Approaches Towards the Inhibition of Anti-Apoptotic Proteins

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Supervised by Prof. David R. Spring

This dissertation is submitted for the degree of Doctor of Philosophy
Declaration

This dissertation is the result of my own work carried out between October 2015 and February 2019. It includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. It does not exceed the word limit specified by the Physics and Chemistry Degree Committee.

Signed,  Date:

_____________________  ____________________

Jessica Iegre

Trinity College, University of Cambridge
Acknowledgments

Firstly, I would like to thank Prof. David Spring for giving me the opportunity to work in his research group, for his help, advice, and encouragement throughout. Your trust and open-mindedness made my PhD a truly enjoyable and stimulating experience. Thanks to Trinity College for funding and for the opportunity to attend national, international conferences, and workshops.

I am very grateful to all the great people I collaborated with throughout my PhD, who taught me that projects are far more interesting when shared! In particular, my thanks go to Dr Paul Brear and Dr Marko Hyvönen for helping me with the biophysics and for letting me go over to their lab and destroy their equipment. I would also like to thank Dr Daniel O’ Donovan, Dr David Baker, and all the wonderful people at AstraZeneca Cambridge for always going out of their way to help me. Thank you, Dr Nicholas Pugh and Niaz Ahmed, for bleeding people every time I wanted to play with platelets and for teaching me that cells can do amazing things even without a nucleus! I feel truly blessed to have been able to collaborate with you all.

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I would like to thank all the past and present Spring group members for their good humour and for making the last years so enjoyable. A special thank goes to my bay-buddy Josie, for her kindnesses, friendship, and for joining the Jossy team. And thank you, Steve, for keeping me company during my whole PhD. We have gone through good and bad days together, but we managed to not kill each other: I guess that means we are real friends!

I would also like to thank my family who has always encouraged me to be the best that I can be. You have always given me the freedom to do what I wanted in life, even if that meant living in different countries – I am incredibly thankful for that, and I love you all.

Last but not least, my gratitude goes to Diego for following me everywhere I go and for his every-day support and tolerance especially in my moodiest days. None of this would have been possible without you by my side – I feel extremely lucky to have you in my life. I dedicate this thesis to you.
Abstract

Anti-apoptotic proteins play a fundamental role in cell survival. Under physiological conditions, such proteins trigger apoptosis in defective or damaged cells only; under pathological conditions, however, they can be dysregulated allowing the cells to survive despite being harmful. Considering the importance of anti-apoptotic proteins in many physio-pathological roles, their specific inhibition is an attractive strategy to develop safe therapeutics.

This thesis describes the inhibition of two classes of anti-apoptotic proteins:

1) Inhibition of the anti-apoptotic protein CK2 to develop novel anti-cancer molecules targeting pockets outside the well-conserved ATP-binding site:

-Using a Fragment-Based-Drug-Discovery (FBDD) approach twelve small molecule inhibitors of CK2 were developed. The lead molecule, 3l, inhibited the catalytic activity of CK2α by binding in the cryptic αD pocket with a Kd of 4 μM. 3l stopped proliferation of colorectal cancer cells with a GI50 of 10 μM and presented improved drug-like properties and selectivity compared to previously reported inhibitors. Remarkably, 3l has the potential to be developed into a potent and selective anticancer drug.

-Using a combination of rational-based approach and peptide stapling, twenty-two conformationally-constrained peptides were generated to target the protein-protein interaction (PPI) of CK2 and affect its function. The lead peptide, P7-F1C5, presented a novel, highly-functionalised constraint that allowed the molecule to become cell-permeable, exert its anti-proliferative activity in cancerous cells, and to become resistant to serum proteases. P7-F1C5 is the first macromolecule reported in the literature that binds to CK2α with sub-micromolar affinity (Kd 150 nM), and that can act as a chemical probe for targeting the PPI of CK2.

2) Inhibition of the anti-apoptotic Bcl-2 proteins to dissect their role in platelet activation and apoptosis.

Bcl-2 proteins regulate cell lifespan; however, their role in non-nucleated platelets is not fully understood. The elucidation of these pathways in platelets is crucial to the development of selective anti-platelet therapeutics.

To this end, this thesis describes the development and the first application of twenty-seven BH3-only peptides in human platelets highlighting how peptides can provide an alternative to conventional methodologies to study PPIs in platelets. The most promising peptide, P9-F5C5, engaged the anti-apoptotic protein Bcl-xL with 26 nM affinity and reviled a new role for the protein Bim in platelet activation.
# Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>1C-PS</td>
<td>one-component peptide stapling</td>
</tr>
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<td>two-component peptide stapling</td>
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<td>Δ</td>
<td>heat</td>
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</tr>
<tr>
<td>$\nu_{\text{max}}$</td>
<td>absorption maximum</td>
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<td>atmosphere solids analysis probe</td>
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<td>n-butyl</td>
<td>normal (primary) butyl</td>
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butyl  tertiary butyl
°C  degrees Celsius
CAMK1  calcium/calmodulin-dependent protein kinase type 1
CAS  computational alanine scanning
CCHP  conformationally-constrained hybrid peptide(s)
CD  circular dichroism
CDK  cyclin-dependent kinase
CDKL  CDK-like kinase
Cft4  chromosome transmission fidelity 4
CK2  casein kinase II
CLIPS  chemical ligation of peptide into scaffolds
CLK  Cdc2-like kinase
cm⁻¹  wavenumbers
COSY  correlation spectroscopy
CPP  cell penetrating peptide(s)
CSF1R  colony stimulating factor 1 receptor
CuAAC  copper-catalysed azido-alkyne click reaction
CVMD  cardiovascular and metabolic diseases
CXCL4  platelet factor 4
Cy  cyclohexyl
Cyt C  cytochrome C
d  deuterated
Da  dalton(s)
Dap  2,3-diaminopropionic acid
Dba  dibenzylideneacetone
DBF  dibenzofulvene
DCE  1,2-dichloroethane
DEAD  diethyl azodicarboxylate
DEPT  distrotionless enhancement polarisation transfer
DIC  N,N′-Diisopropylcarbodiimide
DIMAT  2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DIPEA  N,N-diisopropylethylamine
DISC  death-inducing signalling complex
DMAP  4-dimethylaminopyridine
Dmb  2,4-dimethoxybenzyl
DME  1,2-dimethoxyethane
DMF  N,N-dimethylformamide
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<td>FADD</td>
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<td>FBDD</td>
<td>fragment-based drug discovery</td>
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<td>FBS</td>
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<td>food and drug authority</td>
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<td>fibroblast growth factor receptor</td>
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<td>FITC</td>
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<tr>
<td>G6PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<tr>
<td>GAFF</td>
<td>generalised AMBER force field</td>
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<td>GB</td>
<td>generalised born</td>
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<td>GCN4</td>
<td>general control protein N4</td>
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<td>GI&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>GLP-1</td>
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<td>GPa&lt;sub&gt;IIb&lt;/sub&gt;β&lt;sub&gt;3&lt;/sub&gt;</td>
<td>glycoprotein platelet fibrinogen receptor</td>
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<td>GPCR</td>
<td>G-protein coupled receptor(s)</td>
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<td>G&lt;sub&gt;α&lt;/sub&gt;</td>
<td>G, alpha subunit</td>
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<td>GSK</td>
<td>glycogen synthase kinase</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>h</td>
<td>hour(s)</td>
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<td>1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
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<td>HBA</td>
<td>hydrogen bond acceptor(s)</td>
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<td>HBD</td>
<td>hydrogen bond donor(s)</td>
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<td>HCT116</td>
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<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>Hrk</td>
<td>Harakiri, Bcl-2 interacting protein</td>
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<td>HSP90</td>
<td>heat shock protein 90</td>
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<tr>
<td>HSQC</td>
<td>heteronuclear single quantum correlation</td>
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<td>HTS</td>
<td>high-throughput screening</td>
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<td>Hz</td>
<td>hertz</td>
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<td>IAP</td>
<td>inhibitor of apoptotic protein</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximum inhibitory concentration</td>
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<td>inhibitor of kappa-light-chain enhancer of activated B-cells</td>
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<td>insulin-like growth factor 1</td>
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<td>infrared</td>
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<td>IRAK4</td>
<td>interleukin-1 receptor-associated kinase 4</td>
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<td>ITC</td>
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ivDde  4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3methylbutyl

$J$  coupling constant

JAK  Janus protein kinase

$K_d$  dissociation constant

KHK  ketohexokinase

KIT  mast/stem cell growth factor receptor

L  litre(s)

LCMS  liquid chromatography mass spectroscopy

LE  ligand efficiency

LLE  ligand lipophilicity efficiency

logP  logarithm of the octanol/water partition coefficient

LTA4H  leukotriene-A4 hydrolase

M  molar or mega

m  milli or metre

$m$  meta

MAPK  mitogen activated protein kinase

MBHA  methylbenzhydryl amine

Mcl-1  induced myeloid leukaemia cell differentiation protein

MD  molecular dynamic(s)

Me  methyl

MEP  molecular electrostatic potential

MES  2-Morpholinoethanesulfonic acid sodium salt

min  minute(s)

MM/GBSA  molecular mechanics/generalised born surface area

MMP  mitochondria membrane potential

MNK  MAPK-interacting kinase

mol  mole(s)

MOMP  mitochondria outer membrane permeabilisation

mp  melting point

mRNA  micro ribonucleic acid

$m/z$  mass-to-charge ratio

MW  microwave irradiation

n  nano

NBS  $N$-bromosuccinimide

NF-κB  nuclear factor kappa-light-chain-enhancer of activated B-cells

NHS  $N$-hydroxysuccinimide

NLS  nuclear localisation sequence
NMR  nuclear magnetic resonance
Nopp140  nucleolar phosphoprotein 140
Noxa  phorbol-12-myristate-13-acetate-induced protein 1
NRB  number of rotatable bond(s)
NTA  nitrilotriacetic acid
o  ortho
p  para
p53  phosphoprotein 53
Pbf  2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PBS  phosphate-buffered saline
PCD  programmed cell death
PDB  protein data bank
PDGF  platelet-derived growth factor(s)
PE  phycoerythrin
PE 30-40  petroleum ether (fractions with boiling point between 30-40 °C)
PE 40-60  petroleum ether (fractions with boiling point between 40-60 °C)
PFA  paraformaldehyde
PGE<sub>1</sub>  prostaglandin E1
Ph  phenyl
PI3K  phosphoinositol-3-kinase
PIFA  [bis(trifluoroacetoxy)iodo]benzene
PIP3  phosphoinositol-triphosphate
PK  pharmacokinetic
PLC  preparative liquid chromatography
PPAR  peroxisome proliferator-activated receptor(s)
PPI  protein-protein interaction
ppm  parts per million
PROTAC  proteolysis targeting chimera(s)
PRP  platelet rich plasma
PS  phosphatidylserine
PS  peptide stapling
PSA  molecular polar surface area
PTEN  protein phosphatase and tensin homolog
PTFE  polytetrafluoroethylene
Puma  p53 upregulated modulator of apoptosis
R  unspecified substituent
RCM  ring-closing metathesis
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<td>trityl</td>
</tr>
<tr>
<td>TS</td>
<td>thermal shift</td>
</tr>
<tr>
<td>U2OS</td>
<td>human osteosarcoma cancer cells</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>Wnt</td>
<td>cysteine-rich glycoproteins</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>

Standard one and three letter codes are used for amino acids.
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SECTION I

Introduction
CHAPTER 1:

Current Approaches in Drug Discovery
Until three decades ago, the high-throughput screening (HTS) of libraries of small organic molecules was the gold-standard approach used as a starting point in drug discovery. However, these multimillion-compound libraries were characterised by a high degree of structural similarity and included compounds targeted against known binding sites, hence limiting the chances of engaging previously unexplored biological space. When screened against well-established targets, HTS libraries were particularly effective, but when screened against new or difficult biological systems, they provided very few hits and several false positives.1 In the last few decades, a deeper understanding of biotechnology and biology have expanded the number of ‘druggable’ targets and pushed medicinal chemists to employ new molecular entities beyond traditional small molecules as well as new approaches to discover life-changing medicines.

Fragment-Based Drug Discovery (FBDD), for instance, has emerged as a complementary technique to discover new binding sites and new drugs more efficiently. The establishment of FBDD was made possible thanks to advances made in the field of biotechnology that allow detection of weak bindings such as those of fragments.2

Advances in biology, on the other hand, have led to the realisation that the majority of medicinally-relevant biological systems can rarely be targeted with traditional small molecules. Consequently, pharmaceutical industries have expanded their pipelines to accommodate previously uncharted chemical entities – hereinafter referred to as new modalities.3 New modalities – e.g. peptides, antisense oligonucleotides, proteins, synthetic mRNA, proteolysis targeting chimeras (PROTACs) – feature nowadays at any stage of the drug discovery programs and clinical studies and they are soon-to-become the medicines of tomorrow, able to engage targets considered ‘undruggable’ until not long ago.

For the purpose of this dissertation, an overview of recent advances on both FBDD and therapeutic peptides will be presented in this chapter, since other new modalities fall beyond the scope of the work being described.
1.1. Fragment-Based Drug Discovery

FBDD is a structure-based approach that provides lead compounds to target biological systems. FBDD allows the screening of fewer, low-molecular-weight molecules compared to other approaches employed to obtain chemical leads such as HTS. There are several advantages to using smaller fragment-sized libraries:

- the sampling of the chemical space is much more efficient due to the lower number of possible obtainable molecules;
- being less complex than traditional small molecules, fragments make fewer interactions with the proteins but can bind to a greater number of pockets, on a greater number of targets, resulting in higher hit rates;
- owing to their smaller size, libraries can be assembled and screened by small biotech companies and academic groups.

Although initial hits usually have lower potency, FBDD is considered to be more efficient in the optimisation phases of drug discovery, which are guided by the 3D information of fragment binding modes obtained by means of experimental techniques.

