The Protein Arginine Deiminases:

Inhibitors and Characteristics

By

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Abstract

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Protein arginine deiminases (PADs) are Ca$^{2+}$ dependent enzymes involved in the post-translational modification of proteins through citrullination. Overexpression of PAD2 and PAD4 has been observed in a number of neurodegenerative diseases. In this thesis, two broad investigations were undertaken to look at the expression patterns of endogenous PADs and how they provide means to the development of disease modifying treatments. First, a BODIPY-based fluorescent probe was designed to assess the expression levels in vitro and in vivo of endogenous PAD. Secondly, medicinal chemistry approaches were adopted to design a library of novel non-covalent compounds to establish a structure activity relationship on PADs and identify a potential hit. Results indicated that a BODIPY-based biomarker was a feasible approach to monitor PAD enzymes in vivo. Through structure-activity relationship investigations, it was established that small heterocycles on an amino acid side chain were contributing to the inhibitory activities towards PADs.

Declaration of Work: All synthetic experiments, synthesis of library of compounds, and confocal microscopy studies were performed by Elizabeth J. Curiel Tejeda. Biological assays and evaluations were performed by Ms. Ewa Wasilewski.
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>BAA</td>
<td>Benzoyl arginine amide</td>
</tr>
<tr>
<td>BCO-BODIPY</td>
<td>$N$-benzoyl-$\left(N^5\right)$-(2-chloro-1-iminoethyl)-L-ornithine analog of BODIPY</td>
</tr>
<tr>
<td>BODIPY</td>
<td>4, 4-difluoro-4-borata-3a-azonia-4a-aza-s-indacene</td>
</tr>
<tr>
<td>Cit-MBP</td>
<td>Citrullinated myelin basic protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMT</td>
<td>Disease modifying treatment</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>PAD</td>
<td>Protein arginine deiminase</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>THI</td>
<td>Tetrahedral intermediate</td>
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CHAPTER 1: INTRODUCTION

1.1 Citrullination and Protein Arginine Deiminases

Post-translational modifications (PTMs) are biological events in which modifications to proteogenic amino acids lead to functionally and structurally diverse proteins. From the 250 PTMs known to date, citrullination has gained special attention in neurodegenerative and autoimmune diseases.\(^1\) Citrullination consists of the deimination of peptidyl arginine residues into peptidyl citrulline (Scheme 1), and is known to play an essential role in physiological processes such as skin keratinization, insulation of neuronal axons, gene regulation, and maintenance of plasticity of the central nervous system (CNS).\(^1,2\) Some biochemical properties of this modification are a loss of a 1 Da in mass and overall loss in charge, as the positively charged arginine becomes neutral in citrulline. In turn, this charge change leads to reduced ionic and hydrogen bonding capabilities and changes in protein isoelectric point, leading to disrupted or gained interactions with other proteins and changes in biological pathways.\(^3\)

![Scheme 1. Citrullination of peptidyl arginine by PAD enzymes](image)

Citrullination is a process mainly regulated by Ca\(^{2+}\) dependent amidinotransferase family of enzymes known as protein arginine deiminases (PAD).\(^3\) To date, there are five related isoforms identified in humans, PAD 1-4 and PAD6 which have highly conserved primary structure but
their expression patterns are tissue specific.\(^4\) PAD1 is expressed mainly in the epidermis and uterus and under physiological conditions, is known to citrullinate keratins and keratin-associated protein during terminal differentiation of keratinocytes.\(^5\) PAD2 is the most ubiquitous of these isozymes as it is expressed in multiple organs such as skeletal muscle, brain, spleen, and secretory glands as well as in some macrophages. PAD2 expression is centralized mostly in the CNS with highest expression observed in the hypothalamus and grey matter.\(^6,4\)

PAD3 is expressed in the skin and is involved in citrullination of trichohyalin, a major structural protein of the inner root sheath cells of hair follicles, and mediates aggregation of keratin filaments.\(^7\) PAD4 is the most studied of the isozymes and is mainly expressed in white blood cells. It is also the only isozyme that contains a nuclear localization signal and is known to be involved in histone citrullination and neutrophil extracellular traps (NETs) formation, which is a common mechanism of the innate immune response against foreign bacteria and fungi.\(^8\) Finally, PAD6 is mainly expressed in female reproductive systems, developing embryos, and eggs, and is the least intensively studied isozyme.\(^9\)

PADs are regulated in the body by calcium, whose concentration under normal physiological conditions, range from $10^{-8}$-$10^{-6}$ M, suggesting that these enzymes are only activated during events that lead to high calcium concentrations and that citrullination takes place under such as terminal skin differentiation, immune response, apoptosis, or gene regulation.\(^10,11\)

**1.2 PAD Structure and Biochemistry of PADs**

Extensive structural biology studies have been performed for PAD4 due to its high relevance and involvement in an array of human diseases.\(^12\) PAD4 and generally all PADs exist as dimers in solution. Their overall structure is divided into an N-terminal domain composed mainly of
two immunoglobulin-like subdomains which regulate PAD activity and are also involved in PAD dimerization during catalysis, and a C-terminal domain, which folds into a $\alpha/\beta$ propeller motif and is the location of the catalytic cleft (Fig. 1).$^{12,13}$ The enzyme is also known to bind five calcium ions; two in the catalytic domain and three in the immunoglobulin-like subdomains, and serve for structural purposes.$^{12,13}$

The catalytic active site is composed of a cysteine residue, which is the proposed catalytic nucleophile; a histidine residue, which serves as a general acid and general base, and two aspartic acid residues which help stabilize the tetrahedral intermediates and enzyme bound complexes (Fig. 2)\textsuperscript{13,15}.

**Figure 2.** Key active site residues of PAD enzymes (*humanPAD4* numbering). (Computational design and image obtained from Dr. Lianhu Wei, Kotra group)

Due to the suitable variability in PADs structural sequences, there have been two proposed mechanisms through which PAD enzymes are activated for conversion of peptidyl-arginine into peptidyl-citrulline. The variability comes from enzymatic and structural biology studies against PAD2 and PAD4 which have proposed different activation of the cysteine residue involved in the reaction initiation. Dreyton *et al.*\textsuperscript{14} have suggested that PAD2 uses a substrate-assisted mechanism of catalysis in which the positively charged residue of the guanidinium moiety of the peptidyl-arginine depresses the $pK_a$ of the nucleophilic cysteine and promotes catalysis; while Knuckley *et al.*\textsuperscript{15} suggested that PADs 1, 3, and 4, catalyze citrullination through a reverse
protonation mechanism in which the Cys residue exists as a thiolate in the active form of the enzyme. Upon proper cysteine activation, the mechanism of citrullination proceeds with a nucleophilic attack at the guanidinium carbon of peptidylarginine (Scheme 2, 1) forming a tetrahedral intermediate (THI) stabilized by H-bonding with the two Asp residues (2). Subsequently, the THI breaks down releasing ammonia and the S-alkylthiouonium intermediate (3). A second nucleophilic attack by a water molecule activated by His binds the enzyme-bound intermediate, leading to a second THI adduct (4) which further breaks down and releases the final peptidylcitrulline (5) from the enzyme.

Scheme 2. Proposed Mechanism of citrullination by PAD enzymes.
1.3 Citrullination and PADs in Disease Pathogenesis

The deregulation of this posttranslational modification has been associated with neurodegenerative and immune pathological conditions such as rheumatoid arthritis, Alzheimer's disease, and multiple sclerosis.

1.3.1 Rheumatoid Arthritis (RA)

RA is a chronic autoimmune disease characterized by the inflammation of the peripheral synovial joints which leads to bone erosion and joint destruction, causing pain, disability, and reduced life expectancy. RA pathology is linked to citrullination due to the presence of anti-citrulline peptide antibodies (ACPA), which arise from an immune response to citrullinated proteins in the intracellular and extracellular inflamed synovium during cell damage or apoptosis. Citrullination is believed to be carried out by PAD2 and PAD4, which are expressed in a variety of white blood cells in the joint region near the synovium. Due to the high oxidative stress and disintegration of the synovial cells, these isozymes can infiltrate the extracellular space and become active in calcium rich environments where they can further citrullinate other joint proteins, such as fibrin and collagen, and serve as antigens to ACPA. In combination, both citrullination and subsequent development of these anti-citrulline auto-antibodies trigger an immune response which contributes to the overall chronic inflammation of the joints characteristic of RA.

1.3.2 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative progressive disease characterized by reduction of myelin sheath thickness or the loss of myelin on the axons of the neurons in the CNS, adversely influencing the electrical conduction and communication between nerve cells leading to a progressive loss of motor functions, eventual paralysis and
The main link between MS pathology in both human and MS animal models, and citrullination is the observed biochemical and structural changes on myelin basic protein.\textsuperscript{19} Myelin basic protein (MBP) is a single protein consisting of 170 amino acids and is a key structural protein required for insulation of axons of nerve cells for cell-cell signalling.\textsuperscript{21-22} MBP is a positively charged protein that is able to interact with phosphatidyl serine residues of myelin and allow for proper insulation of axons for electrical conduction.\textsuperscript{22} MBPs contain 18 arginine residues, from which six are normally citrullinated (Arg25, Arg31, Arg122, Arg130, Arg159, and Arg169).\textsuperscript{19-21} Levels of citrullination were increased in MS patients (30\% cit-MBP) when compared to normal individuals; 60\% cit-MBP in chronic MS while in fulminating MS (Malburg’s disease), cit-MBP accounted for 80-90\% of myelin sheath, further demonstrating that hypercitrullination is a potential key factor in MS pathology.\textsuperscript{23, 24}

Hypercitrullination in MS has been linked with the overexpression of both PAD2 and PAD4 in CNS. Moscarello\textit{et al.} observed that PAD2 expression levels were upregulated followed by an increase in cit-MBP in DM20 mice models, further suggesting the role of PAD2 in myelin degeneration, and establishing a precursor-product-type of relationship.\textsuperscript{19} As for PAD4, its direct role in MS pathogenesis is still unclear, albeit of its observed overexpression in MS animal models. Overall, it has been suggested that both overexpression of PAD2 and/or PAD4, leads to hypercitrullination of MBP, altering its tertiary structure in such a way that nerve cell communications are altered where an immune response towards myelin is induced.\textsuperscript{12}

**1.3.3 Alzheimer's Disease (AD)**

The role of citrullination in AD is not fully understood, as research on this area is fairly recent with respect to PADs. Ishigami\textit{et al.} observed that PAD2 levels were at least three-fold higher in AD hippocampus in comparison to normal brain, and in turn, observed elevated levels
of citrullinated proteins in AD patients in comparison to normal brains, specifically vimentin and glial fibrillary acidic protein (GFAP).\textsuperscript{16} These results, even though preliminary, showed remarkable findings suggesting that abnormal accumulation of citrullinated proteins and abnormal expression of PAD2 could be linked to the onset progression of AD, showing the potential role of these isozymes and citrullinated proteins as tools for identifying such disease.

\textbf{1.4. Current Inhibitors for Protein Arginine Deiminases}

Due to their potential role in disease pathogenesis, the development of inhibitors towards PADs for therapeutic use has become of high importance. In the past decade, a series of known drugs in the clinic have shown reversible inhibitory properties towards PAD enzymes such as taxol, streptomycin, and several tetracycline derivatives.\textsuperscript{25,26} However, the above compounds have shown very weak inhibitory activity and poor selectivity. Most of the designed inhibitors described up to date are a series of covalent modifiers which contain a terminal haloacetamidine warhead that mimics the terminal guanidinium moiety of known PAD substrate benzoyl arginine amide (BAA).\textsuperscript{27,28} The haloacetamidine warhead is known to inactivate PAD via the alkylation of the cysteine residue that is implicated in catalysis (Scheme 3).\textsuperscript{12,27} Two mechanisms through which these haloacetamidines inactivate PAD have been suggested. First, is a direct displacement of the halide by the thiol functionality (Scheme 3, A) or through the formation of a sulfonium ring, first by a nucleophilic attack on the iminium carbon by the thiolate, followed by the displacement of the halide. Then, sulfonium ring opening by collapse of THI leads to enzyme bound inhibitor (Scheme 3, B).\textsuperscript{12} Extensive kinetic studies by Causey \textit{et al.} suggested inactivation proceeds through the latter mechanism.\textsuperscript{29} Pharmacological data for these inhibitors show excellent bioavailability towards PAD enzymes and have been observed as good modulators of NET trap formation, transcriptional regulation in cancer, and remyelination...
in MS animal models. Even though these compounds have shown interesting pharmacological activity, they modify the PAD active site irreversibly and are known to have poor selectivity and unspecific reactivity.


