</td>
<td>HPLC / 0.5 mL/min</td>
<td>0 - 18% B over 30 min</td>
<td>2 peaks @ 230 nm</td>
</tr>
<tr>
<td>2</td>
<td>9/04/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>Flash / 12 mL/min</td>
<td>0% B over 4 min, 100% B over 3 min, 100 - 0% B over 2 min</td>
<td>2 peak @ 254 nm one broad, one sharp</td>
</tr>
<tr>
<td>3</td>
<td>9/05/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>Flash / 12 mL/min</td>
<td>0% B over 6 min, 100% B over 4 min, 100 - 0% B over 4 min, 0% B over 1 min</td>
<td>2 peak @ 254 nm one broad, one sharp</td>
</tr>
<tr>
<td>4</td>
<td>9/15/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>Flash / 12 mL/min</td>
<td>0% B over 6 min, 100% B over 6 min, 100 - 0% B over 2 min, 0% B over 1 min</td>
<td>2 peak @ 254 nm 2 sharp*</td>
</tr>
<tr>
<td>5</td>
<td>9/27/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>Flash / 12 mL/min</td>
<td>0% B over 6 min, 100% B over 9 min, 100 - 0% B over 2 min, 0% B over 1 min</td>
<td>1 peak @ 224 nm 1 broad</td>
</tr>
<tr>
<td>6</td>
<td>11/12/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>HPLC 0.5 mL/min</td>
<td>0 - 60% B over 40 min, 60 - 100% B over 20 min, 100% B over 20 min, 100 - 0% B over 20 min, 0% B over 10 min</td>
<td>3 peaks @ 254 nm 1 sharp, 2 broad</td>
</tr>
<tr>
<td>7</td>
<td>11/13/07</td>
<td>DOTA-(tBu)\textsuperscript{3}-D-Phe-OMe</td>
<td>Flash 5 mL/min</td>
<td>0 - 60% B over 40 min, 60 - 100% B over 20 min, 100% B over 20 min, 100 - 0% B over 20 min, 0% B over 10 min</td>
<td>2 peaks @ 212 nm 2 broad</td>
</tr>
<tr>
<td>8</td>
<td>1/03/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>HPLC / 0.25 mL/min</td>
<td>0 - 60% B over 40 min, 60 - 100% B over 20 min, 100% B over 20 min, 100 - 0% B over 20 min, 0% B over 10 min</td>
<td>4 peak @ 254 nm baseline resolution</td>
</tr>
<tr>
<td>9</td>
<td>1/04/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 60% B over 40 min, 60 - 100% B over 20 min, 100% B over 20 min, 100 - 0% B over 20 min, 0% B over 10 min</td>
<td>4 peaks @ 254 nm 2 peaks unresolved</td>
</tr>
<tr>
<td>10</td>
<td>1/07/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 10 mL/min</td>
<td>0 - 60% B over 40 min, 60 - 70% B over 10 min, 100% B over 15 min, 0% B over 15 min</td>
<td>3 peaks @ 220 nm 1 peak w/ wide shoulder</td>
</tr>
<tr>
<td>11</td>
<td>1/08/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 10% B over 2 min, 10% B over 28 min, 10 - 100% B over 20 min, 100% B over 15 min, 0% B over 15 min</td>
<td>2 peaks @ 220 nm 2 wide</td>
</tr>
<tr>
<td>Column #</td>
<td>Date</td>
<td>Product of Interest</td>
<td>Instrument / Flow rate</td>
<td>Method $A = H_2O$, $B = ACN$</td>
<td>Observed Separation</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>--------------------------</td>
<td>------------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>12</td>
<td>1/08/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 25% $B$ over 11 min, 25% $B$ over 28 min, 25 - 100% $B$ over 7 min, 100% $B$ over 8 min, 0% $B$ over 10 min</td>
<td>5 peaks @ 220 nm 4 unresolved</td>
</tr>
<tr>
<td>13</td>
<td>1/09/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 20% $B$ over 15 min, 20% $B$ over 13 min, 20 - 100% $B$ over 2 min, 100% $B$ over 10 min, 0% $B$ over 10 min</td>
<td>5 peaks @ 220 nm 5 wide – almost resolved</td>
</tr>
<tr>
<td>14</td>
<td>1/30/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 10% $B$ over 10 min, 10% $B$ over 16 min, 10 - 62% $B$ over 1 min, 62 - 65% $B$ over 8 min, 100% $B$ over 4 min, 0% $B$ over 10 min</td>
<td>3 peaks @ 220 nm</td>
</tr>
<tr>
<td>15</td>
<td>1/31/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 15% $B$ over 12 min, 15% $B$ over 30 min, 100% $B$ over 14 min, 0% $B$ over 10 min</td>
<td>6 peaks @ 220 nm 2 unresolved</td>
</tr>
<tr>
<td>16</td>
<td>2/15/08</td>
<td>Fmoc-Ser-(tBu)-Pro-OMe</td>
<td>Flash / 5 mL</td>
<td>0 - 60% $B$ over 40 min, 60 - 100% $B$ over 20 min, 100% $B$ over 20 min, 100 - 0% $B$ over 20 min, 0% $B$ over 10 min</td>
<td>4 peaks @ 254 nm 3 unresolved</td>
</tr>
<tr>
<td>17</td>
<td>2/15/08</td>
<td>Fmoc-Ser-(tBu)-Pro-OMe</td>
<td>Flash / 5 mL</td>
<td>0 - 60% $B$ over 40 min, 60% $B$ over 20 min, 100% $B$ over 20 min, 100 - 0% $B$ over 20 min, 0% $B$ over 20 min</td>
<td>4 peaks @ 254 nm 2 unresolved</td>
</tr>
<tr>
<td>18</td>
<td>2/20/08</td>
<td>Fmoc-Ser-(tBu)-Pro-OMe</td>
<td>Flash / 5 mL</td>
<td>0 - 60% $B$ over 40 min, 60% $B$ over 20 min, 100% $B$ over 20 min, 100 - 0% $B$ over 20 min, 0% $B$ over 20 min</td>
<td>3 peaks @ 220 nm fully resolved</td>
</tr>
<tr>
<td>19</td>
<td>2/26/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 25% $B$ over 12 min, 25% $B$ over 28 min, 25 - 100% $B$ over 8 min, 100% $B$ over 10 min, 0% $B$ over 12 min</td>
<td>4 peaks @ 225 nm 3 unresolved</td>
</tr>
<tr>
<td>20</td>
<td>2/27/08</td>
<td>Cyclen carboxymethyl -OBzl</td>
<td>Flash / 5 mL/min</td>
<td>0 - 25% $B$ over 12 min, 25% $B$ over 28 min, 25 - 100% $B$ over 8 min, 100% $B$ over 10 min, 0% $B$ over 12 min</td>
<td>4 peaks @ 220 nm 2 unresolved</td>
</tr>
</tbody>
</table>