In a typical FBDD flow (Figure 1), firstly, a fragment library is assembled, and the fragments are then screened using biophysical techniques. Due to the low molecular weight and potency of the initial fragments, highly sensitive biophysical techniques are required for initial screening, such as Surface Plasmon Resonance (SPR), Isothermal Calorimetry (ITC), Thermal Shift (TS) and functional assays. X-ray or NMR techniques are used to investigate the binding modes of the fragment hits in the protein of interest and, therefore, to characterise the fragments. The third step in a FBDD approach is fragment elaboration. In this stage, the fragments to be progressed are selected according to different criteria such as potency, Ligand Efficiency (LE), Ligand Lipophilicity Efficiency (LLE) and availability of X-ray or NMR structures. The more drug-like fragments showing an IC50 in the mM range and interesting binding modes to the target are selected to be optimised into leads (IC50 in nM range). Such elaboration is achieved via iterative cycles of synthesis, structural analysis, and computer-aided design.

---

* FBDD screens thousands of compounds with a number of heavy atoms ≤ 20; HTS screens millions of compounds with ~ 30 heavy atoms each.
* The LE is defined as the Gibbs free energy of binding (KJ·mol⁻¹ or Kcal·mol⁻¹) divided by the number of non-hydrogen atoms. LE normalises the potency with respect to the number of heavy atoms, and it is of great importance when comparing the potency of compounds with a variety of formula weight (FW). At the fragment stage, an LE of approximately 0.3 or higher is preferred.
* The introduction of lipophilic groups can provide a quick mean of improving the potency of a fragment, but it often leads to problems such as non-specific binding and poor bioavailability. LLE (defined as pIC50 minus cLogP) is a parameter that can be used to ensure that the generated fragments do not gain an excessive hydrophobic character during the elaboration. An LLE of ~5 or higher is advisable.
The fragment elaboration typically occurs via linking, growing and/or merging strategies. The growing strategy consists in using a single vector to grow the fragments to engage in additional interactions (Figure 2a). The merging strategy sees, instead, the best features of the original fragments merged into what is predicted to be a more potent and drug-like molecule (Figure 2b). Alternatively, in the linking strategy fragments binding in different parts of the same pocket or in two adjacent pockets are linked together via linkers (Figure 2c). The linking of fragments has been considered to be the most difficult of the three methods: a linker should be introduced to maintain the optimal binding configurations that have been adopted by the individual fragments, should establish additional interactions with the protein to prevent a loss in LE, and should be synthetically accessible. However, when successful, the linking strategy can provide boosts in potency as high as 300-fold compared to the single fragments. The significant enhancement in binding affinity can be explained with the concept of ‘super-additivity’. Upon binding to the targeted protein, each fragment loses a significant part of its rigid body rotational and translational entropy resulting in an entropic penalty. The linked molecule would account for one entropic penalty only whilst two separate fragments would be affected by two unfavourable entropic terms. Consequently, the linked molecules can have a more favourable binding free energy ($\Delta G$) than the sum of the $\Delta G$ values of the individual fragments.
Chapter 1: Current approaches in drug discovery – FBDD

FBDD has shown some advantages with respect to more conventional drug discovery approaches. In particular:

1) Starting the optimisation with small compounds capable of making high-quality interactions with the protein means that the molecular weight, complexity and physicochemical properties of the molecule can be easily controlled during the elaboration.\(^2\)

2) The high binding energy per atom can be maintained during the optimisation from hit to lead.\(^{16,17}\)

3) The ligand Efficiency (LE) and the Veber ‘rule-of-three’\(^d\) allow easy comparison of lead-likelihood of fragments of different sizes.\(^2\)

4) Compared to HTS, far fewer fragments are required in the initial screening to sample the chemical space (thousands vs millions of compounds).\(^5\)

On the other hand, there are few disadvantages related to FBDD. However, these drawbacks are biotechnology-dependent and can be overcome with further advances in the field. In particular:

1) The low potency of the initial fragments requires the use of sensitive biophysical screening techniques and high fragment concentrations (up to 50 mM). Due to the high concentration, the fragments need to be highly soluble in the hydrophilic solvents used.\(^5\)

---

\(^d\) According to the Veber ‘rule of three’ a fragment to be elaborated into an oral bioavailable lead should satisfy the following criteria: \(FW < 300 \text{ g·mol}^{-1}\), H-bond donor (HBD) \(≤ 3\); H-bond acceptor (HBA) \(≤ 3\); \(c\Log P \leq 3\); Number of rotatable bonds (NRB) \(≤ 3\); Polar surface area (PSA) \(≤ 60 \text{ Å}^2\).\(^{285,286}\)
2) Due to low potency, the fragments are also unsuitable for cell screening and kinetic assays where high concentration will likely lead to false positives.⁵

1.1.1. State of the art of FBDD

FBDD is currently widely used both in industry and academia as a powerful and complementary drug discovery approach. FBDD has proved to be efficient in delivering drug candidates for targets that have been regarded as ‘undruggable’ in the past, such as protein-protein interactions (PPIs).⁵ Currently, around 40 new molecular entities at different clinical stages have been developed using FBDD for a variety of targets. Table 1 lists some of the drug candidates discovered using FBDD currently in phase II, III and approved. In addition, it is estimated that at least 20 molecules are currently undergoing phase I studies.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stage</th>
<th>Company</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vemurafenib</td>
<td>Approved</td>
<td>Plexxikon</td>
<td>B-Raf</td>
</tr>
<tr>
<td>Venetoclax</td>
<td>Approved</td>
<td>AbbVie/Genentech</td>
<td>Bcl-2</td>
</tr>
<tr>
<td>Asciminib</td>
<td>Phase III</td>
<td>Novartis</td>
<td>Bcr-Abl</td>
</tr>
<tr>
<td>Erdafitinib</td>
<td>Phase III</td>
<td>Astex</td>
<td>FGFR1-4</td>
</tr>
<tr>
<td>Lanabecestat</td>
<td>Phase III</td>
<td>AstraZeneca/Lilly/Astex</td>
<td>BACE1</td>
</tr>
<tr>
<td>PLX3397</td>
<td>Phase III</td>
<td>Plexxikon</td>
<td>CSF1R, KIT</td>
</tr>
<tr>
<td>Verubecestat</td>
<td>Phase III</td>
<td>Merck</td>
<td>BACE1</td>
</tr>
<tr>
<td>AT7519</td>
<td>Phase II</td>
<td>Astex</td>
<td>CDK1,2,4,5,9</td>
</tr>
<tr>
<td>AT9283</td>
<td>Phase II</td>
<td>Astex</td>
<td>Aurora, JAK2</td>
</tr>
<tr>
<td>AUY-922</td>
<td>Phase II</td>
<td>Novartis</td>
<td>HSP90</td>
</tr>
<tr>
<td>AZD5363</td>
<td>Phase II</td>
<td>AstraZeneca/Astex/CРUK</td>
<td>AKT</td>
</tr>
<tr>
<td>CPI-0610</td>
<td>Phase II</td>
<td>Constellation</td>
<td>BET</td>
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<tr>
<td>DG-051</td>
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<td>MNK1/2</td>
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<td>Plexxikon</td>
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<td>Lilly/Protherics</td>
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<td>Novitaclax</td>
<td>Phase II</td>
<td>Abbott</td>
<td>Bcl-2/Bcl-xL</td>
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<td>PF-06835919</td>
<td>Phase II</td>
<td>Pfizer</td>
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</table>

Highlighting the importance of FBDD in drug discovery, in 2011, the FDA approved the first fragment-based drug Vemurafenib (Zelboraf®) for the treatment of late-stage melanoma.¹⁹
Vemurafenib was developed from an azaindole derivative fragment (Scheme 1) and targets the ATP binding site of a mutant serine-threonine protein kinase B-Raf (V600E). Using a growing strategy, the initial fragment hit ($IC_{50}$ 100 μM) was elaborated into the 31 nM inhibitor Vemurafenib.

![Scheme 1 - Growing strategy applied to the development of Vemurafenib, the first drug developed using a FBDD approach. The parts of the molecule that represent the growth of the initial fragment are shown in blue.](image)

In 2015, Venetoclax (Venclexta®), a Bcl-2 antagonist, was approved for the treatment of chronic lymphocytic leukaemia, demonstrating the increasing success of the FBDD approach. Venetoclax was developed adopting a linking strategy between two weakly binding fragments engaging two proximal pockets of Bcl-xL (Scheme 2). The fragment elaboration was guided by an ‘SAR-by-NMR’ approach (Structure-Activity-Relationship guided by 2D protein-ligand NMR studies). Importantly, Venetoclax mimics the native BH3 domain of the pro-apoptotic Bcl-2 proteins, thereby acting as one of the first small molecules targeting a difficult PPI.

![Scheme 2 - Chemical structures of Venetoclax and the initial fragments (blue and red) binding to two different binding sites. A linking strategy was adopted to link the two weakly binding fragments together via a sulfonamide (green).](image)

Despite the fact that FBDD emerged only 30 years ago, two drugs have already been approved by the FDA, and there are a great number of molecules currently undergoing clinical studies. This evidence altogether makes FBDD a promising and efficient alternative to discover new drugs, especially those interacting with difficult biological systems.
1.2. Therapeutic peptides

Small molecules (FW < 500 Da) have traditionally been used to target deep and well-defined pockets of biological targets (e.g. proteins, enzymes, receptors, ion-channels etc). Advances in biology, however, brought to light that 80% of the signalling pathways regulating important physio-pathological mechanisms are characterised by the interactions between proteins - often referred to as PPIs. Such interactions are characterised by the contacts between shallow and undefined pockets and, except for a few successful examples, the failed attempts to target PPIs with small molecules are still numerous. Peptides are an ideal alternative to small molecules: they mimic the endogenous portions of the interacting proteins; they show low immunogenicity since they are made of proteinogenic amino acids; they can be easily synthesised; and they have lower production costs compared to other biologics. However, the development of peptide therapeutics is limited by their poor pharmacokinetic (PK) properties. In particular, peptides suffer from poor membrane permeability, poor stability to plasma proteases and high renal excretion often resulting in a short half-life. However, nature provides inspirational examples of a significant number of biologically-active peptides that validate their ability to interfere with both extra- and intra-cellular processes. These successful examples have inspired scientists to develop new methodologies aimed at overcoming the PK limitations of this class of molecules.

1.2.1. Macrocyclised peptides

Peptide cyclisation is probably the most widely adopted strategy to ameliorate the PK properties of peptides, with at least 125 macrocyclic peptides being reported as orally bioavailable. The macrocyclisation process on peptides, assuming it does not affect their secondary structure, is performed to lock the macromolecules in their binding conformation preserving their function and reducing entropic costs. Conversely, other methodologies applied to improve the PK properties of peptides, such as N-alkylation or the introduction of amide isosteres (peptidomimetics), may affect the peptide backbone resulting in changes that could compromise the peptide bioactivity. Peptide macrocyclisation can be achieved in different ways, and a schematic overview of the different techniques is shown in Figure 3.

*The text of this paragraph has been adapted from Iegre et al, Advanced Therapeutics, 2018.*
The head-to-tail cyclisation generally involves the N-terminus and C-terminus of the peptide chain linked together via an amide bond; head-to-side chain and tail-to-side chain cross-link one of the terminus to a side chain of the peptide; side chain-to-side chain macrocyclisation, instead, involves the linkage of two side chains of the peptide sequence with or without the aid of a linker. The intramolecular reaction to cross-link two amino acids of the linear sequence is often referred to as one-component (1C) peptide stapling (PS). On the other hand, intermolecular coupling involving a staple (or linker) and two amino acid side chains is referred to as two-component (2C) PS. It should be noted that the term stapling often refers to macrocyclisations that produce α-helical peptides. When a cyclisation reaction results in a non-helical peptide, the term constraining is generally accepted. In this dissertation, however, the terms stapling and constraining will be used interchangeably to describe macrocyclisations resulting in secondary structures beyond α-helices as well.

In this dissertation, the stapling methodologies only will be presented in detail in the following paragraphs.

1.2.1. One-component peptide macrocyclisation methodologies at a glance

1C-PS is the oldest and simplest of the two classes of peptide stapling methodologies. Disulfide bridge formation between two Cys residues is a common constraining methodology often adopted by nature to stabilise peptides and proteins. Disulfide bonds, however, are susceptible to being reduced in certain environments, such as those found intracellularly; therefore, alternative 1C stapling techniques have emerged to overcome this limitation. The most used 1C stapling technique is the all-hydrocarbon (AH) chemistry which involves a Ring-Closing Metathesis (RCM) reaction between two unnatural amino acids bearing terminal alkenes on the side chains. First reported by Grubbs and Blackwell in the 1990s, the RCM has been widely adopted to stabilise several biologically active and cell-penetrating peptides into helical
The RCM has been thoroughly optimised and, when the appropriate stapling position and amino acids are used, it proved to be successful in enhancing the helicity of peptides, improve the stability to proteases and impart cell-permeability to otherwise impermeable peptides. Another widely used 1C-PS chemistry is lactam formation. Reported for the first time in the late 1980s, lactam cyclisation provided peptides with superior bioactivity compared to the linear analogues. Lactam stapling has been mainly applied to peptides targeting extracellular or membrane-bound proteins, and its applicability to intracellular targets is yet to be determined. Even if more stable to proteolytic degradation than their linear counterparts, peptides stapled using this technique show inferior stability in respect to RCM-stapled peptides. Since the advent of the pioneer 1C-PS methodologies, a plethora of alternative reactions have emerged in the literature, including: thiol-ene reactions between Cys and alkene amino acids, copper-catalysed azido-alkyne cycloaddition (CuAAC) between azido and alkyne groups, C-H activation reaction to cross-link aromatic side chains of natural amino acids, thioether formation, oxime formation, and UV-promoted cycloaddition among others (Figure 4).

General conclusions on the applicability of all the above-mentioned techniques to biologically-relevant targets cannot be drawn considering that a limited number of comparative examples are available in the literature for all these emerging techniques.
The well-established 1C-PS techniques offer the advantage of being carried out on resin, thus saving purification steps. However, 1C-PS methodologies present one main disadvantage: any changes to the staple or the sequence required for peptide optimisation involve the re-synthesis of the amino acid used for stapling, synthesis of the linear peptide sequence, and re-stapling. Significant optimisation may be required for each of these steps, including the insertion of the new amino acid in solid-phase peptide synthesis (SPPS) procedures. To overcome these limitations, research groups have focused on the development of 2C macrocyclisation methodologies.