In 2013, Wei et al. took a novel approach to synthesize novel non-covalent inhibitors by exploiting cationic, anionic, and hydrophobic binding interactions of the active site pocket by in silico design. From these library of inhibitors, hydantoin derivative 3 (Fig. 3, A) showed selective interactions with the key active site residues, specifically with the aspartic acid and arginine residues at the floor of the active site pocket, which helped stabilize the binding interactions and lead to higher potency (Fig. 3, B). In addition, efficacy and toxicity in vivo studies in MOG EAE mouse model showed improvement in clinical scores as well as a reduction in CD3 +ve cells in the treated brain samples in comparison to controls, showing the potential of non-covalent inhibitors as new avenues for disease modifying treatments towards PADs.
Hypothesis and Thesis Rationale

Building on the above discoveries, it is of interest to explore the use of non-covalent inhibitors targeting PAD enzymes to further improve their binding affinity and selectivity towards isozymes. Deriving from this interest, a rational hypothesis was developed forming the basis for this research thesis. Thus, it has been hypothesized that understanding PAD expression in disease pathogenesis and modulation of amino acid side chain functional groups customized to PAD enzymes, will generate potent inhibitors.

For this thesis, two projects were designed to answer the main points of the proposed hypothesis. First, a diagnostic approach was undertaken in which a covalent fluorescent biomarker was designed in order to assess PAD expression levels in vitro and in vivo, and

understand the correlation of PAD expression and hypercitrullination patterns in disease pathogenesis. For the second part of this thesis, a medicinal chemistry study where a library of novel non-covalent inhibitors was created focusing on side chain functional group variability to conduct a structure-activity relationship (SAR) analysis towards PAD isozymes, and instigate on specificity and selectivity for identification of potent inhibitors.

1.6 Biomarkers for Protein Arginine Deiminases (PADs) Using BODIPY Dyes

The Kotra group previously investigated the ligand preferences and the depth of the PADs’ active site by designing a series of novel probes carrying the 2-chloramidine (2CA) moiety tethered to an α-amino acid via a carbon linker (Fig. 4).\(^3^0\) It was determined that a 2CA α-amino acid with a three methylene tether (Fig. 4, n= 3), had higher potency and faster rates of inactivation in comparison to 2CA alone when tested against PAD1, 2, and 4\(^4\) in vitro. Moreover, this compound showed 17 times more selectivity and inactivation rates towards PAD4 specifically.\(^3^1\)

![Figure 4](image)

**Figure 4.** Selected haloamidine probes for PAD active site exploration.

These results sparked the interest of exploring the effects of such ligand \textit{in vivo} to assess its potential use as a biomarker to understand expression levels of PAD enzymes in healthy \textit{vs.}
diseased animal models. This was set to be accomplished by fluorescent imaging using 4,4-difluoro-4-borata-3a-azonia-4a-aza-s-indacene (BODIPY) dyes.

BODIPY dyes were first discovered in 1968 by Treibs and Kreuzer and have gained exponential attention in the past 20 years due to their multidisciplinary use as fluorophores, biological labelling agents, chemosensors, and photodynamic therapeutics. This is due to their substantial chemical photostability, high absorption coefficients and fluorescent quantum yields. In addition, BODIPY dyes have strong UV and emission profiles that can be modified from the green to near-IR ranges by ease of chemical manipulation.

The BODIPY core is described as a boradiazaindacene ring as it is analogous to the tricyclic structure of s-indacene, and follows the numbering system of a typical porphyrinic system, with the 8th-position referred to the meso site (Fig. 5). The main core is composed of a dipyrromethene unit which is complexed to a boron difluoride unit that rigidifies the system and in turn, leads to restrained flexibility, and to said high fluorescent yields. The absorption and fluorescent properties are mainly due to the delocalization of the π-electrons in the central cyanine functionality and the introduction of suitable electron donating and electron withdrawing groups to the pyrrole fragments, leading to a variety of dyes with different spectral properties in a facile manner.

![Figure 5. BODIPY core numbering system](image)

In efforts to apply the use of these BODIPY dyes for identification of PAD enzymes in vivo, two fluorescent biomarkers were proposed. N-benzoyl-(N\(^5\))-(2-chloro-1-iminoethyl)-L-ornithine
analog-BODIPY probes (Fig. 6) were aimed to be synthesized, with the ultimate goal to elucidate the levels of expression of PAD enzymes *in vivo* in different disease animal models. The main purpose will be to further investigate the mechanistic role of these enzymes in disease pathogenesis and create a new avenue for the development of more specific and higher affinity small molecule disease modifying treatments (DMTs).

![Chemical structures](image)

**Figure 6.** Targeted PAD biomarkers; BCO-BODIPY (8) and BCO-distyryl-BODIPY (9).

### 1.7 Novel Non-Covalent Inhibitors against Protein Arginine Deiminases (PADs) as DMTs Targeting Multiple Sclerosis

As indicated above, understanding PAD enzymes expression and the medicinal chemistry approach to design novel non-covalent inhibitors with a variety of functional groups for high specificity, selectivity, and potency, were of interest in relationship to the stated hypothesis.

The basis for designing this library of inhibitors (Fig. 7) is to explore the effects of side chain functional groups on different isozymes. These functional groups were mainly selected from a series of commercially available functional groups and substitutions that can be appended to the core structure that have been published recently in a disclosure of invention by Kotra group. \(^{35}\)
Initially, L-homoserine was set as the backbone for this library as it sets the required length to fit into the active site pocket and accommodates the selected heterocycles at R₂, which replace the chloramidine moiety of covalent modifiers (Fig. 7).³¹ R₂ N-heterocycles were selected to fit within the distances of the key catalytic active residues to maximize anionic, cationic, and hydrophobic interactions.³⁶ N-terminal acyl chlorides and carboxylic acids and C-terminal amines (Fig. 7, R₁ and R₃, respectively), were selected to potentially exploit the hydrophobic, anionic, and cationic interactions of the anchoring peptide regions, and instigate on the effects of such groups on selectivity towards the different PAD isozymes.³⁶ The ultimate goal is to perform inhibition studies against PAD isozymes and conduct a structure-activity-relationship (SAR) study to assess activity of side chain substituents and how selectivity can be improved to design isozyme specific inhibitors.

Figure 7. Proposed library of non-covalent inhibitors towards PAD.
CHAPTER 2: EXPERIMENTAL SECTION

Chemistry

General. All reagents were purchased from Sigma-Aldrich and BACHEM chemicals unless otherwise noted. The compounds were purified by column chromatography using normal silica (60 Å, 70-230 mesh) and reverse-phase (C18) silica cartridges. $^1$H, $^{13}$C, and $^{19}$F NMR spectra were obtained in CDCl$_3$, CD$_3$OD and DMSO-$d_6$, and recorded in a Bruker DPX400, 400 MHz spectrophotometer. Chemical shifts $\delta$ are reported in ppm with trimethylsilane (TMS) as the internal standard. UV-Vis and fluorescence spectra were performed in dioxane and PBS buffer were noted using SpectraMax (Molecular Devices/GE Healthcare) plate reader. LC-MS and mass spectra (ESI) were recorded in a Waters 2545 binary gradient module system and Waters 3100 mass detector, respectively.

2.1 BCO-BODIPY Synthesis

(9[H]-Fluoren-9-yl)methyl-tert-butyl(5-(benzylamino)-5-oxopentane-1, 4-diyl) (S) dicarbamate (11). Fmoc-L-Orn-(Boc)-OH (10) (1.36 g, 2.99 mmol) was dissolved in 30 mL of anhydrous MeCN to which 1.23 g (2.0 eq, 5.97 mmol) of DCC was added and stirred for 20 min at rt. HOBt (457 mg, 2.99 mmol) was added to reaction and stirred for 15 min. Benzylamine (327 µL, 2.99 mmol) was added and reaction was monitored by TLC in 9:1 DCM/MeOH until 10 was consumed. Reaction mixture was filtered and the filtrate was washed with H$_2$O (150 mL), and dried in vacuo to afford 11 as a clear liquid (1.62 g, 94%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.95 (d, $J = 8$ Hz, 2H), 7.74 (d, $J = 8$ Hz, 2H), 7.55 (d, $J = 8$ Hz, 2H), 7.28-7.34 (m, 5H), 7.23 (d, 2H), 6.82 (t, 1H, NH), 4.29 (dd, $J = 4$ Hz, 2H), 4.23 (m, 1H), 4.03 (q, 1H), 2.92 (d, 2H), 1.42-1.96 (m, 4H), 1.37 (s, 9H).
Scheme 4. Synthesis of BCO-BODIPY. Reagents and reaction conditions: (a) BnNH₂ (2.0 eq), HOBt (1.0 eq), DCC (2.0 eq), MeCN, N₂ atm, rt. (b) 20% piperidine, DMF, 2.5 hr, rt; (c) 13 (1.0 eq), 2,4-dimethylpyrrole (2.25 eq), TFA (cat), DCM (anh), N₂ atm, rt, 2.5 hr; (d) DDQ (1.0 eq); (e) Et₃N (16 eq), BF₃·OEt₂ (14 eq), rt, 16 hr; (f) NH₂NH₂·H₂O (1.0 eq), 10% Pd/C, EtOH (abs), N₂ atm, 80 °C, 2 hr; (g) succinic anhydride (1.5 eq), MeCN (anh), N₂ atm, 90 °C, 12 hr; (g) 12 (1.2 eq), DCC (2.0 eq), HOBt (1.0 eq), MeCN, N₂ atm, 16 hr; (i) 30% TFA, DCM, 0 °C; (j) 2-ethyl-
chloroamidinoate (3.5 eq), Et₃N (5.0 eq), EtOH(abs), N₂ atm, 16 hr; (k) BF₃·OEt₂, rt, N₂ atm, 48 hr.

**tert-Butyl (S)-(4-amino-5-(benzylamino)-5-oxopentyl) carbamate (12).** Dicarbamate 11 (1.62 g, 2.98 mmol) was dissolved in 5 mL of 15% (v/v) piperidine in DMF and stirred at rt for 3 hr. Reaction solvent was filtered and filtrate was concentrated. Crude was dissolved in EtOAc (50 mL) and washed with brine (150 mL) and H₂O (150 mL). Organic layer was dried over MgSO₄ and purified by silica gel chromatography in 5-20% MeOH gradient in DCM to afford 12 as a white solid (0.780 g, 63%). ¹H NMR (400 MHz, CDCl₃) δ 8.40 (m, 1H, NH), 7.23-7.34 (m, 5H), 7.34-7.23 (d, J = 8 Hz, 2H), 6.82 (t, 1H, NH), 4.27 (dd, J = 4 Hz, 2H), 3.41 (t, 1H), 2.92 (d, 2H), 1.42-1.96 (m, 4H), 1.37 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 174.9, 156.4, 138.0, 128.5, 127.5, 127.3, 77.8, 53.9, 43.1, 39.5, 28.7, 26.7, 23.7. m.p.: 417-421 °C.

**5,5-Difluoro-1,3,7,9-tetramethyl-10-(4-nitrophenyl)-9a,10-dihydro-5[H]-5λ⁴,6λ⁴-dipyrrolo[1,2-c’:1’,2’-f][1,3,2]diazaborinine (14).** 4-Nitrobenzaldehyde 13 (1.0 g, 6.62 mmol) was dissolved in 20 mL of anhydrous DCM under N₂ atm to which 2,4-dimethylpyrrole (1.42 g, 2.25 eq, 14.89 mmol) was added and stirred vigorously. Catalytic amount of TFA was added to reaction and monitored by TLC in 1:1 EtOAc: hexanes until 12 was completely consumed. DDQ (5.06 g, 6.62 mmol) was added to reaction and stirred for 10 min. Then 4 mL of BF₃·OEt₂ (14 eq) and 4 mL of Et₃N (16 eq) were added quickly and stirred for 2 hr. Reaction solvent was evaporated and remaining crude was dissolved in EtOAc (150 mL) and washed with H₂O (300 mL) and brine (300 mL), and purified by silica gel chromatography in a 10-45% EtOAc gradient in hexanes to afford 14 as a dark-brown solid (2.83 g, 27%). ¹H NMR (400 MHz, CDCl₃) δ 8.13-8.15 (d, 2H, CH, J = 8 Hz), 7.54-7.56 (d, 2H, J = 8 Hz), 6.02 (s, 2H), 2.56 (s, 6H), 1.33 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 142.0, 129.8, 24.5, 122.0, 46.3, 14.8, 8.6. ¹⁹F NMR (376
MH, CDCl$_3$) δ -146.11-146.37 (dd, $J = 36$ Hz, 2F). UV/Vis: $\lambda_{\text{max}}$ 502 nm; $\lambda_{\text{em}}$ 522 nm. Mass (M + H$^+$) calcd 372.15, found 372.14.