*new C18 column used.

The tuning of solvent systems and methods has been an ongoing process. The above listed chromatography trials have yielded mixed results. While further method development is necessary, method translation from the HPLC to flash chromatography are needed as well. Columns 6 and 7 provide an example of this. The baseline resolution using the analytical HPLC in column 6 suggested that similar resolution would be present in the quantitative flash chromatography.
of column 7. This was however not the case and subsequent $^1$H NMR of the wide peak collected from column 7 was inconclusive. Peak resolution, while an important parameter in separation chromatography seems less important than peak shoulder width. This was evident in the results of columns 9-13 where isocratic methods yielded better peak resolutions but overlapping wide shoulders compared to gradient methods with less resolved peaks with smaller shoulder widths.

**Conclusions**

The current thesis research should be considered starting points rather than conclusions. The synthesis of DOTA-(tBu)$_3$-D-Phe-OMe provides a framework for the ideal reaction conditions in coupling the DOTA-(tBu)$_3$-OH and Ser-Pro dipeptide. Synthesizing 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester provides an essential step towards producing gram quantities for the DOTA-based Gd$^{3+}$ complexes needed in future *in vitro* and *in vivo* tests. The solution phase dipeptide synthesis is a pivotal piece of the thesis research and the method was not extensively explored. Given the numerous means of peptide coupling, alternative reaction conditions should be further investigated.

While the science of activatable contrast agents will undoubtedly expand, recent concerns linking gadolinium and nephrogenic systemic fibrosis (NFS) provide a cautionary reminder of balancing clinical utility and patient safety. Once synthesized, the activatable contrast agent described in the current thesis
research will need to undergo rigorous testing both *in vitro* and *in vivo*. The component of intercellular targeting further adds to many environments in which the gadolinium(III) complex will have to remain stable.

The deregulation of Pin1, a peptidyl-prolyl *cis/trans* isomerase, is a proven biomarker in cancer pathogenesis. Neurological taupathies are also characterized by Pin1 deregulation though diagnostic and prognostic correlations have not been established. While Pin1 targeted therapies may evolve in the future, the current knowledge on Pin1 activity and specificity make it an ideal candidate for diagnostic and prognostic exploitation.

The future of *in vivo* molecular imaging holds great promise as more indicative biomarkers are discovered and ‘smarter’ molecular probes are synthesized. The current thesis project presents the initial synthetic chemistry involved in building a molecular probe activated by Pin1 to report on the activity of cancers and formation of taupathies. We hope that this work adds to the efforts of realizing the large scope of *in vivo* molecular imaging and provides powerful tools to fight disease.
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