1.2.1.2. Two-component peptide macrocyclisation methodologies

Unlike the 1C stapling techniques, the 2C-PS offers a considerable advantage: the staple or constraint is optimised independently from the peptide sequence, and it can be used to introduce cell-permeable motifs, fluorescent tags, functionalities useful for biological assays or to improve the PK properties of the overall peptides in a combinatorial manner. Most of the 2C-PS techniques derive from the 1C counterparts and involve both natural and unnatural amino acids. Unlike the 1C macrocyclisations, 2C stapling is often not performed on-resin due to site-isolation issues that penalise the desired intramolecular reaction in favour of the intermolecular reactions (Figure 5).

The most reactive amino acid side chains are suitable for use in peptide macrocyclisation, with cysteine being the most used due to the high nucleophilicity of the sulfhydryl group. With respect to their unnatural counterparts, natural amino acids are cheap, widely available and hence simplify the synthesis of the linear peptide. However, the use of natural amino acids could lead to orthogonality and chemo-selectivity problems. Thiol-ene, in both radical and conjugate
addition, and $S_{N2}$ reactions are the most commonly used reactions for the application of Cys to peptide stapling with a limited number of reports on $S_{N}Ar$.

Greenbaum and Waters pioneered the field with the use of aryl-halide linkers to cross-link two Cys side chains, whilst Timmerman et al. have recently modified the aryl-halide linkers into water-soluble staples to access functionalised stapled peptides (Figure 6). Importantly, the cross-linking between aryl-halides and the thiol groups of Cys residues has been renamed as CLIPS (Chemical Ligation of Peptide into Scaffolds) technology, and it is currently used by start-ups and pharmaceutical companies to access the so-called bicycle and tricycle peptides. Dawson et al. made use of 1,3-dichloroacetone to cross-link between Cys residues via an $S_{N}2$ reaction followed by oxime linkage to introduce functionalities. Likewise, Cramer et al. exploited the $S_{N}2$ reaction between diiodomethane and Cys residues to convert labile disulfide bridges into stable methylene thioacetals (Figure 6). Bernardes et al. have recently reported a biocompatible and chemo-selective methodology which exploits the reaction between cysteine and dibromo-isobutylene to give cell-permeable grafted peptides.

Examples of thiol-ene reactions in peptide stapling are provided by Jiang et al. who used divinyl sulphonamides as linkers for 2C-PS with Cys. Similarly, Chou et al. reported of a light-mediated thiol-ene 2C stapling method utilising bis-alkene linkers. A similar method was developed by Keillor et al. using functionalised maleimides (Figure 6).

$S_{N}Ar$ cysteine stapling chemistry has been reported by Pentelute et al. who exploited the reactivity of sulphhydryl groups towards perfluoroaryl compounds to access stabilised peptides, whilst Derda et al. utilised decafluoro-diphenylsulfone to the same end. Remarkably, these stapling methodologies can be applied to Lys in addition to Cys residues (Figure 6).

Cys-mediated peptide stapling reactions have also been used to generate reversible systems which efficiently pass between rigid and flexible states in a biorthogonal manner, with the aim of elucidating the relationship between binding, biological activity, and secondary structure. To this end, Wilson et al. utilised the nucleophilic substitution reaction between dibromo maleimide linkers and Cys (Figure 6). Similarly, Smith et al. employed an $S_{N}Ar$ reaction to staple the peptide via 3,6-dichloro-1,2,4,5-tetrazine: under UV radiation such a linker would open and provide the uncyclised peptide bearing cyano groups on the cysteine residues. Treatment with an excess of cysteine would return the starting peptide (Figure 6). The 3,6-dichloro-1,2,4,5-tetrazine linker was functionalised via a reverse-electron demand Diels Alder.

A limited number of examples see natural amino acids other than Cys in 2C-PS; namely Trp and Glu (Figure 6). Johannes et al. have developed a method in which two tryptophan residues were reacted with a para-substituted benzaldehyde in a condensation reaction to form
conformationally constrained model peptides with improved proteolytic stability towards proteases compared to the linear analogue.\cite{76} On the other hand, McDowell et al. reported an example of bis-lactamisation in peptide stapling utilising Glu amino acids that, after being protected, were reacted with diaminoalkanes to stabilise model helical peptides (Figure 6).\cite{77}

The only 2C-PS methodology that uses unnatural amino acids is the CuAAC (Figure 6), and it will be discussed in detail in the next paragraph.

1.2.1.2.1. Spotlight on 2C CuAAC peptide chemistry

Pioneer work carried out by Bong et al. reported azido-alanine residues being introduced at an \(i,i+4\) distance to each other and clicked with aliphatic and aromatic dialkyne linkers to stabilise a peptide based on a GCN4 leucine zipper.\cite{78} Inspired by this work, the Spring group expanded the 2C-PS methodology to include stapling positions beyond \(i,i+4\) and, importantly, to introduce functionalisation on the linker. The CuAAC 2C stapling reaction involves an intermolecular
coupling between two azido side chains of a linear peptide and a bis-alkynyl linker to give the cyclised peptide. The optimised macrocyclisation reaction is carried out in aqueous conditions, at low concentrations of peptide (1 mg/mL) and with one equivalent of the linker (Scheme 3).

Among the disadvantages associated with the CuAAC 2C-PS technology there is the use of non-proteinogenic amino acids containing azido side chains, the use of a copper(II) catalyst which leads to air-sensitivity of the catalytic copper-complex, requiring all solvents to be degassed, and the risk of toxicity arising from any residual copper in the final stapled peptides. On the other hand, there are a number of advantages that outbalance the drawbacks of this methodology. In particular, the use of azido side chains provides orthogonality with respect to the other amino acids, leading to the avoidance of chemo-selectivity issues. Furthermore, the azido amino acids can be readily synthesised in few steps, the conditions used in the stapling reaction are mild, and functionalisation of the linkers is relatively easy, allowing the quick and efficient generation of libraries of functionalised stapled peptides. Peptides constrained using the CuAAC 2C-PS methodology have proved to be stable to proteolytic degradation, cell-permeable, biologically active in cells, and have led to the creation of a toolbox of functionalised staples that found application in different targets. A list of selected peptides macrocyclised using the CuAAC 2C-PS chemistry is shown in Table 2.

**Table 2 – List of selected biologically active macrocyclic peptides obtained using the CuAAC 2C-PS methodology.**

<table>
<thead>
<tr>
<th>Position</th>
<th>n</th>
<th>Constraint</th>
<th>Structure</th>
<th>Sequence</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>i,i+4</td>
<td>2</td>
<td></td>
<td>Extended</td>
<td>TNKS REXGDGXE</td>
<td>84</td>
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<td></td>
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<td>i,i+6</td>
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<td>Helix</td>
<td>GNC4 Leucine Zipper RIKQLEEKXGLGXXKIELEKK</td>
<td>78</td>
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Chapter 1: Current approaches in drug discovery – Peptides

Considering the variety of 2C-PS methodologies developed to date, it is clear that the decision of which one to use depends mainly on the structure of the peptide to be constrained and on the availability of the starting materials. The CuAAC 2C-PS methodology is among the most widely applied 2C-PS chemistries.\textsuperscript{78-87} It proved itself compatible with various amino acid sequences, enhanced the activity and proteolytic stability of several biologically active peptides, and it is applicable to both helical and non-helical peptides.\textsuperscript{79-84} Therefore, the CuAAC 2C-PS technique presents itself as a feasible stapling methodology to use when the priority is to functionalise the constrained peptides or study novel functionalised staples since the macrocyclisation reaction itself is well-validated.
1.2.1.3. Discovery of therapeutic macrocyclised peptides

The strategies used to develop therapeutic macrocyclised peptides can be divided into three main groups: natural products, screening of peptide libraries, and target-based rational design (Figure 7).

**Figure 7** – Major approaches adopted by pharmaceutical industries and academic groups to discover cyclic peptides as therapeutics: a) Peptides derived from natural sources; b) biological and chemical peptide libraries; 1) mRNA encoded libraries expressed in vitro and cyclised using specific reagents; 2) phage-display libraries expressed by bacteriophages in host cells and cyclised after phage release either by disulfide bridge formation or by CLIPS technology; 3) genetically selected cyclic peptides expressed in bacteria, cyclised in cell using the SICLOPS technology and tested in functional assays in cells (RTHS: reversed bacteria two-hybrid system); 4) chemical libraries of cyclic peptides synthesised in parallel or using combinatorial chemistry; c) Target-based rational design of cyclic peptides: a generic PPI is shown as pink and red surface, designed peptides as red helices, and staple in grey sticks. Taken from Iegre et al., Advanced Therapeutics, 2018.79

Natural and semi-synthetic cyclic peptides (Figure 7a) pioneered the use of this class of macromolecules as therapeutics; indeed, nine natural product cyclic peptides were approved by the FDA and EMA between 2005 and 2015.80 Examples of this class of molecules are fermentation products of microorganisms - Caspofungin,89,90 Cyclosporin A,91 Daptomycin,92 Anidulafungin,93 Romidepsin,94 Linaclotide and Pasireotide95 among others - hormones and hormones derivatives - Vasopressin, Octreotide and Oxytocin.88 These peptides find application as antifungal agents, antibiotics, chemotherapeutics, treatments for hormonal disorders and offer the advantage of being highly selective and stable \textit{in vivo}.

Peptide libraries can be divided into biological and chemical libraries. Biological peptide libraries (Figure 7b) are synthesised with the aid of bacteriophages (phage-display) or using
cell-free display technologies (mRNA display) and cyclised using some of the methodologies listed herein:

- cysteine amino acids are added to the phage-displayed peptide in a random manner to allow successive disulfide bridge formation. The “CLIPS” cyclisation technology is a variant of this methodology, and it generates bicyclic peptides via conjugation between three cysteine residues and a tris-(bromomethyl)benzene (TBMB) core. More recently, alternative trivalent thiol-reactive linkers have been used to provide bicyclic peptides characterised by diverse conformations and include \( N,N',N''-(\text{benzene-1,3,5-triyl})\text{tris(2-bromoacetamide)} \) (TBAB), \( N,N',N''-\text{benzene-1,3,5-triyltrisprop-2-enamide} \) (TAAB) and \( 1,3,5\)-triacryloyl-1,3,5-triazinane (TATA) (Figure 8a);

- head-to-tail cyclisation performed intracellularly exploiting the protein splicing capability of split inteins (the so-called SICLOPPS technology). This technology implements a reverse two-hybrid system to allow for functional and binding assays (Figure 8b);

- mRNA encoded libraries of linear peptides are translated in vitro in a cell-free system. Cyclisation is then performed using reagents like disuccinimidyl glutarate to connect the \( N \)-terminus and a side chain of a Lys residue or via disulfide bridges across Cys residues (Figure 8c).
Chapter 1: Current approaches in drug discovery – Peptides

Phage-display peptide libraries offer the advantage of providing highly diverse libraries. However, the peptides generated may have low affinity for the target and problems associated with the use of live cells and phages could arise. mRNA encoded libraries may overcome these limitations, therefore representing a valid alternative.\textsuperscript{107} New genetic technologies have recently emerged to allow the incorporation of unnatural amino acids into biological libraries, providing more flexibility in the composition of the peptide sequence.\textsuperscript{108–110} Of particular interest are the mRNA-encoded peptide libraries developed in the Suga group: thioether macrocyclic peptides are generated following spontaneous intramolecular reaction between Cys residues and the side chains of unnatural \(N\)-(chloroacetyl)-D-Trp or \(N\)-(chloroacetyl)-Tyr (Figure 9a).\textsuperscript{111,112} An alternative cyclisation has been achieved by the same group with the introduction of 5-hydroxytryptamine and benzylamine amino acid derivatives followed by fluorogenic oxidative coupling between the two side-chains to afford fluorescent cyclic peptides (Figure 9b).\textsuperscript{113}

![Figure 9 - Macrocyclisations performed using unnatural amino acids in peptide ribosomal synthesis. a) Macrocyclisation obtained using \(N\)-(chloro-acetyl) amino acid derivatives; b) Macrocyclisation performed using \(N\)-benzylamine and hydroxy tryptamine derivatives to obtain fluorescent cyclic peptides. The DNA-RNA hybrid transcript is shown as blue and red helices, linkage of the peptide to the nucleic acid strand as a black, curved line. Generic amino acids are shown as white spheres.](image)

Chemical libraries of cyclic peptides, on the other hand, are synthesised using parallel or combinatorial chemistry. Parallel synthesis is limited in the number of macrocycles that can be simultaneously generated, whilst combinatorial methods allow the generation of a greater number of compounds. Once assembled, the chemical peptide libraries can be screened against a selected target before or after cleavage from the resin support.\textsuperscript{114}
Chapter 1: Current approaches in drug discovery – Peptides

The target-based rational design approach aims to develop cyclic peptides for well-validated targets for which structural information of the protein of interest is available. This approach does not require the synthesis of many biomolecules since the peptides designed feature amino acid sequences that resemble portions of the native protein that is being mimicked. This approach allows for more flexibility for peptide modification and the amount of product synthesised. Furthermore, a target-based rational design approach can be used to optimise hit peptides coming from library screenings.59

In a typical example (Figure 7c), an X-ray crystal structure of the target protein and ideally of the native PPI is desired. In silico molecular modelling can guide the design of the peptide and identify suitable residues for cyclisation to happen.115 Further changes in the peptide sequence are aimed at replacing amino acids to increase the binding affinity and optimise PK properties. Subsequently, the designed peptides are synthesised and assessed in vitro. Iterative cycles of enzymatic, cellular, structural assays and synthesis are performed to build an SAR and guide further modifications until the optimal peptide is developed prior to in vivo testing. This approach offers the advantages of knowing the target in advance, and the synthesis of a smaller number of peptides is required to generate novel synthetic peptides with a high affinity for the desired target and improved PK properties. The main drawback of the target-based rational design approach is the need for detailed structural information of target and natural PPI sequence to guide the design.59