4-(5,5-Difluoro-1,3,7,9-tetramethyl-9a,10-dihydro-5[H]-5$^\alpha$,6$^\alpha$-dipyrrolo[1,2-c:2',1'-ff][1,3,2]diazaborinin-10-yl)aniline (15). Compound 14 (238.20 mg, 1.03 mmol) was dissolved in 30 mL of EtOH to which (6.25 mL, 25.0 eq, 27.73 mmol) of hydrazine hydrate was added. 10% Pd/C (w/w) was dissolved in 1 mL of EtOH and added to the reaction mixture. Reaction was refluxed for 4 hr and reaction solvent was filtered over celite and evaporated. Crude was dissolved in 30 mL of EtOAc and extracted with H$_2$O (100 mL) and brine (100 mL), dried over MgSO$_4$, and purified by silica chromatography in a 15-45% EtOAc gradient in hexanes to afford 15 as an orange solid (0.6 g, 49%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.13-8.15 (d, $J = 8$ Hz, 2H), 6.74-6.76 (d, $J = 8$ Hz, 2H), 5.96 (s, 2H), 3.85 (s, broad, 2H, NH), 2.54 (s, 6H), 1.49 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 154.9, 147.2, 143.2, 142.7, 132.0, 128.9, 124.4, 120.9, 115.3, 14.6, 14.5. $^{19}$F NMR (376 MHz, CDCl$_3$) δ -146.16-146.43 (d, $J = 36$ Hz, 2F). UV/Vis: $\lambda_{\text{max}}$ 499 nm; $\lambda_{\text{em}}$ 540 nm. Mass (M + H$^+$) calcd 342.17, found 342.19.

4-((4-(5,5-Difluoro-1,3,7,9-tetramethyl-9a,10-dihydro-5[H]-5$^\alpha$,6$^\alpha$-dipyrrolo[1,2-c:2',1'-ff][1,3,2]diazaborinin-10-yl)phenyl)amino)-4-oxobutanoic acid (16). Aniline derivative 15 (0.6 g, 1.77 mmol) was dissolved in 70 mL of anhydrous MeCN to which 265.53 mg (1.5 eq, 2.65 mmol) of succinic anhydride was added. Reaction was refluxed at 90 °C for 8 hr. Reaction solvent was removed and resulting crude was dissolved in 50 mL of DCM and extracted with H$_2$O (150 mL) and brine (150 mL), and dried over MgSO$_4$. Crude was purified by reverse phase chromatography in a 30-75% MeCN gradient in H$_2$O. Fractions were evaporated and lyophilized to afford 16 as an orange powder (0.640 g, 52%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.72 (d, $J = 8$ Hz, 2H), 7.21 (d, $J = 8$ Hz, 2H), 5.98 (s, 2H), 2.75 (t, 2H), 2.69 (t, 2H), 2.54 (s, 2H), 2.35 (t, 2H), 2.27 (t, 2H), 2.08 (t, 2H), 1.98 (t, 2H), 1.80 (t, 2H), 1.74 (t, 2H), 1.64 (t, 2H), 1.54 (t, 2H), 1.46 (t, 2H), 1.34 (t, 2H), 1.23 (t, 3H), 1.19 (t, 3H), 1.09 (t, 3H), 1.07 (t, 3H), 1.05 (t, 3H), 1.04 (t, 3H), 1.03 (t, 3H), 0.99 (t, 3H), 0.97 (t, 3H), 0.95 (t, 3H), 0.93 (t, 3H), 0.92 (t, 3H), 0.91 (t, 3H), 0.90 (t, 3H), 0.89 (t, 3H), 0.88 (t, 3H), 0.87 (t, 3H), 0.86 (t, 3H), 0.85 (t, 3H), 0.84 (t, 3H), 0.83 (t, 3H), 0.82 (t, 3H), 0.81 (t, 3H), 0.80 (t, 3H), 0.79 (t, 3H), 0.78 (t, 3H), 0.77 (t, 3H), 0.76 (t, 3H), 0.75 (t, 3H), 0.74 (t, 3H), 0.73 (t, 3H), 0.72 (t, 3H), 0.71 (t, 3H), 0.70 (t, 3H), 0.69 (t, 3H), 0.68 (t, 3H), 0.67 (t, 3H), 0.66 (t, 3H), 0.65 (t, 3H), 0.64 (t, 3H), 0.63 (t, 3H), 0.62 (t, 3H), 0.61 (t, 3H), 0.60 (t, 3H), 0.59 (t, 3H), 0.58 (t, 3H), 0.57 (t, 3H), 0.56 (t, 3H), 0.55 (t, 3H), 0.54 (t, 3H), 0.53 (t, 3H), 0.52 (t, 3H), 0.51 (t, 3H), 0.50 (t, 3H), 0.49 (t, 3H), 0.48 (t, 3H), 0.47 (t, 3H), 0.46 (t, 3H), 0.45 (t, 3H), 0.44 (t, 3H), 0.43 (t, 3H), 0.42 (t, 3H), 0.41 (t, 3H), 0.40 (t, 3H), 0.39 (t, 3H), 0.38 (t, 3H), 0.37 (t, 3H), 0.36 (t, 3H), 0.35 (t, 3H), 0.34 (t, 3H), 0.33 (t, 3H), 0.32 (t, 3H), 0.31 (t, 3H), 0.30 (t, 3H), 0.29 (t, 3H), 0.28 (t, 3H), 0.27 (t, 3H), 0.26 (t, 3H), 0.25 (t, 3H), 0.24 (t, 3H), 0.23 (t, 3H), 0.22 (t, 3H), 0.21 (t, 3H), 0.20 (t, 3H), 0.19 (t, 3H), 0.18 (t, 3H), 0.17 (t, 3H), 0.16 (t, 3H), 0.15 (t, 3H), 0.14 (t, 3H), 0.13 (t, 3H), 0.12 (t, 3H), 0.11 (t, 3H), 0.10 (t, 3H), 0.09 (t, 3H), 0.08 (t, 3H), 0.07 (t, 3H), 0.06 (t, 3H), 0.05 (t, 3H), 0.04 (t, 3H), 0.03 (t, 3H), 0.02 (t, 3H), 0.01 (t, 3H), 0.00 (t, 3H).
tert-Butyl (S)-{(S)-5-(benzylamino)-4-((4-(4-(5,5-difluoro-1,3,7,9-tetramethyl-5[H]-4\(\lambda^4\),5\(\lambda^4\)-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl)amino)-4-oxopentan-2-yl)amino)-N(5)-benzoyl)tetrahydrofuran-2-carbamate (17). Oxobutanoic acid derivative 16 (280 mg, 637.43 µmol) was dissolved in 50 mL of anhydrous MeCN to which 263.04 mg (2.0 eq, 1.27 mmol) of DCC was added and stirred at rt for 20 min. HOBT (146.42 mg, 956.15 µmol) was added to reaction mixture and further stirred for 15 min. Amino intermediate 12 (245 mg, 764.92 µmol) was added to the reaction and stirred at rt for 12 hr. Reaction mixture was filtered and filtrate was concentrated. Resulting crude was dissolved in 50 mL of EtOAc and washed with H₂O (150 mL) and brine (150 mL), dried over MgSO₄, and concentrated in vacuo. Crude was purified by silica chromatography in 2-10% MeOH gradient in DCM to afford 17 as an orange solid (0.145 g, 79%). 

\(^1\)H NMR (400 MHz, CDCl₃) δ 7.68 (d, \(J = 8\) Hz, 2H), 7.39-7.41 (m, 5H), 7.10 (d, \(J = 8\) Hz, 2H), 5.94 (s, 2H), 4.65 (s broad, 1H), 4.40 (d, \(J = 4\) Hz, 2H), 3.04-3.17 (m, 2H), 2.78 (t, 2H), 2.67 (t, 2H), 2.54 (s, 6H), 1.59-1.86 (m, 4H), 1.36 (s, 6H), 1.34 (s, 9H). 

\(^{13}\)C NMR (100 MHz, CDCl₃) δ 174.9, 156.4, 155.2, 143.2, 138.0, 131.5, 129.9, 128.5, 128.4, 127.5, 127.3, 121.1, 119.8, 77.8, 49.2, 49.0, 48.7, 43.1, 39.5, 31.6, 29.5, 29.0, 28.7, 26.7, 23.7, 14.4. 

\(^{19}\)F NMR (376 MHz, CDCl₃) δ -146.13-146.39 (dd, \(J = 36\) Hz, 2F). UV/Vis: \(\lambda_{\text{max}}\) 502 nm; \(\lambda_{\text{em}}\) 522 nm. MS (+ESI): Mass (M + H⁺) calcd 743.56, found 743.38.

(S)-N\(^\lambda^4\)-(5-Amino-1-(benzylamino)-1-oxopentan-2-yl)-N\(^\lambda^4\)-(4-(5,5-difluoro-1,3,7,9-tetramethyl-5[H]-4\(\lambda^4\),5\(\lambda^4\)-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl) succinamide (18). Carbamate 17 (145 mg, 195.24 µmol) was dissolved in 3 mL of cold 25%
(v/v) of TFA in DCM and monitored by TLC 9:1 MeOH/DCM until 17 was consumed. Reaction solvent was evaporated and resulting crude was dissolved in 50 mL of CHCl₃ and washed with 5% NaHCO₃ (150 mL x 3) and brine (150 mL) and dried over MgSO₄. Mixture was carried to next step without further purification. 

\[ \text{H NMR (400 MHz, CDCl₃)} \delta 7.68 (d, J = 8 Hz, 2H), 7.39-7.41 (m, 5H), 7.10 (d, J = 8 Hz, 2H), 5.94 (s, 2H), 4.65 (s broad, 1H), 4.40 (d, J = 4 Hz, 2H), 3.04-3.17 (m, 2H), 2.78 (t, 2H), 2.67 (t, 2H), 2.54 (s, 6H), 1.59-1.86 (m, 4H), 1.36 (s, 6H). \]

\[ \text{F NMR (376 MHz, CDCl₃)} \delta -146.13-146.39 (dd, 2F, J = 36 Hz). \]

UV/Vis: \( \lambda_{\text{max}} \) 502 nm; \( \lambda_{\text{em}} \) 522 nm. Mass (M + H⁺) calcd 643.78, found 643.33.

\( (S)-N¹-(1-(Benzylamino)-5-(2-chloroacetimidamido)-1-oxopentan-2-yl)-N⁴-(4-(5,5-difluoro-1,3,7,9-tetramethyl-5[H]-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)phenyl)succinamide (8). \) Succinamide derivative 18 (320 mg, 498.02 µmol) was dissolved in 8 mL of absolute EtOH to which 1.04 mL (15 eq, 7.47 mmol) of Et₃N was added and stirred for 20 min at rt. Ethyl-2-chloroethanimidoate hydrochloride (275.45 mg, 3.5 eq, 1.74 mmol) was added and stirred for 18 hr at rt. Upon completion, reaction was quenched with H₂O and acidified to pH 2.0 and purified by preparative reverse phase chromatography by LC-MS in 30% MeCN in H₂O to afford 8 as an orange solid (0.085 g, 30%). 

\[ \text{H NMR (400 MHz, CDCl₃)} \delta 7.71 (d, J = 8 Hz, 2H), 7.35 (d, J = 8 Hz, 2H), 7.24-7.32 (m, 5H), 6.30 (s, 2H), 4.41 (d, J = 4 Hz, 2H), 4.36 (m, 1H), 4.32 (d, 2H), 3.37 (t, 2H), 2.59 (m, 2H), 2.54 (m, 2H), 2.42 (s, 6H), 1.7-1.86 (m, 4H), 1.70 (s, 6H). \]

\[ \text{F NMR (376 MHz, CDCl₃)} \delta -146.13-146.59 (dd, J = 36 Hz, 2F). \]

UV/Vis: \( \lambda_{\text{max}} \) (PBS): 502 nm; \( \lambda_{\text{em}} \) (PBS): 522 nm. Mass (M + H⁺) calcd 718.02, found 718.32.
**Quantum Yield Characterization of BCO-BODIPY.** The fluorescence quantum yield of short-wavelength emitting BCO-BODIPY was calculated using a standard solution of uranine (Sigma Aldrich) in 0.1 M NaOH (Φ = 0.92) from following equation 1:

\[
\Phi_x = \Phi_s \left( \frac{A_x}{A_s} \right) \left( \frac{F_x}{F_s} \right) \left( \frac{n_s}{n_x} \right)^2
\]

Equation 1

where the subscripts s and x represent the standard and tested compound, respectively.\(^{37,38}\) Furthermore, Φ is the fluorescence quantum yield, A is absorbance, F is the integrated fluorescence emission curve, and n is the refractive index of the solvents used.

### 2.1.2 Enzymatic and Biological Characterization of BCO-BODIPY

Enzymatic assays, in vitro binding assays, and in vivo pilot animal studies were performed by Ms. Ewa Wasilewski.

**Enzyme Activity Assays** and **Time-dependent binding assay of 11 against PAD4.** PAD activity assays were carried out using a modified procedure from the published report.\(^{36,39}\)

**In vivo pilot studies.** Healthy C/57BL6 female mice were injected with PBS, acyl BODIPY, or 2 μg/mL of BCO-BODIPY and sacrificed at three different time points (30 min, 60 min, and 120 min). Mice were perfused with saline and brain tissue was extracted and frozen. Brain tissues were cut at 6 μm thickness and fixed with paraffin. Frontal cerebral cortex was visualized in a LM700 confocal microscope for fluorescence detection.
2.2 Synthesis of Novel Non-Covalent Inhibitors for PADs

2.2.1 Synthesis of N-methyl piperazine substituted non-covalent inhibitors (Scheme 5)

![Scheme 5. Synthesis of N-methyl piperazine substituted non-covalent inhibitors](image)

Reagents and conditions: (a) 1, DBU (1.0 eq), TBDMSCl (1.5 eq), MeCN (anhyd), N₂ atm, rt, 16 hr; (b) Fmoc-Osu (1.1 eq), Et₃N (1.5 eq), 50:50 MeCN: H₂O, rt, 4 hr; (c) HATU (1.1 eq), DIPEA (3.0 eq), isopropylamine (10.0 eq), DMF (anhyd), N₂ atm, 0 °C → rt, 16 hr; (d) R₁COCl (1.0 eq), DIPEA (3.0 eq), DCM (anhyd), N₂ atm, 0 °C → rt, 2-5 hr; (e) TBAF (1.0 eq), THF (anhyd), N₂ atm, 0 °C - rt, 2 hr; (f) MsCl (1.0 eq), DIPEA (3.0 eq), DCM (anhyd), N₂ atm, 0 °C, 2 hr; (g) N-methyl piperazine (1.0 eq), DIPEA (1.5 eq), DMF (anhyd), N₂ atm, 0 °C → rt, 16 hr.

O-(tert-Butyldimethylsilyl)-L-Homoserine (20). L-homoserine (1 g, 8.4 mmol) and DBU (1.32 mL, 8.8 mmol) were dissolved in 20 mL of anhydrous MeCN and stirred at 0 °C.

Tertbutyldimethylsilyl chloride (1.32 g, 8.8 mmol) was dissolved in 20 mL of MeCN and added dropwise over 5 min. The reaction mixture was warmed to rt and stirred for 16 hr. A white precipitate formed and was washed with MeCN (200 mL), cold H₂O (200 mL) and Et₂O (200 mL), and dried under vacuum to afford 20 as a white powder (1.45 g, 78%). ¹H NMR (400 MHz, CD₃OD) δ 3.87 (t, J = 6 Hz, 2H), 3.71-3.68 (m, 1H), 2.21-1.93 (m, 2H), 0.93 (s, 9H), 0.12 (s, 6H). ¹³C NMR (100 MHz, CD₃OD) δ 61.9, 54.7, 34.4, 26.4, -5.4; m.p: 148-153 °C.
**N-((9[H]-Fluoren-9-yl) methoxy) carbonyl-O-(tert-butyldimethylsilyl)-L-homoserine (21).**

To a solution of 20 (1.45 g, 6.2 mmol) in 100 mL of 5% NaHCO₃ (w/v) in 50% MeCN/H₂O, Fmoc-O-succinimide (2.30 g, 6.8 mmol) was added. The reaction mixture was stirred at rt and monitored by TLC in 100% EtOAc until consumption of 20 was observed. Reaction solvent was removed *in vacuo* and the remaining suspension was acidified to pH 2.0 using 10% (w/v) aqueous citric acid solution. Aqueous layer was extracted in EtOAc (3 × 200 mL) and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 5-40% EtOAc gradient in hexanes to afford 21 as a viscous white semi-solid (2.41 g, 82%). ¹H NMR (400 MHz, CDCl₃) δ 7.57 (d, J = 7.6 Hz, 2H), 7.60 (t, J = 6 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.30 (t, J = 7.2 Hz, 2H), 6.15 (d, J = 7.2 Hz, 2H), 4.21 (t, J = 6.8 Hz, 2H), 3.82 (t, 2H), 2.18-2.05 (m, 2H), 0.91 (s, 9H), 0.09 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 156.4, 143.9, 141.4, 127.8, 127.2, 125.2, 120.1, 67.3, 60.7, 53.1, 47.3, 33.5, 25.9, 18.3, -5.5.

**(S)-2-Amino-4-((tert-Butyldimethylsilyl)oxy)-N-isopropylbutanamide (22).** Compound 21 (1.64 g, 3.6 mmol) was dissolved in 5 mL of anhydrous DMF to which DIPEA (1.88 mL, 10.8 mmol) and HATU (1.51 g, 3.6 mmol) were added and stirred at 4 °C. Isopropylamine (4.56 mL, 36.0 mmol) was added dropwise to the reaction mixture and further stirred at 4 °C. Reaction was monitored by TLC in 7:3 EtOAc: hexanes, until the complete deprotection of Fmoc was achieved. Reaction solvent was evaporated and crude was dissolved in 15 mL of DCM, washed with H₂O (200 mL), brine (200 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in 30-100% EtOAc gradient in hexanes to afford 22 as a yellow oil (0.39 g, 75%). ¹H NMR (400 MHz, CDCl₃) δ 6.68 (m, 1H, NH), 6.15 (m, 1H, NH), 4.10 (m, 1H), 4.03 (septet, 1H), 3.75 (m, 2H), 1.97 (m, 1H), 1.95 (m, 1H), 1.13 (d, J = 6 Hz, 6H), 0.91 (s, 9H),
0.075 (d, J = 4 Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.3, 59.5, 51.1, 40.6, 38.2, 30.7, 25.8, 22.2, 22.1, -5.4.

(S)-N-(4-((tert-Butyldimethylsilyl)oxy)-1-(isopropylamino)-1-oxobutan-2-yl)furan-2-carboxamide (23a). Compound 22 (0.36 g, 1.46 mmol) was dissolved in 5 mL of anhydrous DCM to which DIPEA (762 µL, 4.38 mmol) was added and stirred at 0°C. 2-furoyl carbonyl chloride (215 µL, 2.19 mmol) was added drop-wise to mixture and further stirred at 0°C for 3 hr. Reaction solvent was evaporated and resulting crude was dissolved in DCM (30 mL) and extracted with sat. NaHCO$_3$ (200 mL), H$_2$O (until pH was neutralized), and brine (200 mL), and dried over MgSO$_4$. Resulting crude was purified by silica chromatography in a 0-5% MeOH gradient in DCM to afford 23a as yellow viscous semi-solid (0.14 g, 49%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.77 (d, J = 8 Hz, 1H), 7.12 (d, J = 4 Hz, 1H), 6.70 (d, J = 8 Hz, 1H), 6.50 (s (broad), 1H, NH), 4.73 (m, 1H), 4.09 (septet, 1H), 3.92 (m, 1H), 3.81 (m, 1H), 2.16 (m, 1H), 2.06 (m, 1H), 1.12 (d, J = 4 Hz, 6H), 0.93 (s, 9H), 0.105 (d, J = 4 Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.0, 158.3, 147.7, 144.1, 114.6, 112.2, 61.0, 51.7, 41.6, 34.4, 26.0, 22.6, 18.4, -5.3.

(S)-N-(4-((tert-Butyldimethylsilyl)oxy)-1-(isopropylamino)-1-oxobutan-2-yl)morpholine-4-carboxamide (23b). Compound 22 (0.33 g, 1.20 mmol) was dissolved in 5 mL of anhydrous DCM to which DIPEA (1.05 mL, 6 mmol) were added and stirred at 0°C. Morpholine carbonyl chloride (210 µL, 1.8 mmol) was added drop-wise to the reaction at 0°C. The reaction was monitored and purified as per the procedure described for compound 23a. Target compound 23b was obtained as a white semi-solid (0.245 g, 53%). $^1$H NMR (400 MHz, CDCl$_3$) δ 6.71 (s (broad), 1H, NH), 6.02 (m, 1H, NH), 4.41 (q, 1H), 4.04 (dq, 1H), 3.85 (m, 1H), 3.75 (m, 1H), 3.67 (m, 4H), 3.37 (m, 4H), 1.99 (m, 1H), 1.92 (m, 1H), 1.15 (d, J = 4 Hz, 6H), 0.91 (s, 9H),
$0.085 \ (d, \ J = 4 \ Hz, \ 6H)$. $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.4, 157.5, 66.5, 61.4, 53.0, 44.5, 44.1, 35.3, 26.1, 22.8, 18.5, -5.1.

**(S)-N-(4-((tert-Butyldimethylsilyl)oxy)-1-(isopropylamino)-1-oxobutan-2-yl)cyclohexanecarboxamide (23c)**. Compound 22 (0.33 g, 1.20 mmol) was dissolved in 5 mL of anhydrous DCM to which DIPEA (1.05 mL, 6 mmol) was added and stirred at 0 °C. Cyclohexyl carbonyl chloride (240 µL, 1.8 mmol) was added drop-wise at 0 °C. The reaction was monitored as per procedure described for 23a to obtain the target compound. Compound 23c was obtained as a white solid (0.2 g, 45%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.61 (s (broad), 1H, NH), 5.85 (s (broad), 1H, NH), 4.40 (q, 1H), 4.05 (dq, 1H), 3.89 (m, 1H), 3.77 (m, 1H), 3.33 ( m, 4H), 2.03 (m, 1H), 1.91 (m, 1H), 1.59-1.54 (m, 6H), 1.12 (d, $J = 4$ Hz, 6H), 0.91 (s, 9H), 0.085 (d, $J = 4$ Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.4, 157.3, 61.4 53.0 47.8 44.8 41.2 34.8 25.9 25.5 24.3, 22.6, 22.5, -5.3. m.p: decomposes at 310 °C.

**(S)-N-(4-Hydroxy-1-(isopropylamino)-1-oxobutan-2-yl) furan-2-carboxamide (24a).**

Compound 23a (0.14 g, 379.87 µmol) was dissolved in 5 mL of anhydrous THF to which TBAF (165 µL, 569.81 µmol) was added and reaction was stirred at 0 °C for 30 min. Reaction solvent was evaporated and resulting crude purified by silica chromatography in 0-10% MeOH gradient in DCM to yield 24a as a yellow viscous semi-solid (0.075 g, 81%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.53 (d, $J = 7.2$ Hz, 1H), 7.45 (m, 1H, NH), 7.13 (d, 1H), 6.75 (d, $J = 7.3$ Hz, 1H), 6.51 (m, 1H, NH), 4.75 (m, 1H), 4.08 (septet, 1H), 3.72 (m, 2H), 2.09 (m, 1H), 1.83 (m, 1H), 1.16 (d, $J = 4$ Hz, 6H).

**(S)-N-(4-Hydroxy-1-(isopropylamino)-1-oxobutan-2-yl) morpholine-4-carboxamide (24b).**

Compound 24b was synthesized from 23b (0.245 g, 632.11 µmol) following reaction procedure
described for 24a. Compound 24b was afforded as a white solid (0.103 g, 63%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.04 (d, 1H, NH), 5.75 (m, 1H, NH), 4.45 (septet, 1H), 4.21 (br. s, 1H, OH), 4.05 (m, 1H) 3.69 (m, 6H), 3.34 (m, 4H), 1.97 (m, 1H), 1.65 (m, 1H), 1.17 (d, $J = 4$ Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.4, 157.5, 66.5, 61.4, 53.0, 44.5, 44.1, 35.3, 26.1, 22.8, 18.5, -5.1. m.p: decomposes at 210 °C.

(S)-N-(4-Hydroxy-1-(isopropylamino)-1-oxobutan-2-yl) cyclohexanecarboxamide (24c).

Compound 24c was synthesized from 23c (0.2 g, 519.98 µmol) following procedure described for synthesis of 24a. Compound 24c was afforded as a white semi-solid (0.11 g, 78%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.61 (d, 1H, NH), 5.85 (d, 1H, NH), 4.40 (q, 1H), 4.05 (dq, 1H), 3.89 (m, 1H), 3.77 (m, 1H), 3.33 (m, 4H), 2.03 (m, 1H), 1.91 (m, 1H), 1.59-1.54 (m, 6H), 1.12 (d, $J = 4$ Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.6, 157.3, 61.40, 53.0, 47.8, 44.8, 41.2, 34.9, 25.9, 25.5, 24.3, 22.6.

General procedure for the preparation of the corresponding L-R$_1$-Hse-OMs (25).

Corresponding compound 24a-24c (0.05 g) was dissolved in approximately 5 mL of anhydrous DCM to which DIPEA (3.0 eq) was added and stirred at 0 °C. Methanesulfonyl chloride (2.5 eq) was added drop wise to the reaction at 0 °C. Reaction was monitored by TLC in 95:5 DCM:MeOH until starting material was consumed. Solvent was evaporated and crude was dissolved in 10 mL of DCM, and washed with dilute NH$_4$Cl (150 mL), brine (200 mL), and dried over MgSO$_4$. Solvent was evaporated to afford the corresponding mesylate. Intermediates were used immediately for the next reaction without further purification.