1.2.1.4. Cyclised peptides in advanced stages of the pipeline

Twenty-six cyclic peptide therapeutics have been approved by the FDA thus far.116 Of these, twenty were cyclised via intramolecular formation of disulfide bridges, four via intramolecular amide bonds and two via intramolecular formation of other types of chemical bonds (ester and carbamate).116 Vasopressin, the first cyclic peptide marketed, was approved in 1962 for the treatment of anti-diuretic hormone deficiency, and it is constrained via an intramolecular disulfide bridge. Macrocyclisation techniques besides disulfide bridging and lactamisation only became popular in the late 1990s, and it is therefore not surprising that the cyclic peptide drugs approved thus far do not feature more modern type of macrocycles, considering the long timelines of drug discovery and clinical studies. Noteworthily, most of the cyclic peptides with therapeutic applications approved to date derive from natural products. Thanks to the advances in biotechnology, the cyclic peptides that are currently undergoing clinical development feature both classic and modern cyclisation chemistries and have been discovered using directed-evolution techniques, rather than being inspired by natural products.117 An overview of some notable cyclic pharmaceutical peptides with public structures available currently in clinical studies and approved drugs is shown in Figure 10.
Chapter 1: Current approaches in drug discovery – Peptides

Historically, cyclic peptides found application in a wide variety of primary disease areas, with the majority of the drugs approved to treat infertility and obstetric/gynecologic disorders, cardiovascular and metabolic diseases (CVMD), and endocrinology-related diseases (Figure 11a). This fact has now been challenged by the cyclic peptides currently under clinical development, where oncology features as the main player in the therapeutic areas’ field, closely followed by respiratory, inflammatory and autoimmune (RIA) diseases (Figure 11). It should be noted that the interest of pharmaceutical companies working on cyclic peptides goes beyond oncology and RIA, with discovery pipelines focusing on the development of cyclic peptides for complement-mediated diseases (Ra Pharmaceuticals), CVMD, haematology, ophthalmology, and anti-infectives diseases (Bicycle Therapeutics), and antimicrobials (Polyphor). It is therefore evident that in the next decades, the primary therapeutic area landscape is bound to change significantly again. In addition, considering that cyclic peptides are receiving the attention of an increasing number of pharmaceutical companies and academic groups, further therapeutic areas will most likely engage this new drug modality.
CHAPTER 2:
Anti-Apoptotic Proteins
Cellular-self destruction or programmed cell death (PCD) is a fundamental process that allows the cells to be eliminated should they become defective, old, and potentially harmful to the living organism. Apoptosis (type I PCD) is the major form of PCD, followed by autophagy (type II) and necrosis (type III). Considering that PCD is of crucial importance to homeostasis, the cells are equipped with a multitude of finely regulated mechanisms that allow them to escape apoptosis under physiological conditions and trigger it when potentially harmful alterations are detected. These mechanisms consist of complicated cross-signalling between proteins and messengers that occur both intracellularly and extracellularly. The pro- and anti-apoptotic signalling pathways are extremely complicated and, in some parts, not fully understood. The overall cascades can be divided into four major classes: tumour necrosis factor (TNF) pathway, survival pathway, DNA damage and stress pathway, and death ligands pathway as exemplified in Figure 12.

The TNF pathway is considered an anti-apoptotic cascade: the cell signalling protein tumour necrosis factor α (TNFα) binds and activates the TNF receptors expressed on the cell membrane. The receptor, in turn, activates the complex-1 which triggers the NF-κB protein complex initiating the transcription of anti-apoptotic genes. The survival pathway is activated by a variety of extracellular ligands including hormones, chemokines, survival factors, collagen and laminin. Binding of these ligands to their receptors activates cytosolic kinases which all converge on the phosphorylation and activation of the AKT/PKB pathways resulting in the inhibition of the pro-apoptotic Bcl-2 proteins (such as Bad). When the pro-apoptotic Bcl-2 proteins are inhibited, the cell survival is maintained.
inhibited, the anti-apoptotic proteins of the same family are activated and prevent the release of the cytochrome C with consequent activation of caspases, resulting in inhibition of apoptosis. The action of the survival pathway can be reinforced by the activation of the inhibitor of apoptosis proteins (IAPs) which inhibit the caspases, thereby preventing apoptosis from happening. Conversely, the phosphatase and tensin homolog protein PTEN can block the activation of the AKT/PBK pathways by PI3K kinase, inducing apoptosis.\textsuperscript{122} On the other hand, stress and DNA damage trigger apoptosis by activating the pro-apoptotic Bcl-2 proteins and by activating p53, among other effectors, in the nucleus.\textsuperscript{126} Activation of pro-apoptotic Bcl-2 proteins (BH3-only proteins) means that mitochondria lose their outer membrane potential, the cytochrome C is released, and caspases are activated to exert apoptosis. Finally, apoptosis can be triggered by the so-called death ligands, namely FasL and TRAIL. The latter can indirectly stimulate the death-inducing signalling complex (DISC) to activate caspase 8 and therefore apoptosis.\textsuperscript{126} Anti-apoptotic proteins such as FAIM3 and FLIP prevent the apoptosis by inhibiting components of this cascade. The death ligands pathway is often referred to as extrinsic apoptosis to differentiate it from the intrinsic apoptosis, which is mitochondria-associated.\textsuperscript{123,124} Dysregulations that break the equilibrium between the activity of pro- and anti-apoptotic proteins are linked to the development and progression of pathological conditions, the most studied of which is cancer. Alterations that result in the cells becoming able to escape apoptosis include: activation or up-regulation of mitogenic signals (\textit{i.e.} Erk1/2, AKT), inactivation or downregulation of pro-apoptotic proteins (\textit{i.e.} Bax and Bak), and up-regulation of genes encoding for anti-apoptotic proteins (\textit{i.e.} Bcl-2 proteins).\textsuperscript{122}
Chapter 2: Anti-apoptotic proteins

2.1. Inhibition of anti-apoptotic proteins

Considering the central role of anti-apoptotic proteins in regulating cell lifespan, it is not surprising that their dysregulation is implied in a variety of diseases. Restoring the naturally-occurring apoptotic activity in diseased organisms by acting on anti-apoptotic proteins has been reported to be a more successful strategy than targeting pro-apoptotic proteins.\textsuperscript{122} Anti-apoptotic proteins play important roles in neurodegenerative conditions including Alzheimer’s and Parkinson’s disease, as well as during heart attacks or ischemic strokes.\textsuperscript{127-130} Under these pathological conditions, there is a decreased activity of anti-apoptotic proteins in favour of a more pronounced apoptotic activity. On the contrary, in a number of physio-pathological conditions cells escape apoptosis to a much larger extent due to increased anti-apoptotic activity. Examples of such conditions include auto-immune diseases, inflammation, animal hibernation, and cancer.\textsuperscript{123,124,131,132} Resistance to PCD is the hallmark of cancer cells, and inhibition of anti-apoptotic proteins is a well-exploited strategy in oncology. Such inhibition is achieved by targeting the apoptotic cascade at any level: mitochondrial outer membrane proteins (\textit{i.e.} Bcl-2), proteasomes, nonproteolytic death effectors, death receptors, nuclear factors, stress kinases (Figure 12).\textsuperscript{133} Depending on the desired target, apoptosis is induced utilising different approaches: for instance, anti-apoptotic gene transcription can be switched off; alternatively mRNA degradation can be induced using antisense oligonucleotides, then again small molecules, peptides, and antibodies can be developed to either inhibit the activity of a specific protein, or to prevent the cross-talk between proteins (PPIs).\textsuperscript{133}

The aforementioned pro- and anti-apoptotic pathways are the subject of intense study and scrutiny. Many of the proteins involved have been the subject of drug discovery campaigns, or probe-designing efforts, but several of their mechanisms still require elucidation. In an effort to analyse and comprehend these mechanisms better, two main proteins will be the focus of this dissertation: CK2 and Bcl-2.
2.1.1. Anti-apoptotic CK2 protein kinase

CK2 (formerly named protein Casein Kinase 2) is a protein kinase which plays crucial roles in multiple intracellular pathways including the regulation of cell proliferation and cell growth; it is also believed to be an anti-apoptotic protein in both healthy and cancer cells.\textsuperscript{134,135} It is, however, overexpressed in various cancer cell lines including breast, lung, prostate, colorectal, renal, leukaemia and glioblastoma brain tumours.\textsuperscript{136,137}

CK2 is a heterotetrameric enzyme composed of two catalytic subunits (α and/or α')\textsuperscript{f} and two regulatory subunits (β) (Figure 13).

\textbf{Figure 13 - Human CK2 holoenzyme (PDB code: 1JWH).\textsuperscript{138} Catalytic subunits are shown in cyan (α) and green (α'), regulatory subunits in yellow and purple (β).}

CK2 is constitutively active and uses ATP or GTP as co-substrate.\textsuperscript{139} Interestingly, these characteristics are not common to other eukaryotic protein kinases (EPKs),\textsuperscript{139} and the mechanisms that control its catalytic activity are unique to CK2.\textsuperscript{140}

Formation of the holoenzyme occurs via dimerisation of the β subunits followed by complexation with the N-terminal domain of CK2α. The surface contact between the α and the β domains is relatively small compared to other PPIs (832 Å\textsuperscript{2}). Thereby, the activity of the CK2 enzyme can be affected either by acting on the α subunits or by preventing the formation of the holoenzyme.

\textbf{2.1.1.1. CK2α subunit}

CK2α is composed of two domains, the N- and C-terminal domain with the ATP active site located between them (Figure 14).\textsuperscript{140} The N-terminal domain (red-orange) contains five stranded β-sheets (β1-β5) and one α-helix (αC) situated next to the inter-domain cleft. The αC-helix contains basic lysine residues and has, therefore, a preference for acidic substrates.\textsuperscript{138} The N- and C-terminal segments are linked via the hinge/αD region, and the αD pocket that will be

\textsuperscript{f}The α subunit presents 20 additional amino acids at the C-terminus that are absent in the α' subunit.\textsuperscript{144}
discussed in this work is located in close proximity to the αD helix. The C-terminal segment (green, blue and purple) is formed of α-helices and two double-stranded β-sheets at the inter-domain cleft and contains the activation loop. The conformation of the activation segment, as well as the glycine-rich loop (β1β2-loop), is important for the catalytic activity of the protein.140

The mechanisms that control the catalytic activity of CK2 are distinct from most other EPKs, namely the catalytic activity of CK2 is not controlled by phosphorylation (within the activation loop) or by interaction with regulatory proteins.140 Instead, the conformation of the DWG motif (Asp175-Trp176-Gly177) in the activation segment and the adopted active conformation of the αC-helix make CK2α constitutively active. In this conformation, the activation segment is open and allows for substrate binding and phosphor-transfer reactions. Specifically, the N-terminal segment establishes extensive contacts with the αC-helix and the activation loop, stabilising CK2α in a constitutively active conformation.140,142 The ability of CK2α to maintain the activation segment in this active form, unlike most EPKs, has also been attributed to differences in its sequence.140 Most EPKs contain a DFG motif, whereas CK2α feature a DWG motif. In EPKs, such as CK2’s closest analogue CDK2, the central Phe is dynamic and variable between the active and inactive states (green in Figure 15). In CK2 (purple in Figure 15) an additional hydrogen bond between the indole-nitrogen of the Trp176 and the backbone carbonyl of Leu173 is established, thereby maintaining the active state conformation.140
Upon complexation with CK2β, a major conformational change occurs in CK2α: the β4β5-loop of CK2α, which is in the open form in X-ray crystal structures of the holoenzyme, switches to the closed form in the monomeric form.143

2.1.1.2. CK2β

The CK2β subunit is smaller than the catalytic α domains (~28 kDa and ~48 kDa respectively) and, although it is not required to activate CK2α, it regulates the activity of the kinase. In particular, CK2β increases the thermostability of CK2 and enhances its catalytic activity. In addition, intracellular localisation of CK2 is influenced by the presence of CK2β which is required for the protein shuttling between intracellular compartments. Moreover, CK2β acts as a docking station for some CK2 substrates (i.e. Nopp140, p53, FAF-1, topoisomerase II, FGF-2, and eIF2β)144 by bringing the α subunit into proximity with them. In addition, CK2β is needed to dock to and penetrate the nuclear membranes.145 CK2β is present as a homodimer in solution and comprises of a body and a tail (Figure 16). The body includes the N-terminus and the dimerisation domain, which comprises the zinc binding site. The zinc is anchored by three-stranded anti-parallel β sheets and four cysteine residues; The tail includes the C-terminus, and it is formed by a β-turn and a short α-helix.

The β-turn includes the hydrophobic hotspot residues which are essential for the docking to the CK2α subunit. In particular, the lack of residues Arg186, Tyr188, Phe190, and His193 is detrimental to the holoenzyme formation. Unlike for CK2α, no major conformational changes occur in CK2β upon holoenzyme assembly, as documented by the X-ray structures of the subunit in isolation and complexed with CK2α.138
Chapter 2: Anti-apoptotic proteins - CK2

Figure 16 – Structure of CK2β (PDB code: 1JWH).\textsuperscript{138} a) CK2β homodimer: the two CK2β subunits are shown as blue and green ribbons, the zinc ions as orange spheres. b) Details of the CK2β domain: N-terminal domain is shown as yellow-orange-green and contains the dimerisation domain; the C-terminal domain (purple) contains the portion of CK2β that binds to CK2α.

2.1.1.3. Signalling pathways involving CK2

CK2 is involved in a multitude of subcellular pathways at different levels and has a substantial impact on cell growth, proliferation, and apoptosis.\textsuperscript{146} The importance of CK2 in cell proliferation and growth is evident considering the increase in its expression level in healthy cells during cell proliferation. Moreover, CK2α-CK2β knockout mice models led to mice death, emphasising the essential role of this protein in cell survival.\textsuperscript{134} The mechanisms through which CK2 acts as an apoptosis-suppressor involve several signalling pathways, including the secreted cysteine-rich glycoproteins (Wnts), IAPs, reactive oxygen species (ROS), caspases, TNF pathway, and PI3K/AKT thereby acting on multiple pathways of the apoptotic cascades (Figure 17).\textsuperscript{146} For example, CK2 promotes degradation of the NF-κB-inhibitory-protein IκB and consequent activation of the transcription factor NF-κB.\textsuperscript{147} In addition, CK2 activates different components of the Wnt pathway, such as the dishevelled protein (Dvl) via phosphorylation: it phosphorylates, stabilises, increases the transcriptional activity of β-catenin and recruits other Wnt regulators. When activated, Wnt signalling inhibits the so-called disruption-complex, preventing apoptosis.\textsuperscript{148} As reported by Piazza et al., PIP3 (phosphatidylinositol 3,4,5-triphosphate) is generated by the kinase PI3K and causes the activation of several downstream protein kinases of the survival pathway (vide supra), including AKT/PKB, which critically regulates cell survival, proliferation, and oncogenesis. Moreover, PTEN is a lipid and protein phosphatase able to dephosphorylate PIP3, and it is an essential tumour suppressor. CK2 phosphorylates PTEN in the C-terminal region, stabilising the protein against ubiquitin-mediated proteasomal
degradation, and enhancing *PTEN* activity. In addition to its direct regulation of *PTEN*, CK2 is able to activate AKT through direct phosphorylation and prevent dephosphorylation of its active form *via* an indirect mechanism.\(^{148}\)

**Figure 17** - Overview of the central role of CK2 in the apoptotic pathways. Anti-apoptotic proteins are shown in red. Activation is depicted as an arrow, inhibition as a dash at the end on the line.