(S)-N-(1-(Isopropylamino)-4-(4-methylpiperazin-1-yl)-1-oxobutan-2-yl)furan-2-carboxamide (26). Compound 25a (0.05 g, 182.93 µmol) was dissolved in 2 mL of anhydrous DMF and
stirred at 0 °C. 1-methylpiperazine (18.15 µL, 163.62 µmol) was added drop wise at 0 °C and reaction was allowed to warm up 60 °C and stir for 2 hr. Upon reaction completion, solvent was removed in vacuo and resulting crude was dissolved in DCM (50 mL) and washed with H$_2$O (150 mL), brine (150 mL) and dried over MgSO$_4$. Crude was purified by silica chromatography in a gradient of 0-10% MeOH in DCM to afford 26 as a clear viscous semi-solid (0.019 g, 25%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.89 (s (broad), 1H, NH), 7.49 (d, $J = 8$ Hz, 1H), 7.16 (d, $J = 4$ Hz, 1H) 6.78 (m, 1H, NH), 6.52 (dd, $J = 8$ Hz, $J = 4$ Hz, 1H) 4.47 (m, 1H), 4.28 (m, 1H), 3.69 (m, 4H), 3.39 (m, 4H), 2.79 (m, 2H), 2.20 (m, 2H), 2.12 (s, 3H), 1.21 (d, $J = 4$ Hz, 6H).

(S)-N-(1-(Isopropylamino)-4-(4-methylpiperazin-1-yl)-1-oxobutan-2-yl)morpholine-4-carboxamide (27). Compound 27 was synthesized from 25b (0.14 g, 243.85 µmol) following reaction protocol described for 26. Compound 27 was obtained as a viscous clear semi-solid (0.056 g, 27%). $^1$H NMR (400 MHz, CDCl$_3$) δ 4.68 (m, 1H), 4.06 (septet, 1H), 2.73 (m, 2H), 2.43 (s, 3H), 2.37 (m, 8H), 1.12 (d, $J = 4$ Hz, 6H).

(S)-N-(1-(Isopropylamino)-4-(4-methylpiperazin-1-yl)-1-oxobutan-2-yl)cyclohexanecarboxamide (28). Compound 28 (0.05 g, 80.4 µmol) was synthesized from 25c following procedure described for synthesis of 26. Compound 28 was as a white solid (0.006 g, 30%). $^1$H NMR (400 MHz, CDCl$_3$) δ 6.13 (d, 1H, NH), 4.54 (m, 1H), 4.19 (m, 1H), 4.125 (q, 2H), 2.85 (m, 1H), 2.17 (m, 1H), 2.14 (s, 3H), 1.91-1.37 (m, 10H), 1.23 (d, $J = 4$ Hz, 6H). m.p.: 418-420 °C.
2.2.2 Synthesis of N-imidazole substituted non-covalent inhibitors (Scheme 6)

Scheme 6. Synthesis of N-imidazole substituted non-covalent inhibitors. Reagents and conditions: (a) 20 (1.0 eq), Boc₂O (1.1 eq), Et₃N (1.5 eq), 50:50 MeCN:H₂O, rt, 4 hr; (b) HATU (1.10 eq), DIPEA (3.0 eq), benzylamine (1.1 eq), DMF (anh), N₂ atm, 0 °C→rt, 16 hr; (c) TBAF (1.0 eq), THF (anh), N₂ atm, 0 °C→rt, 2 hr; (d) CBr₄ (1.5 eq), PPh₃ (1.5 eq), DCM (anh), N₂ atm, 0 °C→rt, 2.5 hr; (e) imidazole (2.5 eq), DIPEA (1.5 eq), DMF (anh), N₂ atm, 0°C→rt, 16 hr; (f) 30% TFA, DCM (anh), N₂ atm, 0 °C, 45 min; (g) for R₂COCl: DIPEA (3.0 eq), DCM (anh), N₂ atm, 0 °C, 4 hr; for R₂COOH: HATU (1.1 eq), DIPEA (3.0 eq), MeCN (anh), N₂ atm, 0 °C→rt, 16 hr.

N-(tert-Butoxycarbonyl)-O-(tert-butyldimethylsilyl)-L-homoserine (29). Compound 20 (2.36 g, 10.11 mmol) was dissolved in a 50 mL solution of 1:1 acetone:H₂O. Et₃N (2.12 mL, 15.17 mmol) and Boc-anhydride (2.43 g, 11.12 mmol) were added to reaction and stirred at rt for 6 hr. Reaction was monitored by TLC in 95:5 DCM:MeOH until 20 was completely consumed. Reaction solvent was evaporated in vacuo and remaining aqueous layer was acidified to pH 2.0 with 10% citric acid. The aqueous layer was extracted with EtOAc (3 x 200 mL) and dried over MgSO₄. Compound 29 was obtained as a viscous clear oil (2.72 g, 81%). ¹H NMR (400 MHz, CDCl₃) δ 5.92 (d, 1H, NH), 4.33 (q, 1H), 3.81 (m, 2H), 2.07 (m, 2H), 1.441 (s, 9H), 0.910 (s, 9H), 0.08 (d, J = 4 Hz, 6H).

tert-Butyl (S)-(1-(benzylamino)-4-((tert-butyldimethylsilyl)oxy)-1-oxobutan-2-yl)carbamate (30). Compound 29 (2.72 g, 8.16 mmol) was dissolved in 15 mL of anhydrous MeCN to which
DIPEA (4.26 mL, 24.47 mmol) and HATU (3.41 g, 8.97 mmol) were added and stirred at 4 °C. Benzylamine (980.0 µL, 8.97 mmol) was added drop wise to reaction mixture and further stirred at 4 °C for 6 hr. Reaction solvent was evaporated *in vacuo* and resulting crude was dissolved in 15 mL of EtOAc and extracted with H$_2$O (150 mL), brine (150 mL), and dried over MgSO$_4$. Crude was purified by silica chromatography in a 5-30% gradient of EtOAC in hexanes to afford **30** as clear oil (2.29 g, 85%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.37 (m, 5H), 7.10 (d, 1H, NH), 6.20 (d, 1H, NH), 4.55 (dd, $J =$ 4 Hz, 2H), 4.41 (m, 1H), 3.85 (m, 2H), 2.12 (m, 2H), 1.52 (s, 9H), 0.99 (s, 9H), 0.15 (d, $J =$ 4 Hz, 6H).

**tert-Butyl (S)-(1-(benzylamino)-4-hydroxy-1-oxobutan-2-yl)carbamate (31).** Compound **30** (1.13 g, 2.67 mmol) was dissolved in 10 mL of anhydrous THF and stirred at 0 °C. TBAF (774.17 µL, 2.67 mmol) was added drop wise to reaction mixture and further stirred 0 °C for 30 min. Reaction was monitored by TLC 3:2 EtOAc: hexanes until **30** was consumed. Reaction solvent was removed and remaining crude was purified by silica chromatography in a 10-80% EtOAc gradient in hexanes to afford **31** as a white solid (0.680 g, 82%).$^1$H NMR (400 MHz, CDCl$_3$) δ 7.59 (d, 1H, NH), 7.30-7.20 (m, 5H), 5.86 (d, 1H, NH), 4.38 (dd, $J =$ 4 Hz, 2H), 4.31 (m, 1H), 3.60 (m, 2H), 1.98-1.69 (m, 2H), 1.40 (s, 9H). m.p.: 330-333 °C.

**tert-Butyl (S)-(1-(benzylamino)-4-bromo-1-oxobutan-2-yl)carbamate (32).** Compound **31** (0.25 g, 810.71 µmol) and carbon tetrabromide (0.318 g, 1.22 mmol) were dissolved in 10 mL anhydrous DCM and stirred at 0 °C. Triphenylphosphine (0.403 g, 1.22 mmol) was dissolved in 5 mL of anhydrous DCM and added dropwise to the reaction mixture over a 5 min period. Reaction was stirred at 0 °C for 10 min and then allowed to warm to rt. Reaction was monitored by TLC in 3:7 EtOAc: hexanes until **31** was consumed. Reaction solvent was evaporated and crude was purified by silica chromatography in a 10-40% EtOAc gradient in hexanes to afford
as a white powder (0.173 g, 58%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.59 (d, 1H, NH), 7.22 (m, 5H), 6.09 (d, 1H, NH), 4.41 (dd, $J = 4$ Hz, 2H), 4.31 (m, 1H), 3.39 (m, 2H), 2.42-2.14 (m, 2H), 1.40 (s, 9H). m.p.: 328-330 °C.

tert-Butyl(S)-(1-(benzylamino)-4-(1[H]-imidazol-1-yl)-1-oxobutan-2-yl)carbamate (33). Compound 32 (0.52 g, 1.40 mmol) was dissolved in 3 mL of anhydrous DMF and stirred at 0 °C. A solution of imidazole (0.238 g, 3.50 mmol) and DIPEA (365.94 µL, 2.10 mmol) in DMF was added drop wise to bromide mixture and further stirred at 0 °C. Reaction was monitored by TLC in 9:1 DCM: MeOH until 32 was consumed. Reaction solvent was evaporated in vacuo and resulting crude was dissolved in EtOAc (100 mL) and extracted with H$_2$O (300 mL) and brine (300 mL), and dried over MgSO$_4$. Resulting crude was purified by silica chromatography in a 30-70% EtOAc gradient in hexanes and then in a 0-10% MeOH gradient in DCM to yield 33 as a yellow semi-solid (0.38 g, 76%). $^1$HNMR (400 MHz, CDCl$_3$) $\delta$ 8.17 (s, 1H), 7.32 (d, $J = 4$ Hz, 1H), 7.21 (m, 5H), 6.87 (d, $J = 4$ Hz, 1H), 5.94 (d, 1H, NH), 4.38 (dd, $J = 4$ Hz, 2H), 4.22 (m, 1H), 3.97 (m, 2H) 2.24-1.99 (m, 2H), 1.43 (s, 9H).

(S)-2-Amino-N-benzyl-4-(1[H]-imidazol-1-yl)butanamide (34). Compound 33 (0.38 g, 1.40 mmol) was dissolved in a solution of anhydrous DCM with 30% TFA and stirred at 0 °C. Reaction was monitored by TLC in 95:5 DCM: MeOH until 33 was consumed. Reaction solvent was diluted with toluene and evaporated in vacuo to remove excess TFA to yield 34 as yellow crystalline solid (0.356 g, quantitative yield).

(S)-N-(1-(Benzylamino)-4-(1[H]-imidazol-1-yl)-1-oxobutan-2-yl) furan-2-carboxamide (35). Compound 34 (0.095 g, 367.76 µmol) was dissolved in 3 mL of anhydrous DCM to which DIPEA (320.29 µL, 1.84 mmol) was added and stirred at 0 °C. 2-furoyl chloride (36.26 µL,
367.76 µmol) was added drop wise at 0 °C. Reaction was stirred at 0 °C for 30 min and allowed to warm up to rt and stir for 12 hr. Reaction solvent was evaporated and resulting crude was dissolved in 15 mL of EtOAc and extracted with sat. NaHCO₃ (200 mL), H₂O (until pH was neutralized), brine (200 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 0-5% MeOH gradient in DCM to afford 35 as a yellow viscous semi-solid (0.025 g, 20%). ¹H NMR (400 MHz, CDCl₃) δ 8.41 (d, J = 4 Hz, 2H), 7.69 (s, 1H), 7.47 (s, 1H), 7.28 (m, 5H), 7.09 (d, J = 4 Hz, 1H), 6.92 (d, 1H), 6.50 (dd, J = 4 Hz, 1H), 5.53 (d, 1H, NH) 4.72 (m, 1H), 4.40 (dd, J = 4 Hz, 2H), 3.97 (m, 2H) 2.24-1.99 (m, 2H).

(S)-N-(1-(Benzylamino)-4-(1[H]-imidazol-1-yl)-1-oxobutan-2-yl) piperidine-1-carboxamide (36). Compound 34 (0.095 g, 367.7 µmol) was dissolved in 3 mL of anhydrous DCM to which DIPEA (320.29 µL, 1.84 mmol) was added and stirred at 0 °C. 1-piperidine carbonyl chloride (46 µL, 367.76 µmol) was added drop wise at 0 °C and stirred at rt for 4 hr. Reaction solvent was evaporated in vacuo and crude was dissolved in 10 mL EtOAc and extracted with H₂O (100 mL) and brine (100 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography 0-10% MeOH gradient in DCM to afford 36 as a white semi-solid (0.120 g, 65%). ¹H NMR (400 MHz, CDCl₃) δ 7.82 (s, 1H), 7.57 (d, 1H, NH), 7.33 (m, 5H), 6.59 (d, 1H, NH), 4.40 (dd, J = 4 Hz, 2H), 4.29 (m, 1H), 3.66 (m, 2H), 3.6 (m, 2H), 2.50 (m, 1H), 2.23(m, 1H), 1.74 (p, 4H), 1.54 (m, 2H).

(S)-N-(1-(Benzylamino)-4-(1[H]-imidazol-1-yl)-1-oxobutan-2-yl) 1-methyl-1[H]-pyrrole-2-carboxamide (37). N-methylpyrrole-2-carboxylic acid (0.046 g, 367.76 µmol) was dissolved in 2 mL of anhydrous DMF to which DIPEA (320.29 µL, 1.84 mmol) and HATU (0.139 g, 367.76 µmol) were added and stirred at 0 °C for 20 min. Compound 34 (0.095 g, 367.76 µmol) was dissolved in 1 mL of DMF and added to reaction mixture and stirred for 1 hr at 0 °C and then
warmed to rt and stirred for 12 hr. Reaction solvent was evaporated and crude was dissolved in 50 mL EtOAc and extracted with H₂O (100 mL) and brine (100 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 0-15% MeOH gradient in DCM to afford **37** as a white semi-solid (0.02 g, 27%). ¹H NMR (400 MHz, CDCl₃) δ 8.18 (s, 1H), 7.53 (d, J = 8 Hz, 1H), 7.35 (s, 1H), 7.34 (d, J = 4 Hz, 1H), 7.27 (m, 5H), 7.08 (d, J = 4 Hz, 1H), 6.92 (s, 1H), 6.74 (s, 1H), 6.09 (d, 1H, NH), 5.54 (d, 1H), 4.63 (m, 1H), 4.40 (dd, J = 4 Hz, 2H), 4.00 (m, 2H), 3.88 (s, 3H), 2.24-1.99 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 179.4, 171.1, 162.2, 138.1, 128.8, 127.7, 127.6, 113.1, 107.7, 50.2, 43.62, 3.91, 34.3.

**(S)-N-(4-(1H-imidazol-1-yl)-1-(isopropylamino)-1-oxobutan-2-yl) furan-2-carboxamide** (**38**). A solution of imidazole (0.0536 g, 788.20 µmol) and DIPEA (0.0612 g, 472.92 µmol) in 2 mL of anhydrous DMF was prepared and stirred at rt for 30 min. Compound **23a** (0.1 g, 315.28 µmol) was dissolved in 1 mL of anhydrous DMF and added drop wise to pre-stirring mixture at 0 °C. Reaction was monitored by TLC in 3:1 EtOAc: hexanes until **23a** was consumed. Reaction solvent was evaporated in vacuo and resulting crude was dissolved in 50 mL of EtOAc and washed with H₂O (100 mL), and brine (100 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 30-100% EtOAc gradient in hexanes to afford **38** as a yellow viscous semi-solid (0.025 g, 35%). ¹H NMR (400 MHz, CDCl₃) δ 7.89 (s, 1H), 7.70 (s, 1H), 7.49 (d, J= 7.2 Hz, 1H), 7.19 (d, J = 4 Hz, 1H), 7.14 (d, J = 7.3 Hz, 1H), 7.03 (d, 1H, NH), 6.78 (m, 1H, NH), 6.52 (dd, J = 7.2 Hz, J = 7.3 Hz, 1H ), 6.15 (d, 1H, NH), 4.59 (m, 1H), 4.11 (m, 2H) 4.08 (m, 1H), 2.41 (m, 1H), 2.21 (m, 1H), 1.18 (d, J = 4 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 171.3, 161.3, 137.8, 133.1, 128.7, 128.1, 126.0, 120.6, 113.3, 111.8, 50.3, 43.2, 38.5, 35.8.
(S)-N-(4-((Cyanomethyl)-1[H]-imidazol-1-yl)-1-(isopropylamino)-1-oxobutan-2-yl)furan-2-carboxamide (39). Compound 39 was synthesized from 23 (0.14 g, 213.3 µmol) following procedure described for 38 to obtain compound 39 as a yellow-brown semi-solid (0.035 g, 27%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.55 (s, 1H), 7.17 (d, $J = 7.2$ Hz, 1H), 7.07 (d, 1H, NH), 7.03 (m, $J = 4$ Hz, 1H), 6.54 (dd, $J = 7.2$ Hz, $J = 4$ Hz, 1H), 4.55 (m, 1H), 4.05 (m, 2H), 3.74 (m, 1H), 3.67 (s, 2H), 2.34 (m, 2H), 2.18 (m, 2H), 1.15 (d, $J = 4$ Hz, 6H).

2.2.3 Synthesis of 1H, 2, 3-triazole substituted non-covalent inhibitors (Scheme 7)

**Scheme 7.** Synthesis of 1H,2,3-triazole substituted non-covalent inhibitors. Reagents and conditions: a) 31 (1.0 eq), MsCl (1.1 eq), DIPEA (3.0 eq), DCM$_{\text{anh}}$, N$_2$ atm, 0 °C, 45 min; b) NaN$_3$ (4.0 eq), DMF$_{\text{anh}}$, N$_2$ atm, 60 °C, 2.5 hr; c) Cul (1.0 eq), TEMED (1.0 eq), Et$_3$N (1.0 eq), TMS-acetylene (2.5 eq), THF$_{\text{anh}}$, N$_2$ atm, rt, 18 hr; d) TBAF (1.5 eq), THF$_{\text{anh}}, 10\%$ AcOH, N$_2$ atm, 0 °C→rt, 16 hr; e) 30% TFA, DCM$_{\text{anh}}$, N$_2$ atm, 0 °C, 45 min; f) for R$_2$COCl: DIPEA (3.0 eq), DCM$_{\text{anh}}$, N$_2$ atm, 0 °C, 4 hr; for R$_2$COOH: HATU (1.1 eq), DIPEA (3.0 eq), ACN$_{\text{anh}}$, N$_2$ atm, 0 °C→rt, 16 hr.

tert-Butyl (S)-(4-azido-1-(benzylamino)-1-oxobutan-2-yl)carbamate (40). Compound 31 (1.75 g, 5.67 mmol) was dissolved in 5 mL of anhydrous DCM and stirred at 0 °C. DIPEA (2.97 mL, 17.02 mmol) and MsCl (483.16 µL, 6.24 mmol) were added dropwise and further stirred at 0 °C until 31 was consumed. Reaction solvent was then evaporated and crude was re-dissolved in 30 mL of EtOAc and washed with dilute NH$_4$Cl (150 mL) and brine (150 mL), and dried over MgSO$_4$. Organic layer was evaporated and resulting yellow solid was re-dissolved in 5 mL of anhydrous DMF. Sodium azide (1.47 g, 22.61 mmol) was added to reaction and stirred at 60 °C.
for 2.5 hr. Reaction solvent was evaporated and resulting crude was dissolved in 30 mL EtOAc and washed H₂O (150 mL) and brine (150 mL), dried over MgSO₄, and purified by silica chromatography in a 10-60% EtOAc gradient in hexanes to yield 40 as a clear viscous semi-solid (1.04 g, 55%). ¹H NMR (400 MHz, CDCl₃) δ 7.32-7.24 (m, 5H), 6.68 (s (broad, 1H, NH), 5.26 (d, 1H, NH), 4.40 (dd, J = 4 Hz, 2H), 4.21 (septet, 1H), 3.41 (m, 2H), 2.09-2.01 (m, 1H), 1.96-1.92 (m, 1H), 1.42 (s, 9H).

**tert-Butyl (S)-1-(benzylamino)-1-oxo-4-(4-(trimethylsilyl)-1H-1,2,3-triazol-1-yl)butan-2-yl)carbamate (41).** To a suspension of CuI (0.245 mg, 1.28 mmol) in 5 mL of anhydrous THF, TEMED (193 µL 1.28 mmol), Et₃N (180 µL, 1.28 mmol), and TMS-acetylene (918 µL, 6.45 mmol) were added and stirred at rt. Compound 40 (0.860 g, 2.58 mmol) was dissolved in 10 mL of anhydrous THF and immediately added to reaction mixture and allowed to stir at rt for 18 hr. Reaction was quenched with H₂O and extracted in 60 mL EtOAc. Organic layer was then washed with brine (150 mL) and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 30-60% EtOAc gradient in hexanes to afford 41 as a white semi-solid (0.430 g, 62%). ¹H NMR (400 MHz, CDCl₃) δ 7.57 (s, 1H), 7.51 (m, 1H, NH), 7.28-7.19 (m, 5H), 5.87 (d, 1H, NH), 4.47 (dd, J = 4 Hz, 2H), 4.39 (m, 2H), 4.30 (septet, 1H), 2.47-2.39 (m, 1H), 2.30-2.25 (m, 1H), 1.40 (s, 9H), 0.29 (s, 9H).

**tert-Butyl (S)-1-(benzylamino)-1-oxo-4-(1[H]-1,2,3-triazol-1-yl)butan-2-yl)carbamate (42).** Compound 41 (0.7 g, 1.62 mmol) was dissolved in 10 mL of anhydrous THF containing 1M AcOH and stirred at 0 °C. TBAF (470 µL, 1.62 mmol) was added at 0 °C and reaction was allowed to warm up to rt and stir for 16 hr. Reaction solvent was evaporated in vacuo and crude was purified by silica chromatography in a 0-5% MeOH gradient in DCM to yield 42 as a white semi-solid (0.435 g, 75%). ¹H NMR (400 MHz, CDCl₃) δ 7.65 (s, 1H), 7.56 (s, 1H), 7.32-7.26
(m, 5H), 6.74 (s (broad), 1H, NH), 5.32 (d, 1H, NH), 4.47 (dd, J = 4 Hz, 2H), 4.43 (m, 2H), 4.11 (m, 1H), 2.47-2.2.40 (m, 1H), 2.33-2.26 (m, 1H), 1.42 (s, 9H).

(S)-2-Amino-N-benzyl-4-(1H)-1, 2,3-triazol-1-yl)butanamide (43). Compound 42 (0.430 g, 1.196 mmol) was dissolved in 10 mL of anhydrous DCM with 30% TFA and stirred at 0 °C for 45 min. Reaction solvent was diluted with toluene and evaporated to remove excess TFA and afford 43 yellow viscous solid. Crude was used for next reaction without further purification (0.620 g, quantitative yield).

(S)-N-(1-(Benzylamino)-1-oxo-4-(1H)-1,2,3-triazol-1-yl)butan-2-yl)furan-2-carboxamide (44). Compound 43 (0.305 g, 1.18 mmol) and DIPEA (614.63 µL, 3.53 mmol) were dissolved in 7 mL of anhydrous DCM and stirred at 0 °C for 45 min. 2-Furoyl chloride (116.31 µL, 1.18 mmol) was added to reaction mixture dropwise at 0 °C and allowed to further stir for 10 min. Reaction was allowed to warm up to rt and stir over 16 hr. Reaction solvent was evaporated and resulting crude was dissolved in 20 mL of EtOAc and washed sat NaHCO₃ (200 mL), H₂O (until pH was neutralized), brine (200 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 0-10% MeOH gradient in DCM to afford 44 as a light brown viscous semi-solid (75 mg, 45%). ¹H NMR (400 MHz, CDCl₃) δ 7.77 (m, 1H, NH), 7.70 (d, J = 7.2 Hz, 1H), 4.56 (s, 1H), 7.49 (d, J = 4 Hz, 1H), 4.45 (s, 1H), 7.29-7.21 (m, 5H), 7.03 (dd, J =7.2 Hz, J = 4 Hz, 1H), 6.48 (s (broad), 1H, NH), 4.70 (m, 1H), 4.49 (dd, J = 4 Hz, 2H), 4.45 (m, 2H), 2.38-2.33 (m, 1H), 2.43-2.36 (m, 1H). ¹³C NMR (400 MHz, CDCl₃) δ 170.6, 157.9, 146.9, 143.8, 136.9, 129.2, 128.8, 126.7, 112.1, 111.1, 55.7, 46.8, 43.2, 30.1.

(S)-N-(1-(Benzylamino)-1-oxo-4-(1H)-1,2,3-triazol-1-yl)butan-2-yl)-1-methyl-1H-pyrrole-2-carboxamide (45). N-methylpyrrole-2-carboxylic acid (147.17 mg, 1.18 mmol) was dissolved in
3 mL of anhydrous DMF to which HATU (491.95 mg, 1.29 mmol) and DIPEA (614.63 µL, 3.53 mmol) were added and stirred at 0 °C for 45 min. Compound 43 (0.305 g, 1.18 mmol) was dissolved in 2 mL of DMF and added drop wise to mixture at 0 °C. Reaction was allowed to warm to rt and stir for 16 hr. Reaction solvent was evaporated in vacuo and resulting crude was dissolved in 30 mL EtOAc and washed with H₂O (200 mL) and brine (200 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 0-10% MeOH gradient in DCM to yield 45 as a white semi-solid (0.120 g, 62%). ¹H NMR (400 MHz, CDCl₃) δ 7.97 (s, 1H), 7.76 (d, J = 7 Hz, 1H), 7.63 (s, 1H), 7.47 (d, J = 4 Hz, 1H), 7.29-7.19 (m, 5H), 6.77 (d, J = 7 Hz, 1H, NH), 6.05 (d, J = 4 Hz, 1H), 5.30 (d, 1H, NH), 4.62 (septet, 1H), 4.52 (dd, J = 4 Hz, 2H), 4.40 (m, 2H), 3.86 (s, 3H), 2.91-2.79 (m, 1H), 1.83-1.67 (m, 1H).