The link between CK2 and ROS has been investigated by Ahmad et al. using various prostate cancer models.\(^ {149}\) The research group discovered that when CK2 is inhibited, the intracellular levels of \( \text{H}_2\text{O}_2 \) increased leading to the release of pro-apoptotic signals such as cytochrome c, activation of caspase 3, downregulation of IκB, translocation of NF-κB, and DNA fragmentations. Moreover, expression levels of the anti-apoptotic IAPs are diminished when CK2 is inhibited.\(^ {149}\)

### 2.1.1.4. Approaches towards the inhibition of CK2 in oncology

Among other features, cancer cells present dysregulated proliferation and deregulated apoptotic activity, leading to uncontrolled cell growth.\(^ {150}\) The well-established overexpression of CK2 in cancer cells has been attributed to a new basal level of CK2 protein expression taking place when healthy cells morph into cancer cells. The new and dysregulated basal level disrupts the cell homeostasis and makes the cells more sensitive to CK2 inhibition.\(^ {150}\) In healthy cells, however, the level of CK2 expression increases only during controlled proliferation.\(^ {146}\) The ability of CK2 to affect cell proliferation and apoptosis in both normal and cancer cells has been validated using several *in vivo* and *in vitro* experimental models.\(^ {134,135}\) Therefore, as reported by Trembley et al.,\(^ {146}\) the importance of targeting CK2 for anti-cancer purposes can be understood.
considering:

- CK2 appears to be largely affected by mitogenic signals;
- Downregulation of CK2 expression has a strong influence on inflammation, angiogenesis, and drug efflux pathways to the benefit of cancer cell elimination;
- Dysregulated expression of CK2 in cancer cells is an index of the pathological status of the tumour;
- CK2 downregulation results in inhibition of cell growth, proliferation, and an increased apoptotic activity;
- CK2 is crucial for cell survival, and there appear to be no redundant pathways to compensate for its downregulation.

All this evidence together makes CK2 inhibition an attractive target for cancer therapy.\textsuperscript{146}

A wide variety of approaches have been used to inhibit CK2, and both RNA knockdown and small molecules strategies have been embraced. The first antisense oligonucleotide was developed by Faust \textit{et al.}\textsuperscript{151} Although the oligodeoxynucleotide was able to cause an antisense-mediated disruption of CK2 leading to apoptosis, it proved less efficacious in reducing the expression level of the protein.\textsuperscript{152} Therefore, small molecules were preferred to induce CK2 inhibition as they have the potential to be more selective, with different strategies being applied and reported herein (Table 3).

The most common one is the inhibition of CK2 with small molecules that bind at the ATP-binding site; however, despite their efficiency in providing low nM-range inhibitors, they suffer the drawback of having off-target activity towards other kinases with a similar ATP-binding sites.\textsuperscript{135,152} Several ATP-competitive inhibitors showing a range of potency and selectivity have been developed either in industry or in academia – \textit{e.g.} tetrabromobenzimidazole derivatives (TBB, TBI, DMAT),\textsuperscript{153} condensed polyphenolic derivatives,\textsuperscript{154} indoloquinazoline-based compounds\textsuperscript{155} and pyrazolo[1,5-a]pyrimidine derivatives.\textsuperscript{156} Among these, the ATP-competitive CK2 inhibitor CX4945, Silmitasertib, developed by Cyclene Pharmaceutical, represents the first orally bioavailable inhibitor of CK2 currently undergoing clinical trials.\textsuperscript{139} CX4945 inhibits CK2 with an IC\textsubscript{50} of 1 nM, and its selectivity was screened against a panel of 238 kinases; 7 of these were inhibited at more than 90% showing that CX4945 is able to engage kinases other than CK2.\textsuperscript{139}
Table 3 - An overview of some inhibitors of CK2 grouped by type of inhibition.

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<td>Displacer of CK2α substrates</td>
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<td>Inhibitors of the PPI between CK2β and its substrates</td>
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<tr>
<td>Allosteric inhibitors with unknown binding mode</td>
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<td>Inhibitors of the α/β PPI</td>
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Due to potential selectivity issues linked to targeting the highly conserved ATP-binding site, an
increasing interest has arisen in the development of non-ATP-competitive and allosteric inhibitors.\textsuperscript{a,157} CK2 inhibition exploiting binding sites outside the catalytic ATP pocket has been achieved using a variety of approaches (Table 3).

An interesting approach towards non-ATP-competitive inhibition is the one used by Perera et al. displacing the CK2 substrates. CK2 targets present an acidic phosphoro-acceptor site which allows for phosphorylation, and peptide P15 was designed to bind to these appendices to inhibit substrate phosphorylation in a non-ATP-competitive fashion.\textsuperscript{158}

Other approaches target the CK2β regulatory subunit. For instance, peptide P1 binds to the N-terminal domain of the β subunit, triggering cell apoptosis by preventing key interactions of the α subunit with specific anti-apoptotic protein substrates.\textsuperscript{159}

An additional interesting non-ATP-competitive strategy is the disruption of the α/β subunit interaction. Inhibition of the PPI between the α catalytic subunit and the β regulatory subunit results in prevention of the holoenzyme assembly, with negative consequences on substrate recognition, protein shuttling between different intracellular compartments, and the stability of the catalytic subunit, thereby impacting the cell cycle and cell progression. The inhibition of the holoenzyme assembly by targeting the interface has been reported to be a more selective and elegant way of affecting the activity of CK2, especially in the light of the fact that the catalytic α subunit remains active towards some of its substrates and that the protein interface is not conserved among other kinases.\textsuperscript{160}

The interface of the isolated CK2 protein was successfully targeted using small molecules such as the podophyllotoxin indole analogue W16\textsuperscript{160} or using the cyclic disulfide bridged peptide Pc.\textsuperscript{161} Interestingly, the small molecule W16 inhibited the catalytic activity of CK2α in an allosteric manner whilst Pc resulted in an enhancement of the catalytic activity.\textsuperscript{160,161} The potency of these two compounds in a cellular context is unknown. Similarly, Raaf and et al. showed that the known CK2 inhibitor DRB was also able to bind at the interface although, with a low binding affinity insufficient to cause a biological response.\textsuperscript{162} Lately, a 12-mer peptide, named B2 was identified using phage-display technology. B2 is able to disrupt both the PPI and the catalytic activity with an identical IC\textsubscript{50} of 0.8 μM; however, it is not clear how the 12-mer peptide binds to CK2α and how it inhibits the catalytic activity of the protein.\textsuperscript{163} Recently, a cyclic peptide analogue of Pc was reported in the literature: the Pc peptide was conjugated with a TAT cell-penetrating peptide to gain cell-permeability, and the disulfide bond in Pc was replaced with a

\textsuperscript{a} Allosteric inhibition aims to develop a ligand for a site different from the active site, but able to cause a change in the protein structure which will impede catalysis.\textsuperscript{247}
head-to-tail lactam. Although cell-permeable, there are no reports of the binding mode or target engagement of TAT-Pch.\textsuperscript{164} Data on the stability of the complex under physiological conditions are also missing. It is therefore clear that, despite recent advances, selective, stable, and potent inhibitors acting at the CK2 PPI are still missing. The discovery of molecular probes able to disrupt the PPI of CK2 in cells and, potentially, \textit{in vivo} would shed light on the regulatory mechanisms of the protein and the importance of the holoenzyme to cancer progression.

\textsuperscript{164} Cell-penetrating peptide derived from the transactivator of transcription domain (TAT) of human immunodeficiency virus. The sequence of TAT peptide is GRKRRQRRRPQ.
2.1.2. Anti-apoptotic Bcl-2 proteins

Bcl-2 proteins are a family of proteins with well-established roles in apoptosis. The family comprises membrane-associated and cytosolic proteins with both pro- and anti-apoptotic functions acting on mitochondria outer membrane permeabilization (MOMP), thereby representing an essential component of the intrinsic apoptotic cascade.\(^{165}\) The proteins of the Bcl-2 family can be divided as follows: pro-apoptotic (Bim, Bad, Bid, Bmf, Noxa, Puma, Hrk, Bik); anti-apoptotic (Bcl-x<sub>L</sub>, Bcl-w, Bcl-2, Mcl-1, and A1); effectors of apoptosis (Bax and Bak).\(^{165}\) They share the so-called Bcl-homology (BH) domains that can be grouped as follows: BH1, BH2, BH3, and BH4.\(^{166}\) The Bcl-2 family members interact with each other via a series of PPIs made possible by the BHs. The BHs, in fact, are necessary for the formation of homo- and hetero-complexes. The pro-apoptotic proteins are often referred to as BH3-only proteins since they conserve the BH3 domain only. The anti-apoptotic proteins, on the other hand, can present all four homology domains, with BH1 and BH2 necessary to interact with the effectors of apoptosis Bax and Bak.\(^{166}\) The exact mechanisms by which apoptosis is induced by the Bcl-2 family members is highly debated and not fully understood. It is widely accepted that the pro-apoptotic proteins form a heterodimer with the anti-apoptotic proteins thereby inducing conformational changes that displace the effectors of apoptosis from their complex with the anti-apoptotic proteins (Figure 18a). Once freed, Bax and Bak become able to trigger apoptosis by affecting the MOMP. Alternatively, the BH3-only proteins bind and activate Bax and Bak directly: proteins that can interact with the effectors of apoptosis directly are called activators (Bid, Bim, Puma), whereas proteins that bind to the anti-apoptotic proteins are called sensitisers (Bad, Bmf, Bik, Noxa, Hrk) (Figure 18a).
It should be noted that the activators can also act as sensitisers, but sensitisers do not act as activators. When active, Bax and Bak oligomerise on the mitochondria membranes by means of the BH3-domain, resulting in the formation of pores, and hence triggering apoptosis.\textsuperscript{165–168} It should be noted that members of the Bcl-2 family may also interact with proteins outside of their group. In addition, the PPI between pro- and anti-apoptotic Bcl-2 proteins occur in a very selective manner, with BH3-only proteins interacting with specific Bcl-2 anti-apoptotic proteins only as exemplified in Figure 18b.

2.1.2.1. The function of Bcl-2 proteins in the survival of nucleated cells

As for all the anti-apoptotic proteins, the Bcl-2 family members act as guardians of cell death and survival; therefore, alteration in gene expression or activity of Bcl-2 proteins leads to pathological conditions. For example, the expression levels of Bcl-2 anti-apoptotic proteins have been found to be particularly elevated in a number of cancers.\textsuperscript{169} Cancerous cells over-expressing anti-apoptotic Bcl-2 proteins can easily escape apoptosis and therefore are not eliminated by the organism. Elevated expression levels of Bcl-2 anti-apoptotic proteins can be attributed to the loss of endogenous mRNAs that represses Bcl-2 gene expression under physiological conditions, to changes to the gene structures, the number of copies, and gene hypomethylation.\textsuperscript{169} It is not surprising that inhibition of anti-apoptotic proteins of the Bcl-2 family is an attractive, well-exploited strategy used in oncology to restore the physiological level of apoptosis, and hence allowing the organism to eliminate defected, cancerous cells \textit{via} the PCD.
2.1.2.2. Approaches towards the inhibition of anti-apoptotic Bcl-2 proteins

The role of the anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1) is to sequester the effectors of apoptosis Bax and Bak, therefore, preventing their pro-apoptotic activity. Any molecules that can antagonise the action of the anti-apoptotic proteins by displacing or liberating the effectors of apoptosis are known as Bcl-2 inhibitor. Since the BH3 only proteins act as inhibitors of the anti-apoptotic Bcl-2 proteins, the majority, if not all of the Bcl-2 inhibitors known to date are BH3 mimetics: they bind to the hydrophobic groove of the anti-apoptotic proteins in the same way that BH3-only proteins do with their α-helical BH3 domain (Figure 19).[^170]

![Figure 19](image_url) - Typical binding mode of BH3-only proteins to Bcl-2 anti-apoptotic proteins. The interaction between Bim and Bcl-2 is shown here (PDB: 1PQ1).[^171] a) View from the top of the BH3 domain of the Bim protein (green) complexing with Bcl-xL. b) Side-view of the complex showing the binding of the helical Bim protein into the hydrophobic groove of Bcl-xL. Protein surface is coloured according to the electrostatic potential: grey (hydrophobic), blue (negatively charged) and red (positively charged).

All the BH3 mimetics developed to date can be divided into two categories: true BH3 mimetics – characterised by high affinity for the target and no off-target effects – and putative BH3 mimetics – showing low affinity for the anti-apoptotic targeted protein and several off-target effects.[^170] Although non-selective, putative BH3 mimetics are often used to investigate resistance mechanisms in cancer cells.[^170] BH3 mimetic therapeutics comprise both small molecules and peptides, and they all find application as anti-cancer drugs. Table 4 lists some notable compounds inhibitors of the anti-apoptotic Bcl-2 proteins that were promoted to the late stages of the drug discovery process.
### Table 4 - Non-comprehensive list of notable Bcl-2 inhibitors in advanced phases of drug discovery.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Structure</th>
<th>Target</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-737</td>
<td><img src="image" alt="ABT-737" /></td>
<td>Bcl-2, Bcl-xi, Bcl-w</td>
<td>Stopped after pre-clinical studies</td>
</tr>
<tr>
<td>Navitoclax (ABT-263)</td>
<td><img src="image" alt="Navitoclax" /></td>
<td>Bcl-2, Bcl-xi, Bcl-w</td>
<td>Stopped after phase II studies</td>
</tr>
<tr>
<td>Venetoclax (ABT-199)</td>
<td><img src="image" alt="Venetoclax" /></td>
<td>Bcl-2</td>
<td>Approved</td>
</tr>
<tr>
<td>Gossypol</td>
<td><img src="image" alt="Gossypol" /></td>
<td>Bcl-2, Bcl-xi, Bcl-w</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Obatoclax</td>
<td><img src="image" alt="Obatoclax" /></td>
<td>Bcl-2, Bcl-xi, Bcl-w, A1, Mcl-1</td>
<td>Stopped after phase II</td>
</tr>
<tr>
<td>SHABa Bad</td>
<td><img src="image" alt="SHABa Bad" /></td>
<td>Bcl-2, Bcl-xi, Bcl-w</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>
ABT-199 (Venetoclax) is the first marketed Bcl-2 inhibitor. ABT-737 and ABT-263 (Navitoclax) are analogues of Venetoclax whose clinical trials were stopped due to a dose-limiting thrombocytopenia. Venetoclax was developed to overcome this limitation by interacting with the anti-apoptotic Bcl-2 protein selectively. The thrombocytopenia caused by ABT-737 and Navitoclax inspired scientists to investigate the role of the Bcl-2 proteins in platelets.

2.1.2.3. The function of Bcl-2 proteins in platelets apoptosis and activation

Platelets are short-lived (8-10 days in humans), anucleated cells produced from megakaryocytes in the bone marrow, spleen, and foetal liver, and they constitute a key component of the blood. Even though platelets do not have a nucleus, they contain the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, and some mRNAs. Platelets play essential roles in pathophysiological conditions including haemostasis, cardiovascular thrombotic events, cancer metastasis, inflammation and autoimmune diseases. In particular, platelets maintain fluidity of the circulating blood and become active to trigger clot formation in the event of wounds, thereby preventing the organism from bleeding to death. During cardiovascular thrombotic events, the number of platelets in circulation increases and, in addition, platelets are considered responsible for the formation of atherosclerotic plaques, inflammatory processes, immune responses, and play a role in neurodegenerative conditions. Similarly, platelets are important in cancer metastasis, where they help to tether circulating cancer cells, therefore increasing their invasiveness.

Platelet activation and lifespan are regulated by complex PPIs, the understanding of which has received increasing attention in recent years due to the relevance of this type of cell to many pathological conditions.

The proteins of the Bcl-2 family have a key role in regulating intrinsic apoptosis and the lifetime of platelets. The role of Bcl-2 proteins in platelet survival has been investigated using transgenic animal models, and more recently, BH3-only small molecule mimetics ABT-737, Navitoclax and ABT-199, that find application in oncology. Interested in the dose-limiting
CHAPTER 2: Anti-apoptotic proteins – Bcl-2

thrombocytopenic side-effect of ABT-737 and its orally available analogue Navitoclax, Debrincat et al. investigated the effect of these Bad-like BH3 mimetics on platelet apoptosis. They found that the anti-apoptotic protein Bcl-xL is indispensable to platelet survival and its inhibition causes platelet death. On the contrary, ABT-199, a selective Bcl-2 inhibitor, does not cause platelet apoptosis. Similarly, concomitant depletion in mice of Bcl-2 and Mcl-1 was found not to be detrimental to platelet survival.

It is interesting to note that activated platelets go through apoptotic-like changes, and the apoptotic and activation pathways converge on the loss of mitochondrial membrane polarisation (MMP) and the occurrence of phosphatidylserine (PS) exposure (Figure 20). It is the essential roles of Bcl-2 proteins in regulating MMP that suggested they might have a central role in both platelet apoptosis and activation. However, the mechanistic differences between the apoptotic and activation signalling are poorly understood. The main divergences between the two pathways are described by Vogler et al. as follows, and these conclusions derived from the use of ABT-737 in platelets:

- Bcl-2-mediated apoptosis is slower than Bcl-2-mediated platelet activation;
- Activation of caspases is essential for apoptosis but not for activation, at least in the first stages of platelet activation to a pro-coagulant phenotype;
- Platelet activation is a calcium-dependent mechanism, whilst apoptosis is not.

Figure 20 - Exemplified differences between apoptosis and activation in platelets. Apoptosis (pink) is induced by ageing, stress, hypoxia etc. and activates the intracellular intrinsic pathway that culminates with loss of MMP, the release of cytochrome c from the mitochondria, activation of caspases, PS exposure. Activation (yellow) in response to agonist or collagen overlaps to some degree with the apoptotic pathway. Once active, the platelets change shape, release granules and expose glycoprotein receptors to facilitate aggregation at a later stage. The factors released by pro-coagulant platelet trigger the coagulation phase (green) that culminates with the clot formation enabled by fibrin.
In addition, Vogler et al. have shown that inhibition of the anti-apoptotic proteins Bcl-2 and Bcl-x\textsubscript{L} by the Bad mimetic ABT-737 inhibits platelet activation whilst resulting in platelet apoptosis, supporting the hypothesis of distinct roles for the Bcl-2 proteins in platelet activation and apoptosis.\textsuperscript{188,190}

Once platelets are activated by agonists or adhesion to the collagen exposed on the blood vessel following an injury, they secrete granules (\textit{i.e.} \(\alpha\)- and dense granules) necessary to trigger the coagulation cascade, change shape, and expose glycoproteins (\textit{i.e.} GP\textsubscript{IIb/IIIa}). All these events are necessary for the fibrinogen binding and clot formation (Figure 20).

Whilst the role of some Bcl-2 proteins in platelet survival and activation has been elucidated (Bad, Bcl-2), a deep understanding of the involvement of all the members of the Bcl-2 family in platelet apoptosis and activation is still missing.

The lack of a nucleus in platelets makes the use of conventional recombinant techniques ineffective and hampers the study of PPIs, and hence the elucidation of intracellular pathways. As for nucleated cells, the use of small molecules to study PPIs can lead to selectivity problems due to the lack of specificity among related proteins.\textsuperscript{25} In addition, as highlighted earlier, the design and development of small molecules targeting a shallow protein-protein interface can be challenging.\textsuperscript{191} On the other hand, an alternative way to study PPIs in platelets is provided by the use of transgenic animal models. Nevertheless, the generation of such models is time-consuming, expensive, may be prone to side-effects during the development of the embryo, and may not be representative of the human situation.\textsuperscript{192}

The understanding of PPIs in human platelets could aid the discovery of selective and specific anti-platelet therapeutics which could find use in the treatment of cardiovascular diseases, inflammation and cancer therapy. Therefore, there is an unmet need for new, complementary and efficient approaches to study medicinally-relevant PPIs that can be used in human platelets directly overcoming the above-mentioned limitations. Macrocyclic peptides may be a suitable solution; however, there are no reports of their use in platelets.\textsuperscript{193}
2.1.3. Brief considerations on selectivity

The main problem associated with targeting anti-apoptotic proteins is selectivity. Whilst overexpressed in diseased cells, anti-apoptotic proteins are also present in healthy cells, and therefore, side-effects may be an issue. Off-target effects and unspecific cytotoxicity can be minimised by exploiting any small structural differences between the targeted protein in the diseased and healthy cells or by taking advantage of specific receptors on the surface of the targeted cell-type that may facilitate the entrance of the therapeutic agent. Biologics are often used to this end due to their high specificity, however, they suffer from poor PK properties, have high development costs, and can lead to immunogenicity;\textsuperscript{194,195} small molecules, on the other hand, can be more easily tuned to optimise their PK properties, but they often lack the high specificity of more complex macromolecules.\textsuperscript{24} This can lead to off-target effects. Such off-target effects can be minimised by taking advantage of allosteric sites that are not well conserved in a family of proteins.\textsuperscript{157} Short peptides sit in between these two categories of drugs, sharing the high specificity of biologics, and, if properly modified, the PK properties of small molecules. They can act at the interface of protein signalling, disrupting PPIs, thus targeting shallow and relatively large surfaces (800-2000 Å\textsuperscript{2}), instead of deep binding pockets.\textsuperscript{3,25,40}
SECTION II

Results and discussion
CHAPTER 3:

A Fragment-Based Approach Towards the Inhibition of the Anti-Apoptotic Protein CK2
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

3.1. Summary

CK2 (formerly Casein Kinase II) protein kinase plays crucial roles in cell growth, cell proliferation, and apoptosis. It is overexpressed in a wide range of cancer cells, and due to the lack of compensatory mechanisms, CK2 inhibition is an attractive strategy used in oncology: the small molecule CX4945 is currently under phase II clinical studies.\textsuperscript{139,146}

This chapter describes the discovery of novel CK2 inhibitors targeting poorly-conserved binding sites within the α catalytic subunit to overcome the limitations of existing inhibitors namely, lack of specificity for CK2 and poor drug-like properties.

Using a fragment-based drug-discovery (FBDD) approach, \textbf{3l} and \textbf{3g} were developed. \textbf{3l} is the first selective CK2α inhibitor, with drug-like properties, that engages the cryptic αD pocket of CK2α and does not interact with the highly-conserved ATP-binding site (Figure 21). \textbf{3g} is the first fragment-sized molecule that binds at the interdomain surface of CK2 and that could act as a starting point for the development of novel inhibitors of the holoenzyme assembly (Figure 21).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure21.png}
\caption{Previous and current approaches to develop CK2 inhibitors. Structures of compounds \textbf{3g} and \textbf{3l} are shown at the bottom of the figure.}
\end{figure}
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

3.2. Project background

The Spring group and collaborators at the University of Cambridge became interested in developing novel CK2 inhibitors acting outside the ATP-binding site to engage this exciting target. Prior to the work described in this thesis, pioneering studies on CK2 inhibition using a FBDD approach were conducted by Dr Chris Stubbs in the Abell Group (Department of Chemistry, University of Cambridge)\textsuperscript{196} and Dr Paul Brear in the Hyvönen Group (Department of Biochemistry, University of Cambridge). An initial FBDD approach was adopted, and X-ray crystallography used to screen 400 fragments from the 'NMR fragment library' developed in the Abell Group. The screening provided promising preliminary results, yielding several hits that showed promiscuity for several binding sites within the α catalytic subunit; interestingly, some fragments were found to bind at the α/β interface of the protein, whereas others revealed a new pocket in proximity to the αD helix, referred to as the αD pocket.\textsuperscript{197,198} It was therefore decided to optimise the fragments for the two above-mentioned binding sites in an independent manner in order to improve the binding affinity of the initial hits and, ultimately, avoid undesired promiscuity. Optimisation of the fragments binding at the protein interface was predominantly carried out by Dr Kathy Hadje Georgiou during her PhD in the Spring Group\textsuperscript{197} whereas, optimisation of fragments binding in the hidden αD pocket was performed by Dr Claudia De Fusco and Dr Laura Carro, Postdoctoral fellows in the Spring Group.

3.2.1. Preliminary work on fragments binding in the αD pocket

Although the αD pocket was mentioned in work by Kinoshita and co-workers,\textsuperscript{199} the full size of this pocket was only revealed upon binding of the fragments synthesised in the Spring Group (Figure 22). Considering the αD helix of CK2α is less conserved than the ATP site amongst related protein kinases of the CMGC\textsuperscript{j} family,\textsuperscript{4} it was thought that this pocket could be exploited further to develop selective CK2α inhibitors.

\textsuperscript{i} Library assembled for NMR screening of other targets. In this project, the library was screened by X-ray crystallography.

\textsuperscript{j} Family of kinases including CDK, CDKL, CK2, CLK, DYRK, GSK, MAPK.
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

Following initial screening, the fragments that bound at the αD pocket, as confirmed by electron density seen in X-ray co-crystallography, were elaborated using iterative cycles of design, synthesis, and biological testing. The structure and binding affinity ($K_d$ values obtained by ITC) of some of the fragments are shown in Figure 23.

In summary, using a FBDD approach the initial hit NMR154L was successfully elaborated into F6 with a $K_d$ of 41 μM and a ligand efficiency (LE) of 0.38 Kcal mol$^{-1}$ heavy atom$^{-1}$. 
Although the optimisation of the fragments improved the affinity due to their ability to expand the αD pocket, inhibition of CK2 activity was not observed. X-ray crystal structures of the molecules bound to CK2α showed that the residue Met163 did not flip to the ‘out’ conformation and did not displace the ATP from its binding site hence resulting in no inhibition of phosphorylation (Figure 22). Such a residue-flip is thought to be essential for the inhibition of the catalytic activity of CK2α. It was envisioned that growing the fragments towards the ATP-binding site would allow for ATP displacement by triggering the Met163 flip while keeping the selectivity over other kinases by the interacting with the poorly-conserved αD pocket. To this end, a series of compounds bearing aliphatic, aromatic and heteroaromatic moieties were synthesised and tested. The most promising compound, CAM4066 inhibited phosphorylation of CK2 substrates by successfully linking fragment F5 to a weakly binding ATP fragment via a flexible linker (Figure 24).

The ability of CAM4066 to arrest cell proliferation in cancer cells was comparable to that of the clinical candidate CX4945 (GI50 8.8 μM vs 4.8 μM respectively). However, CAM4066 proved to be more selective than the ATP-competitive inhibitors reported in literature: unlike CX4945, none of the closely related kinases of the CMGC family were inhibited by CAM4066.

Although CAM4066 was the first CK2 inhibitor to bind to the cryptic αD pocket, the compound presented several limitations. In particular, the molecule featured a high number of amide bonds, rotatable bonds, hydrogen-bond donors (HBDs) and acceptors (HBAs), and a drop-off of activity in cells which required the formulation as a methyl ester pro-drug (pro-CAM4066) to

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8 Synthesis performed by Dr Claudia De Fusco, Dr Kathy Hadje Georgiou, Dr Laura Carro; biological evaluation performed by Dr Paul Brear.
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

gain cell entry due the presence of the carboxylic acid. In addition, the compound still included a weakly-binding portion interacting with the highly-conserved residues of the ATP binding site.