(S)-N-(1-(Benzylamino)-1-oxo-4-(1H)-1,2,3-triazol-1-yl)butan-2-yl)-4-fluorobenzamide (46).
p-Fluorobenzoic acid (0.148 g, 1.0605 mmol), was dissolved in 5 mL of anhydrous DMF to which HATU (403.24 mg, 1.065 mmol) and DIPEA (510 µL, 2.892 mmol) were added and stirred at 0 °C for 45 min. Compound 43 (0.250 g, 0.964 mmol) was dissolved in 2 mL of DMF and added drop wise to mixture at 0 °C. Reaction was allowed to warm to rt and stir for 16 hr. Reaction solvent was evaporated and resulting crude was dissolved in 30 mL of EtOAc and washed with H₂O (200 mL), brine (200 mL), and dried over MgSO₄. Resulting crude was purified by silica chromatography in a 0-10% MeOH gradient in DCM to yield 46 as a white solid (0.125 g, 62%). ¹H NMR (400 MHz, CDCl₃) δ 7.99 (t, 1H), 7.95 (t, 1H), 7.77 (td, 2H), 7.65 (d, 1H), 7.53 (s, 1H), 7.27-7.19 (m, 5H), 7.01 (td, 2H), 4.79 (septet, 1H), 4.46 (dd, J = 4 Hz, 2H), 4.38 (d, 2H), 2.55-2.48 (m, 1H), 2.45-2.39 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 170.9, 166.7, 163.7, 137.7, 129.7, 128.6, 127.5, 115.7, 115.4, 51.1, 46.9, 43.6, 33.3. ¹⁹F NMR (376 MHz, CDCl₃) δ -107.14 (s, 1F). m.p.: 180-185 °C.
2.2.4 Enzymology

Enzyme activity assays and biological characterization studies were performed by Ms. Ewa Wasilewski (University Health Network) following protocol for enzymology studies as previously described. 36
CHAPTER 3: RESULTS AND DISCUSSION

3.1.1 Synthesis and Spectral Characterization of BCO-BODIPY

The synthesis of fluorescent biomarker BCO-BODIPY 8 (Scheme 4) to assess the expression levels of PAD enzymes in animal models was achieved successfully. The synthesis of BCO-BODIPY consisted of 11 steps, first with the construction of the inhibitor portion of the biomarker. α and γ amino-protected L-ornithine 10 was coupled with benzylamine to afford intermediate 11, which was then deprotected with 15% piperidine to afford amino intermediate 12. Construction of the acyl-BODIPY core 16 was carried out using a procedure similar to that reported by Cui et al. Briefly, nitro-BODIPY 14 was synthesized in a one-pot reaction, first by the formation of the dipyrromethane core which arose from the aldol condensation between 2, 4-dimethylpyrrole and 4-nitrobenzaldehyde catalyzed by trifluoroacetic acid (TFA). Addition of 2, 3 dichloro-5, 6-dicyano-1, 4-benzoquinone (DDQ) led to the oxidation of the dipyrromethane intermediate to afford dipyrromethene and subsequent treatment with boron trifluoride dietherate (BF$_3$•OEt$_2$) and triethylamine (Et$_3$N), led to the complexation of BF$_2$. Further reduction of 14 with hydrazine hydrate yielded amino-BODIPY 15 which was acylated with succinic anhydride to afford the final acyl-BODIPY 16. 16 coupled with ornithine derivative 12 yielded intermediate 17 and Boc-deprotection with TFA and condensation with ethyl-2-chloroethanimidoate yielded BCO-BODIPY 8 in 30% yield. Spectroscopic data for this probe were obtained in phosphate buffer (PBS) at pH 7.4 (Fig. 8) and $\lambda_{max}$ of 500 nm and $\lambda_{em}$ of 522 nm were measured. A quantum yield value of $\Phi = 0.56$ in ethanol was also calculated and is within range of the expected spectroscopic characterizations of the parent BODIPY probe.
Figure 8. Spectroscopic characterization of BCO-BODIPY in phosphate buffer at pH 7.4. Absorption (red) and Emission (blue).

3.1.2 Time Dependent Inactivation of hPAD4 by BCO-BODIPY.

Preliminary time dependent inactivation assays were conducted for BCO-BODIPY with concentrations 0 to 1 mM against hPAD4 (Fig. 9). Results showed lost of hPAD4 activity over a 60 minutes period of time with increasing concentrations of BCO-BODIPY. Partial decrease in activity at time 0 suggests concentration dependence of the enzyme for inhibition. The constant remaining activity levels over 60 minutes observed with concentrations from 0 µM to 100 µM BCO-BODIPY suggested that rate of catalysis for this enzyme is rather slow. As concentration of BCO-BODIPY increases from 250 µM to 1 mM, there is an observable decrease in hPAD4 activity over time, suggesting the time dependent characteristics of the chloramidine type of inhibitors. Overall, these preliminary results show that BCO-BODIPY has inhibitory properties.
towards hPAD4. Further enzymatic analysis and kinetic characterizations are required for this probe.

![Graph showing time-dependent inactivation of hPAD4 by BCO-BODIPY](image)

**Figure 9.** Time-dependent inactivation of hPAD4 by BCO-BODIPY (Graph obtained from Ms. Ewa Wasilewski).

### 3.1.3 *In vitro and in vivo Binding Studies of BCO-BODIPY against hPAD4*

An *in vitro* labeling assay by SDS-PAGE gel was performed to observe the degree of fluorescent labeling of hPAD4 with BCO-BODIPY to investigate the targeting properties of this biomarker (Fig. 10). Results showed an increase in fluorescence from 0 min to 60 min, in both lanes 3 and 4 when hPAD4 is incubated with 2mM of BCO-BODIPY in comparison to the control (no BCO-BODIPY, lane 2), suggesting that this probe has both the binding and fluorescent properties required to detect PAD *in vivo.*
Figure 10. *In vitro* binding assay of BCO-BODIPY against hPAD4. 500 µM of hPAD4 was incubated for 30 to 60 min with 2mM BCO-BODIPY and ran for 34 min at 200V in a 15% SDS-PAGE. Gel was visualized in a Typhoon300 fluorescence gel reader. Lane 1: Pre-stained protein ladder; lane 2: hPAD4 (no BCO-BODIPY); lane 3: hPAD4 (t= 30 min + BCO-BODIPY); lane 4: hPAD4 (t= 60 min+ BCO-BODIPY).

A pilot study in which healthy female mice were injected with BCO-BODIPY was carried out to assess BCO-BODIPY targeting to PAD *in vivo*. Mice were injected with a 2 mg/mL solution of BCO-BODIPY and sacrificed at 30, 60, or 120 min time points. The frontal cerebral cortex was analyzed under a confocal microscope to detect the levels of fluorescence and PAD binding (Fig. 11). An increase in fluorescence over time was observed in the mice that were injected with BCO-BODIPY in comparison with the controls. Also, it was noted that incubating the model with BCO-BODIPY for 120 min prior to sacrificing, seemed to provide the best signal of PAD bound to the probe in this region of the brain.
Figure 1. In vivo labeling of PADs with BCO-BODIPY in healthy mice. Healthy mice were injected either with PBS, BCO, acyl-BODIPY, and BCO-BODIPY. Mice were sacrificed at three time points (30 min, 60 min, and 120 min) and perfused. Brain tissues were cut at 6 µm thickness and fixed with paraffin. Frontal cerebral cortex was visualized in a LM700 confocal microscope.

Furthermore, the increase in fluorescence in a patchy pattern over time further suggested PAD labeling, as this is the characteristic expression pattern of PAD in this region of brain.\textsuperscript{19}
Overall, these preliminary results suggested that this probe does target and inhibit PAD, further showing its potential for \textit{in vivo} studies in diseased animal models.

\textbf{3.1.4 Attempts to Synthesize BCO-Distyryl-BODIPY}

The synthesis of BCO-distyryl-BODIPY \textit{9} (Fig. 6) was undertaken to create a long emission probe for \textit{in vivo} studies as BCO-BODIPY had an emission range of 500-522 nm which overlapped with the region of tissue auto fluorescence from biological samples and presented difficulties when differentiating the probe's signal from background noise.\textsuperscript{41} Therefore, the synthesis of a long wavelength emitting near IR probe was necessary. The methyl groups at positions 3' and 5' in the parent BODIPY core can serve as nucleophilic active centers where conjugation of the aromatic system can be extended by condensation with different aromatic aldehydes through Knoevenagel reactions.\textsuperscript{42} For the distyryl condensation, two different approaches were undertaken to protect intermediate \textit{15} (Scheme 8). Phtalamide protected amino-BODIPY \textit{47} was successfully obtained, however, condensation did not proceed as described due to solubility issues in a variety of solvents.\textsuperscript{42} Further, the use of methyl carbamate afforded intermediate \textit{48} and condensation afforded \textit{50} in a 40\% yield. The removal of the Moc-protecting group was carried out over several conditions described in Table 1; however, Moc-deprotection seemed unsuccessful, mainly due to the instability of the distyryl-BODIPY core to the reaction conditions described.
Scheme 8. Attempts to synthesize BCO-distyryl-BODIPY from intermediate 50.

Table 1. Conditions for Moc-deprotection of intermediate 50.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Reaction Physical Appearance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH_2NH_2•H_2O/KOH/ethylene glycol</td>
<td>Decomposed</td>
<td>43</td>
</tr>
<tr>
<td>Reflux; 12 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat. K_2CO_3/DCM</td>
<td>No Reaction</td>
<td>44</td>
</tr>
<tr>
<td>Rt; 12 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBAF/THF 0°C</td>
<td>Decomposed</td>
<td>45</td>
</tr>
</tbody>
</table>

Another attempt was to synthesize 9 from intermediate 17 (Scheme 9), where 51 was obtained successfully. However, attempts to proceed to 52 under the reaction conditions stated in Table 2 were unsuccessful due to either the inactivity of the carbamate to reaction conditions stated or the instability of the boron-complex of the BODIPY core to the Bronsted and Lewis acids used.\(^\text{46}\) An alternative route to use a more suitable amino protecting group with milder and selective conditions was designed by a colleague where an azide protecting group was used instead of Boc on intermediate 17, and BCO-distyryl-BODIPY was obtained successfully in a 32% yield.
Scheme 9. Synthesis of BCO-distyryl-BODIPY.

Table 2. Conditions for Boc-deprotection of intermediate 51.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Reaction physical appearance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% TFA /DCM 4 °C; 6 hrs</td>
<td>Decomposed</td>
<td></td>
</tr>
<tr>
<td>AcOH/DCM 0 °C→rt; 24-48 hr</td>
<td>No Reaction</td>
<td></td>
</tr>
<tr>
<td>H2O 100 °C; 24 hr</td>
<td>No Reaction</td>
<td>47</td>
</tr>
<tr>
<td>Neat 80 °C; 10 min</td>
<td>Decomposed</td>
<td>48</td>
</tr>
<tr>
<td>4M TMSCl (2.5 eq)/ 4M Phenol (2.5 eq) 0 °C; 20 min</td>
<td>Decomposed</td>
<td>49</td>
</tr>
<tr>
<td>BF3•OEt2 /DCM/ 4Å MS 0 °C→ rt; 24 hr→48 hr</td>
<td>No Reaction</td>
<td>50</td>
</tr>
<tr>
<td>1M HCl/Et2O 4 °C; 30 min</td>
<td>Decomposed</td>
<td>51</td>
</tr>
</tbody>
</table>

3.2.1 Synthesis of Novel Non-Covalent Inhibitors

The synthesis of a library of novel non-covalent inhibitors was designed to understand how changes in amino acid chain substituents influence specificity and selectivity towards PAD isozymes to create potent inhibitors. From the proposed library, 11 compounds were successfully synthesized and characterized (Fig. 12). The synthesis of these derivatives was shown to be difficult; specifically the side chain substitutions (R2) as the nucleophilicity of the
selected heterocycles tended to be very poor for direct substitution reaction conditions. For this reason, several schemes were developed for appropriate synthesis of these inhibitors. The synthesis of N-methyl piperazine substituted inhibitors was based on scheme 5, first with the protection of the primary alcohol of L-homoserine by silylation with TBDMSCl and followed by amino protection with Fmoc carbamate as previously reported, to afford intermediate 21. Treatment of 21 with excess isopropylamine under HATU amino acid coupling conditions afforded intermediate 22 which was then treated with three different acyl chlorides to afford the respective intermediates 23a-23c. Desilylation followed by mesylation and direct substitution with N-methyl piperazine afforded compounds 26, 27, and 28 with yields varying from 25-30%. 