3.2.2. Preliminary work on fragments binding at the α/β interface

It emerged from a literature search at the time that only three classes of molecules are reported to bind at the α/β CK2 interface:

- DRB, a small molecule binding at the interface and at the ATP binding site;\(^{162}\)
- W16 and azonaphthalene derivatives for which no x-ray structural information is available;\(^{160,201}\)
- Pc peptide and its analogues, cyclic peptides with poor drug-like properties that can disrupt the interaction between the α and β subunits.\(^{161,164}\)

The limitations of these molecules regarding activity and drug-likeness led the Spring group to further elaborate on NMR154L. Such a fragment was identified as hit from the initial X-ray screening and it was found to bind at the protein interface as well as in the αD pocket. The ultimate goal was to deliver drug-like chemical tools to study regulatory mechanisms of CK2 by acting at the interface. This fragment elaboration was carried out prior to the work described in dissertation, and in parallel to the optimisation of the αD fragments. Seventy fragments were synthesised\(^{202}\) elaborating from the initial molecule NMR154L, and some of them are represented in Figure 25.

\(^{1}\) For chemical structures vide 2.1.1.4
\(^{202}\) Synthesis carried out by Dr Kathy Hadje Georgiou and part III students Alexandra Lubin and William Green
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Some of the elaborated fragments made by previous members of the Spring group to develop CK2 inhibitors of the α/β interface. In yellow, substituents on the amine motif of NMR154L, in green substituents of the 4-chlorine atom, in pink substituents of the ethylamine group.

Unfortunately, despite the many fragment elaboration steps carried out in the group, none of the molecules showed affinity below the high μM range.
3.3. Project aim and overview

In this dissertation, the FBDD approach towards CK2 project aimed to overcome the limitations of CAM4066 to develop selective second-generation CK2 inhibitors targeting the αD pocket. In particular, the project aimed to develop drug-like molecules with a reduced number of rotatable bonds, HBDs, HBAs, able to inhibit CK2α without interacting with the well-conserved ATP binding pocket and that could exert their biological activity without needing to be administered as a pro-drug.

This chapter describes the rational design, synthesis, and biological testing of the elaborated fragments binding to the αD pocket of CK2. We envisioned that the need for the interaction with the ATP binding site could be avoided and selectivity enhanced by increasing the affinity of the αD-binding motif of CAM4066. To this end, various substituents on the lower and upper aromatic rings of F5 were explored to interact with vacant parts of the αD pocket. The combination of the best substitution patterns and a fragment merging strategy resulted in the development of a second-generation CK2 inhibitor (3l) with improved drug-like properties.

During the initial optimisation of the fragments for the αD pocket, some molecules were found to bind predominantly at the α/β interface of the CK2 protein. Their synthesis, biological testing, and X-ray crystallographic structures are described in this chapter.

An overview of the molecules subject of this chapter is provided in Scheme 4.
Growing strategy to explore vacant part of the pocket and/or push the α helix

Scheme 4 - Overview of the molecules discussed in this chapter. Compounds shown in black were found to bind in the αD site, compounds in grey were found to bind predominantly at the protein interface. The compounds are grouped according to the rationale behind their design and synthesis.
3.4. Results and discussion

All of the compounds were synthesised and co-crystallised with CK2α, and the results are reported in this section. The fragments that showed electron density in the αD pocket or at the α/β interface were further tested in assays to determine their affinity for the protein as well as a phosphorylation assay to assess their ability to inhibit the catalytic activity of the protein. Ligand Efficiency (LE) was also calculated in order to assess the relative-quality of the fragments. Biological testing and X-ray crystallography were carried out by our collaborator Dr Paul Brear in the Hyvönen group, Biochemistry Department, University of Cambridge.

The molecules whose synthesis and biological evaluation are described in the following paragraphs were synthesised according to the generic synthetic route adapted from previous reports and outlined in Scheme 5.

![Scheme 5](image)

The appropriately substituted aryl derivative 1 underwent a Suzuki cross-coupling reaction with the desired aryl boronic acid or ester to afford the biaryl of generic structure 2. Intermediate 2 was reduced in its nitrile or aldehyde functionality to afford the desired final amine of generic structure 3.

3.4.1. Fragments exploring vacant parts of the αD pocket

3.4.1.1. 1-Methanamine, 3-chloro biaryl fragments: 3a-3e

The X-ray structure of F5 and F6 (synthesised by Dr Kathy Hajde Georgiou and Dr Laura Carro prior to this work) co-crystallised with CK2α provided the basis for the design of compounds 3a-3e. In particular, functional groups with different properties were employed with the aim of exploring the vacant part of the αD pocket (Figure 26). It was envisioned that the affinity of the

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*a The work described in this chapter has been published in scientific journals. In particular, the synthesis and biological evaluation of compounds 3a-3d and 3i-3l have been reported in Legre et al., ChemSci, 2018 and the synthesis and biological evaluation of compound 3g has been published in Brear et al., BMC, 2018. Part of the text and figures presented in this chapter has been adapted from the above-mentioned publications.
fragments could be enhanced by engaging additional interactions within the said pocket. The availability of the starting materials was also a factor influencing the design.

![Figure 26](image)

*Figure 26 – Chemical structures (a) and co-crystal structures of F5-F6 in the αD pocket showing the pocket expansion going from F5 (b) to F6 (c).*

The first step of the synthetic route leading to the 1-methanamine, 3-chloro biaryl fragments was envisioned to be a Sukuki-Miyaura cross-coupling between 2-chloro benzonitrile of generic structure 1 and the appropriate boronic acid to afford the biaryl derivative 2. Nitrile reduction of 2 would yield the desired amine 3 (Scheme 6).¹⁹⁸

![Scheme 6](image)

*Schematic 6 – Generic synthesis of 1-methanamine, 3-chloro biaryl fragments of generic structure 3.*

The Suzuki-Miyaura step required optimisation as initial attempts to synthesise the first biaryl intermediate 2a following previously reported procedures¹⁹⁸ resulted in low yields (Table 5).

3-chloro-4-bromo-benzonitrile and 2-isopropylphenyl boronic acid were employed as substrates in the presence of Pd(PPh₃)₄, Na₂CO₃, and DME according to literature precedent.²⁰³ However, the reaction did not proceed to complete conversion, and several unidentified impurities were formed, which proved inseparable from the desired product via column chromatography. We attempted to reduce the amount of by-products by varying reaction time (between 1 and 3 h), catalyst equivalents (0.005 and 0.010) or type of heating (microwave irradiation and thermal). Pleasingly, the desired product 2a was obtained in good yield (85%) when the reaction was performed using the triflate derivative of 1 (1a hereinafter) and thermal heating (entry 6, Table 5). The same reaction performed under MW irradiation gave the desired product 2a in poorer yield (entry 4, Table 5).
Table 5 - Optimisation of Suzuki coupling conditions to obtain compound 2a.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Catalyst equivalents</th>
<th>Heating</th>
<th>Temperature</th>
<th>Reaction time</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-Br</td>
<td>0.01</td>
<td>MW</td>
<td>130 °C</td>
<td>3 h</td>
<td>Aryl bromide/ unidentified impurity</td>
</tr>
<tr>
<td>2</td>
<td>-Br</td>
<td>0.005</td>
<td>MW</td>
<td>130 °C</td>
<td>1 h</td>
<td>Aryl bromide/ unidentified impurity</td>
</tr>
<tr>
<td>3</td>
<td>-Br</td>
<td>0.005</td>
<td>MW</td>
<td>110 °C</td>
<td>2 h</td>
<td>Unidentified impurities</td>
</tr>
<tr>
<td>4</td>
<td>-OTf</td>
<td>0.005</td>
<td>MW</td>
<td>130 °C</td>
<td>2 h</td>
<td>20% conversion to 2a</td>
</tr>
<tr>
<td>5</td>
<td>-Br</td>
<td>0.005</td>
<td>Thermal</td>
<td>90 °C</td>
<td>3 h</td>
<td>Unidentified impurities</td>
</tr>
<tr>
<td>6</td>
<td>-OTf</td>
<td>0.005</td>
<td>Thermal</td>
<td>90 °C</td>
<td>3 h</td>
<td>85% conversion to 2a</td>
</tr>
</tbody>
</table>

These optimised cross-coupling conditions employing 1a were successfully applied to the synthesis of the biaryl fragments shown in Table 6. Compound 1a was obtained in 91% yield by treating the commercially available 4-hydroxy-3-chloro-benzonitrile with Tf$_2$O in pyridine (Table 6).
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

Table 6 - Compounds obtained using the same Suzuki-Miyaura reaction conditions shown in entry 6, Table 5.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound number</th>
<th>Ar, R</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2b</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>2c</td>
<td></td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>2d</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>2e</td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>

Nitrile precursor 2c was obtained via Suzuki cross-coupling using the same conditions as described above with the exception that the corresponding pinacol boronic ester was used instead of the boronic acid for commercial availability reasons. In all Suzuki couplings described in Table 6, by-products tentatively assigned to be the result of incorporation of phenyl groups from PPh₃ in the products were obtained in small amounts, as previously reported. Traces of the de-boronated derivative of the boronic acid were also found in most cases.

The nitrile groups of the obtained biaryl fragments (generic structure 2, Scheme 6) were reduced to the corresponding primary amines 3 by treatment with LiAlH₄ and AlCl₃ in Et₂O. For compounds stable in acidic conditions, the corresponding amines were converted into hydrochloride salts using 4 M HCl in 1,4-dioxane. The salt proved to have greater kinetic solubility in the aqueous buffer used in biological testing than the corresponding free amines (Table 7).
CHAPTER 3: A fragment-based approach towards the inhibition of the anti-apoptotic protein CK2

Table 7 - Results of the nitrile reduction steps (entries 3 and 5) and treatment with HCl (entries 1, 2 and 4).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound number</th>
<th>Ar</th>
<th>R</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a</td>
<td></td>
<td>-NH₃⁺Cl⁻</td>
<td>77⁺</td>
</tr>
<tr>
<td>2</td>
<td>3b</td>
<td></td>
<td>-NH₃⁺Cl⁻</td>
<td>43⁺</td>
</tr>
<tr>
<td>3</td>
<td>3c</td>
<td></td>
<td>-NH₂</td>
<td>55⁻</td>
</tr>
<tr>
<td>4</td>
<td>3d</td>
<td></td>
<td>-NH₃⁺Cl⁻</td>
<td>32⁺</td>
</tr>
<tr>
<td>5</td>
<td>3e</td>
<td></td>
<td>-NH₃⁺CF₃COO⁻</td>
<td>54⁻</td>
</tr>
</tbody>
</table>

*overall yield of nitrile reduction and salt formation; »yield of the nitrile reduction only

Moderate yields could be attributed to the formation of the de-chlorinated analogues as observed by ¹H-NMR analysis of the crude reaction mixture, as well as a small amount of unreacted starting material and loss of mass during the purification step.²

Treatment of 3c with HCl led to degradation of the compound, most likely by polymerisation at the indole 3-position as shown by the lack of signal of the corresponding proton of the indole (NMR analysis of crude mixture).

²The amines synthesised were purified either on silica gel or aluminium oxide gel by flash chromatography. Silica gel was chosen over aluminium oxide due to its better separation potential. When this purification method was chosen, silica was deactivated using various percentages of 7 M NH₃ in MeOH in the eluent. However, due to the nature of silica gel and the polarity of the amine, an amount of product was inevitably lost in the purification due to the streaking of the compound through the column. For purifications where the difference between Rf of the impurities and the product was greater than 0.05, aluminium oxide was used as stationary phase and the streaking of the compounds was minimal.
Compound 3e was submitted for crystallisation as a trifluoroacetate salt since its corresponding amine was purified via preparative HPLC using acidic conditions (0.05-0.1% TFA). Purification of this compound using reversed-phase preparative HPLC was possible due to its increased polarity.

The so-obtained compounds were co-crystallised with CK2α. The electron density for compound 3a was observed at both the αD pocket and at the α/β interface showing promiscuity and partial occupancy of each site (Figure 27a-b). However, its binding mode in the αD pocket suggested the possibility of exploiting a space that opened up around the 6’ position of the bottom ring in addition to the usual 2’ position (Figure 27a).

The X-ray structures of 3b and 3d showed the compounds adopting the typical binding modes for these fragments in the αD pocket. Unlike the other fragments, the top aromatic ring of 3d seems to rotate as shown by the electron density for the chlorine atom observed at both the 3 and 5 positions (Figure 27c).

![Figure 27 - Crystallographic structure of 3a (PDB code: 5057), 3c, 3d (PDB code: 5058) and 3e binding to CK2α. a) 3a in the αD pocket; b) 3a at the interface; c) 3d in the αD pocket; d) 3c showing two different binding modes (blue and green) in the αD pocket; e) Fragment 3e binding at the interface. The protein surface is represented in grey, secondary structure as cyan ribbons and ligand in green or blue. The H-bonds between the ligands and the Val162 are represented as white dashed lines.](image)

The amine remains in the same position as the other fragments creating an H-bond interaction with the carbonyl of Val162 indicating that this is a strong interaction and an anchor point for all the fragments (Figure 27c). Interestingly, the X-ray structure of 3c showed two different binding modes in the αD site (Figure 27d). One of these poses (blue, Figure 27d) sees the indole motif closer to the αD helix while the other (green, Figure 27d) resembles the binding mode
characteristic of the biphenyl fragments previously synthesised in the Spring group. The combination of these two poses suggested that exploring different aromatic substituent patterns on the benzylamine core such as 3-indole-phenyl-methanamine derivatives might be beneficial (vide infra 3.4.1.3).

Biaryl fragments bearing H-bond donor/acceptor substituents at the 2’ methyl position such as 3e appeared to bind weakly at the α/β interface, and no electron density was observed at the αD site (Figure 27e). X-ray crystallography for compounds 3a-3d showed electron density in the αD pocket, and therefore, their binding affinity was determined via isothermal titration calorimetry (ITC). The results are shown in Table 8.