N-imidazole substituted inhibitors were obtained by following scheme 6, as attempts to synthesize these compounds from a mesylate intermediate showed to be unfavourable and the β, γ-unsaturated by-product 53, was the predominant species (Scheme 10, 53). Amino protection of intermediate 20 was done with Boc instead of Fmoc due to the instability of the latter to the desilylation conditions normally used, and intermediate 29 was afforded. Coupling under HATU conditions with benzylamine was carried out instead of isopropylamine due to the difficulties presented during bromination in the presence of this functional group and intermediate 30 was obtained. Desilylation and bromination with carbon tetrabromide and triphenylphosphine afforded intermediate 32. Substitutions of N-imidazole was optimized when 1H-imidazole was treated with one equivalent of DIPEA and stirred for one hour in DMF at 0 °C and added drop-wise to intermediate 32 to afford 33 in 65% yield and 53 as a minor product. Treatment of 33 with TFA afforded amino 34 which was reacted with three different acyl chlorides and carboxylic acids to afford compounds 35, 36, 37, and 38 in 25-35% yields. The synthesis of compounds 44-47 was carried using a specially designed scheme 7, as the direct substitutions of 1H, 2, 3- and 1H, 2, 4- triazoles with either a mesylate or a bromo intermediate were
unsuccessful, as once again, 53 was the predominant compound. Intermediate 31 was converted into azide intermediate 40, first by activation of the alcohol by mesylation, followed by treatment with sodium azide. The formation of 1H, 2, 3- triazole proceeded with the treatment of intermediate 40 with TMS-acetylene in the presence of a copper catalyst to afford 41 in 66% yield. The reaction mechanism for this triazole synthesis proceeds through a [1+3] Sharpless modified Huisgen cycloaddition mechanism (Scheme 10). This mechanism proceeds first with the formation of a copper acetylide intermediate followed by a ligand substitution by the corresponding azide (Scheme 8, (1)). An unusual short lived six membered copper (III) metacycle species is formed (2) which then rearranges into a more stable triazolyl-copper species (3), and upon proteonolysis, affords the corresponding triazole in 66% yield (4). Desilytation and decarbamation yielded intermediate 43 which was coupled with the corresponding acyl chlorides and carboxylic acids to afford compounds 44, 45, and 46 in 40-55% yields.


3.2.2 Attempts to Synthesize Substituted Imidazoles Non-Covalent Inhibitors

Synthesis of (4-Imidazolyl)acetonitrile 39 was achieved from the bromo intermediate in a very low yield (7%). Several conditions to improve the direct substitution of 4-substituted
imidazoles on side chain were explored (Scheme 11). Direct substitutions of 4-Methyl-imidazole, 4-(5)-(Hydroxymethyl)imidazole, and (4-Imidazolyl)acetonitrile were carried out with the mesylate intermediate 31, however, no observable product conversions were observed as the major product obtained was 53. Further, reported Mitsunobu reaction conditions for direct imidazole substitutions were carried out as reported; however, no product conversion was observed, presumably due to the presence of electron donating groups in the substituted imidazoles that could potentially increase the pKa of the imidazole, and make them unsuitable nucleophiles for the reaction conditions stated. Therefore, a more rigorous and specific route, where building of substituted imidazole heterocycles will be developed, as direct substitution techniques were not efficient.

Scheme 11. Synthesis of substituted-imidazoles non-covalent inhibitors
Figure 12. Library of successfully synthesized non-covalent PAD inhibitors

Table 3. Enzyme inhibition kinetics for non-covalent compounds \( h \text{PAD1}, m \text{PAD2}, \) and \( h \text{PAD4} \) and \( IC_{50} \) CHO cell toxicity assays for synthesized non-covalent inhibitors. N/A refers to data unavailable (Assays carried out by Ms. Ewa Wasilewski). Experimental logP and \( pKa \) values were obtained using ChemBioDraw Ultra v. 13.0.

<table>
<thead>
<tr>
<th>#</th>
<th>( K_i (\mu M) )</th>
<th>( IC_{50} ) CHO Cells (( \mu M ))</th>
<th>Predicted side chain pKa</th>
<th>Predicted logP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( h \text{PAD1} )</td>
<td>( m \text{PAD2} )</td>
<td>( h \text{PAD4} )</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>N/A</td>
<td>1838 ± 2421</td>
<td>488 ± 47</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>27</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>
3.2.3 In Vitro Enzyme Inhibition Studies and Structure-Activity-Relationship Analysis

Enzymatic assays were carried with hPAD1, mPAD2 and hPAD4 and inhibition constants for the compounds were calculated along with experimental logP and pKa values (Table 3). Substitutions on the side chain amino acids as well as the groups on the amino and carboxy termini influenced the inhibitory activities of the synthesized PAD inhibitors. A preliminary SAR analysis was carried out to interpret the results from the inhibition data to elucidate on the effects of the side chains on overall inhibition as well as their implication in selectivity and specificity against the tested isozymes (Table 4). It is important to note, however, due to the limited number of compounds, a complete and rigorous SAR analysis cannot be obtained but a general interpretation was carried out nonetheless. Compounds 26, 27, 28, and 46, did not show
any overall inhibition towards any of the isozymes ($K_i > 500 \mu M$). These inhibitors carried different N-terminal six membered aliphatic and aromatic cyclic moieties which presumably are too bulky to be accommodated in this anchoring region of the isozymes. Compounds 26, 27, 28 with N-methyl piperazine side chain substituents did not show any favourable binding, suggesting that six membered aliphatic heterocycles do not fit the proposed model described in the thesis rationale, presumably due to their larger volume and flexibility. For this reason, it was decided to focus on five membered heterocycles to explore non-covalent interactions at the active site. Compounds 36, 38, 39, and 45 showed moderate activity towards PADs ($K_i > 200$-$500 \mu M$). Compounds 38 and 39 carried the N-terminal furoyl moiety, the C-terminal isopropylamine, and the corresponding substituted imidazoles. 38 showed greater than 5-fold affinity for $h$PAD4 in comparison to $h$PAD1 and $m$PAD2 suggesting specificity and selectivity towards this isozyme. On the other hand 39, showed at least 3-fold affinity towards $h$PAD1 in comparison to other isozymes, suggesting that active site pocket of $h$PAD1 might have key amino acid residues that promote dipole-dipole or H-bonding interactions, thus promoting higher binding affinity. Substituted imidazole 37 and triazole 44 showed good activities towards PADs ($K_i < 200 \mu M$) with at least 2-fold selectivity towards $m$PAD2 in comparison to $h$PAD1 and $h$PAD4. The presence of N-methyl pyrrole seemed to improve activity in comparison to furoyl substituted inhibitors, suggesting that possible van der Waals and π-stacking interactions are preferred in this anchoring region.

Interestingly, moderate to good activity was observed in 38 which contains the furan and imidazole moiety on the N-terminal and side-chain, respectively. However, inhibition data could not be fully interpreted as enzyme inactivation due to the inhibitory properties of this compound as it was contraindicated by CHO cells IC$_{50}$ studies, which showed that 38 was toxic. Therefore, it was presumed that the inherent activity observed with this compound was more of a toxicity-
mediated inactivation rather than a reflection of inhibition. To further support this finding, it was proposed that toxicity effects might be due to the presence of the isopropyl amide moiety as compound 35, with benzyl amide moiety, lacked activity against all isozymes and no signs of toxicity in CHO cells were observed.

Overall, compounds 37 and 44, with N-methyl pyrrole moiety, and imidazole and triazole as side chain substituents, respectively, showed improved inhibition profiles across all the isozymes with overall selectivity towards mPAD2, suggesting that the presence in five membered heterocycles might be involved in H-bonding, ionic, or dipole-dipole interactions with active site residues.

A preliminary in vivo study with compound 37 was carried in EAE animal models (n = 7) to observe the physiological effects of this hit in a disease animal model. EAE induced C57BL/6 female mice (n = 7) were injected with a 5 µg/µL solution of 37 after 12 days of immunization. Their EAE scores were measured and compared against the control group (PBS, n = 5) to analyze the pharmacological effects of 37 on motor function regeneration. The results showed a significant decrease in EAE scores in the treatment group in comparison to PBS over the course of treatment, suggesting that 37 has potential in re-establishing motor functions in diseased models (graph not shown). Further biochemical and biological analysis of brain and spinal cord samples are to be carried out to have a thorough understanding of the effects of this inhibitor in myelin regeneration.
**Table 4.** SAR table for novel non-covalent inhibitors towards PAD enzymes. Table shows the overall activity of each compound towards PAD isozymes; not active ($K_i > 500 \, \mu{M}$), moderately active ($K_i \, 200-500 \, \mu{M}$), and active ($K_i < 200 \, \mu{M}$). Selectivity was assessed by calculating the ratios of inhibition between each isozyme, for each isozyme, where ratios greater than 2-fold suggest selectivity.

<table>
<thead>
<tr>
<th>#</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>Overall PAD Activity</th>
<th>$hPAD1$</th>
<th>$mPAD2$</th>
<th>$hPAD4$</th>
</tr>
</thead>
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<tr>
<td>26</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td>Not active</td>
<td>0.3 (PAD4)</td>
<td>3.7 (PAD2)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td>Moderately active</td>
<td>0.6 (PAD2)</td>
<td>1.7 (PAD2)</td>
<td>6.3 (PAD2)</td>
</tr>
<tr>
<td>39</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td>Moderately active</td>
<td>2.8 (PAD2)</td>
<td>0.6 (PAD4)</td>
<td>0.6 (PAD1)</td>
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<tr>
<td>35</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
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<td>N/A</td>
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<td>3.9 (PAD1)</td>
<td>0.4 (PAD1)</td>
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<td><img src="image18" alt="Image" /></td>
<td>Active</td>
<td>0.4 (PAD2)</td>
<td>2.6 (PAD1)</td>
<td>0.9 (PAD1)</td>
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<tr>
<td>44</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
<td>Active</td>
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<td>1.6 (PAD1)</td>
<td>1.8 (PAD1)</td>
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<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
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<td>N/A</td>
<td>N/A</td>
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<tr>
<td></td>
<td>Structure 1</td>
<td>Structure 2</td>
<td>Activity</td>
<td>Value 1 (PAD2)</td>
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<td>Value 3 (PAD1)</td>
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<tr>
<td>28</td>
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<td>36</td>
<td><img src="image3.png" alt="Structure 1" /></td>
<td><img src="image4.png" alt="Structure 2" /></td>
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<td>9.2 (PAD2)</td>
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</tr>
</tbody>
</table>
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research thesis was to understand how PAD expression levels are implicated in disease pathogenesis and how modulation of amino acid side chain functional groups customized to PAD enzymes, generated potent non-covalent inhibitors as new disease modifying treatments.

From the preliminary results stated above for BCO-BODIPY, the potential of these biomarkers as essential tools for analyzing endogenous PAD expression patterns in vivo can be appreciated. For future studies, in addition to fluorescence imaging, a secondary staining method such as antibodies targeting PAD2 and PAD4 and anticitrulline antibodies will be required as a proof-of-concept study to confirm and prove that the observed staining patterns with the synthesized probes actually correlate to PAD binding, further confirming the effectiveness of this fluorescent biomarkers PAD targeting agents. In addition, in vivo studies in healthy and diseased animal models will be required with BCO-distyryl-BODIPY in order to obtain a better quality image by reducing the autofluorescence issues observed in the pilot animal study performed with BCO-BODIPY and thus obtain a more quantitative analysis of PAD expression.

The design of novel non-covalent inhibitors with a focus on the side chain substituents opened a new avenue to understand novel non-covalent interactions with PADs. The main outcomes from the preliminary SAR studies suggested that the C-terminyl anchoring region might have preferable π stacking and hydrophobic interactions where aromatic groups are better accommodated in comparison to bulky aliphatic chains. N-terminal anchoring region might be comprised of non-polar amino acid residues, presumably aromatic amino acids, as Van der Waals and π stacking interactions seemed to improve activity across isozymes. Focusing on side
chain substitutions, both imidazole and triazole substituted inhibitors showed moderate to good activity for all the isozymes, suggesting that five membered heterocycles with H-bonding, dipole-dipole or ionic interactions capabilities, would be suitable groups for future design of potent selective inhibitors.

For future studies, more rigorous synthetic schemes should be designed in order to expand the current library of inhibitors with the proposed substitutions to obtain a more rigorous SAR study. In addition, a computational model or a crystal structure of the most suitable hits against the PAD isozymes should be obtained to visualize the binding interactions of these inhibitors with active site residues. In addition, it will be of interest to analyze how this inhibitors' mode of binding differs across isozymes to elucidate on selectivity and specificity and improve the design of such inhibitors. Nonetheless, preliminary inhibition data and in vivo studies identified a set of hits that can be further exploited into second generation inhibitors where selectivity and specificity aspects can be more rigorously exploited for the design of more potent inhibitors.

Overall, the present results and proposed future studies in this thesis research project open a new door for elucidating the mechanism of action of PADs in disease pathogenesis and create an interesting avenue for the development of DMTs towards PAD enzymes.
CHAPTER 5: REFERENCES


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