Table 8 - ITC and LE of the biaryl fragments F5, 3a-3d.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound number</th>
<th>Fragment</th>
<th>LE</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F5</td>
<td></td>
<td>0.32</td>
<td>267</td>
</tr>
<tr>
<td>2</td>
<td>3a</td>
<td></td>
<td>0.27</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>3b</td>
<td></td>
<td>0.38</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>3c</td>
<td></td>
<td>&gt;0.25</td>
<td>&gt; 500</td>
</tr>
</tbody>
</table>
Pleasingly, fragment 3b showed a significant enhancement in binding affinity compared to F5 with a $K_d$ of 17 μM representing the fragment with the highest binding affinity and LE of the whole series. Unfortunately, fragments 3a and 3c did not show improved binding affinity compared to F5 whilst 3d provided a slight improvement. However, their binding modes proved to be useful for the design of other fragments. The binding affinity of 3e for CK2α was not measured due to the weak electron density observed in the X-ray structure.

### 3.4.1.2. Biaryl compound bearing a 5-membered heterocyclic substituent: 3f

X-ray structures of commercially available and previously synthesised fragments – i.e. 3b and F7 – led to the proposal of a new biphenyl fragment bearing a 2-methyl-5-membered heterocycle on the bottom aromatic ring (Figure 28). By merging the two classes of compounds, it was envisioned that new interactions with the protein could be engaged. Docking studies were performed to establish the likelihood of these compounds to bind in the αD pocket. The results showed that the proposed compound 3f may lead to the heterocyclic ring expanding the αD pocket.

![Figure 28 - Fragment merging strategy. a) Overlap of compound 3b (blue) and F7 (green) made by Dr. C. De Fusco. b) Docking pose of 3f (purple) as a result of the merging of the previously mentioned fragments. CK2α is shown as a cross-section of the αD pocket. Protein surface is shown in grey.](image)

Structures of the other compounds docked can be found in the appendix A.3.
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With the docking study in hand, synthesis of 3f was commenced. In the first synthetic route attempted (Scheme 7) the synthesis of 3f started from a cross-coupling reaction between the commercially available 2-N-Boc-methanaminebenzeneboronic acid and compound 1a to afford biaryl nitrile intermediate 2f.

![Scheme 7 - Synthetic route attempted to synthesise the heterocycle derivative 3f.](image)

Compound 2f was then treated with HCl to remove the Boc group, and the reaction mixture was basified to obtain compound 2f’ as the free amine. The biphenyl compound was subjected to Clauson-Kaas reaction\(^{207}\) in the presence of 2,5-dimethoxytetrahydrofuran and glacial acetic acid to afford the corresponding pyrrole derivative 2f” in a 15% yield. Unfortunately, this proved to be unstable in the presence of AlCl\(_3\), which was used as a Lewis acid catalyst in the subsequent attempted nitrile reduction. Reduction in the absence of the Lewis acid also failed to yield the desired product 3f and only starting material was returned. An alternative nitrile reduction was carried out using H\(_2\) in the presence of Raney Nickel to afford trace amounts of impure 3f.

Considering the elevated cost of the boronic acid required and the low yield of the final two steps of the synthetic route, an alternative pathway was used to introduce a 5-membered heterocycle into the 2’ position of biphenyl fragments (Scheme 8). The new synthetic pathway commenced with a Suzuki coupling between 1a and (2-(hydroxymethyl)phenyl)boronic acid to afford biaryl compound 2e, which was subjected to nucleophilic substitution in the presence of N-bromosuccinimide.\(^{208}\) The corresponding bromide derivative 2f”” was synthesised in large
amounts to allow for potential subsequent diversification. Compound 3f was obtained from 2f” upon reaction of 2f”” with pyrrole in the nucleophilic substitution, followed by nitrile reduction using Raney Nickel. Due to the potential instability of pyrrole product 3f under acidic conditions, the compound was submitted for testing as the free amine.

![Scheme 8 - Synthetic route to compound 3f.](image)

Unexpectedly, X-ray crystallography of compound 3f revealed that only weak electron density was observed at the interface and no binding was detected in the αD pocket. Considering these results, the biological evaluation of compound 3f was not pursued, and the synthesis of analogues not attempted.

### 3.4.1.3. 3-Indole-phenyl-methanamine derivatives: 3g and 3h

The two poses found in the X-ray structure of 3c (Figure 27d) suggested investigating a new design set. Docking studies were performed to explore different aromatic substituent patterns on the benzylamine core and their ability to push the αD helix outwards. This strategy was regarded as promising since the flexibility of the α-helix is unique to CK2, and therefore this may help improve the selectivity of the compounds. For compound 3g docking gave promising...

*Structures of the other compounds docked can be found in the Appendix A.3.*
results, the docked compound overlapped with the X-ray structure of compound 3c, and the nitrogen of the indole was able to H-bond the carbonyl of the Met255 via a water molecule (Figure 29).

![Figure 29 - Docked 3g (purple) and X-ray structure of 3c (ligand in blue and protein in cyan).](image)

Compound 3g was synthesised in the hope that this could give information on the tolerability of the indole in the 5-position of the aryl ring prior to synthesis of more hindered compounds.

The synthesis of 3g (Scheme 9) commenced with a Suzuki-Miyaura cross-coupling between indole-3-boronic acid pinacol ester and 3-chloro-5-bromobenzonitrile to afford 2g in 48% yield. A slightly higher yield (55%) was achieved when the corresponding boronic acid was used in the presence of Pd\(_2\)(dba)\(_3\), PCy\(_3\) and K\(_3\)PO\(_4\). The choice of reaction conditions was determined on the basis of substrate similarity with literature procedures.\(^{210}\) The nitrile group in 2g was then reduced with LiAlH\(_4\) and AlCl\(_3\) to yield the desired product 3g in 79% yield.

![Scheme 9 - Synthesis of compound 3g commencing from the commercially available starting material.](image)

Attempted purification using silica gel on flash chromatography or preparative HPLC of the final compound led to degradation of the molecule. It was hypothesised that the degradation was due to the acidic nature of the silica gel and mobile phases of the HPLC respectively. Therefore, purification was successfully performed via flash chromatography on aluminium oxide under basic conditions. The methylamine 3g was submitted for testing as the free-base because, as seen for 3c, treatment with acidic solution led to polymerisation at the 3-position of the indole.
as shown by lack of signal of the proton at the 3-position of the indole (NMR analysis of the crude mixture).

Unexpectedly, co-crystallisation of compound 3g with CK2α showed electron density at the interface, with the chlorine atom buried in the lipophilic Phe pocket, much deeper than the fragments binding at the interface studied thus far (Figure 30). Weak electron density only was observed in the αD pocket.

![Figure 30 - Overlay of the X-ray structures of 3g (green, PDB code: 6GIH) and 3a (blue, PDB code: 5OS7) showing how 3g binds deeper into the Phe pocket.](image)

The fact that the chlorine atom of 3g bound deeply in the Phe pocket resulted in a considerable boost in potency. In addition, the compound was able to displace a CK2β-like probe in a Fluorescent Polarisation assay (FP) with an IC₅₀ of 44 μM. Pleasingly, compound 3g represented the highest affinity fragment binding at the α/β interface developed during our FBDD research.

3g appeared to be the first CK2 inhibitor acting at the α/β interface with fragment-like properties reported in the literature, and therefore, it could be further developed into a drug-like molecule with high potency and selectivity against CK2 (Table 9).

### Table 9 - Fragment-like properties of 3g and other known small molecules acting at the α/β interface of CK2. Adapted from Brear et al., BMC, 2018.

<table>
<thead>
<tr>
<th>Property</th>
<th>Ideal Range</th>
<th>DRB</th>
<th>W16</th>
<th>3g</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW</td>
<td>&lt;300</td>
<td>319</td>
<td>611</td>
<td>257</td>
</tr>
<tr>
<td>TPSA</td>
<td>≤ 60</td>
<td>87.7</td>
<td>133</td>
<td>41.8</td>
</tr>
<tr>
<td>HBA</td>
<td>≤3</td>
<td>6</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>HBD</td>
<td>≤3</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>NRB</td>
<td>≤3</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

FW: formula weight in g/mol, TPSA: polar surface area in Å², HBA: hydrogen-bond acceptors, HBD: hydrogen-bond donors, NRB: number of rotatable bonds, ATP: does the compound bind in the ATP pocket of CK2. Green is within the ideal range; amber is within 15% of ideal range; red is over 15% from ideal range.
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Considering the binding mode of 3c showed the potential of 3g pushing the αD helix outwards, it was decided to synthesise compound 3h – analogue of compound 3g bearing a trifluoromethyl group as replacement of the chlorine. In addition, compound 3g showed weak electron density in the αD pocket, and it was envisioned that the chlorine-to-trifluoromethyl in 3h would abrogate the binding of the molecule at the interface in favour of an enhanced binding in the αD pocket considering previous reports.197

As reported in Scheme 10, synthesis of 3h followed the same synthetic steps and conditions as 3g except for the nitrile reduction which was performed using hydrogenation with Raney Nickel212 due to –CF₃ lability in the presence of LiAlH₄ and AlCl₃.

![Scheme 10 - Synthesis of compound 3h starting from the commercially available starting material.](image)

Suzuki cross-coupling between the commercially available 3-bromo-5-(trifluoromethyl)benzonitrile and indole-3-boronic acid in the presence of Pd₂(dba)₃ and PCy₃ afforded intermediate 2h in 35% yield; 2h was then reduced to the desired compound 3h using H₂ and Raney Ni (28% yield).

The introduced trifluoromethyl group decreased the binding affinity for the α/β interface, as shown by the poor electron density observed in the X-ray structure, yet, no electron density for the compound was observed in the αD pocket. Other compounds binding in the αD pocket gave more promising results than the 3-indole-phenyl-methanamine derivatives, and therefore, it was decided not to pursue the synthesis of this series of compounds further.

### 3.4.1.4. Dichloro-biaryl derivatives: 3i and 3j

The crystal structures of fragments F5, F6 and 3d binding to the αD pocket showed two binding poses that only differ by the position of the chlorine atom, suggesting that slow rotation of the biaryl bond occurs. It was envisioned that 3,5-dichloro-biphenyl fragments might result in a reduction of the entropy of binding by preventing free rotation around the biaryl bond and therefore a decrease in binding energy.

The first dichloro derivative synthesised was 3i, an analogue of F5, whose synthesis was achieved as shown in Scheme 11.
Compound 1b was obtained from the commercially available phenol derivative using Tf₂O and pyridine; the same conditions successfully used to synthesise compound 1a.²⁰⁴ However, the yield of the phenol triflation (74%) was lower than for 1a (91%) probably due to the steric bulk around the hydroxyl group. The cross-coupling of 1b with phenylboronic acid to produce the essential precursor 2i proved challenging, and a few Suzuki coupling conditions were screened as reported in Table 10.

**Table 10 - Suzuki coupling conditions screened to obtain 2i.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Catalyst</th>
<th>Base</th>
<th>Reaction time</th>
<th>Outcome</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Boronic acid</td>
<td>Pd(PPh₃)₄</td>
<td>Na₂CO₃</td>
<td>16 h</td>
<td>Very little desired product, 3 unknown impurities</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>2</td>
<td>Pinacol boronic ester</td>
<td>Pd(PPh₃)₄</td>
<td>Na₂CO₃</td>
<td>30 min</td>
<td>2 unknown aromatic by-products</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>Boronic acid</td>
<td>PdCl₂(dpdpf) K₃PO₄</td>
<td>2 h (MW)</td>
<td>Desired product, aromatic impurity</td>
<td>24%</td>
<td></td>
</tr>
</tbody>
</table>

The use of PdCl₂(dpdpf)·CH₂Cl₂ in the presence of K₃PO₄ in DME, EtOH and H₂O (entry 3, Table 10) appeared to be the best set of conditions.²¹³ The low yield observed was attributed to the loss of the desired product during purification. The Rf of the desired product was very similar to an unidentified impurity formed during the reaction. Flash chromatography using different solvent systems failed to separate the two molecules, which were partially separated using preparative TLC.

Nitrile reduction of 2i with LiAlH₄ and AlCl₃, followed by treatment with HCl afforded 3i in 48% yield (Scheme 11).

In parallel, it was decided to synthesise the dichloro analogue of the highest affinity fragment 3b. The synthesis of 3j started from the 3,5-dichloro-4-hydroxybenzaldehyde which underwent treatment with Tf₂O and pyridine in CH₂Cl₂ to afford 1c in 57% yield. Attempts using the corresponding nitrile in the Suzuki coupling failed. Coupling using 1c and 2-ethyl-phenylboronic acid afforded the intermediate 2j that could not be isolated due to its Rf similar to that of unidentified impurities formed. The so-obtained crude compound underwent a reductive Boc
amination in the presence of butylcarbamate and Et₃SiH in TFA to afford 3j in 37% overall yield (Scheme 12).

![Scheme 12 - Synthetic steps leading to the dichloro derivative 3j.](image)

Pleasingly, in crystallisation studies compounds 3i and 3j were the first fragments able to open the αD pocket of the more rigid construct of the protein. In the X-ray structures (Figure 31), the bottom ring is twisted 90° relative to the upper aromatic ring. As expected, only one binding mode was detected for these compounds suggesting that the two chlorine atoms help to lock the molecule in a twisted conformation reducing the rotational freedom of the aromatic rings.

![Figure 31 – a) X-ray structures of compound 3i (blue, PDB code: 5OTR) and 3j (green, PDB code: 5OTZ) co-crystallised with CK2α. b) Zoomed-in section showing the fragments are not able to flip the Met163 and displace the ATP (blue) from its binding site. Protein is shown as cyan ribbon and grey surface.](image)

Pleasingly, the biological evaluation of compounds 3i and 3j showed a remarkable boost in binding affinity compared to the fragments tested thus far (Table 11). Moreover, the LE was also improved.

---

For more details about the different constructs used vide 8.1.1. CK2α_KA presents a more flexible β4-β5 motif and αD helix whereas CK2α_FP is more rigid.
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Table 11 - LE and $K_d$ of dichloro derivatives 3i and 3j.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>LE</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3i</td>
<td><img src="image" alt="Structure 3i" /></td>
<td>0.42</td>
<td>12</td>
</tr>
<tr>
<td>3j</td>
<td><img src="image" alt="Structure 3j" /></td>
<td>0.39</td>
<td>6.5</td>
</tr>
</tbody>
</table>

3.4.2. Fragments growing towards the ATP binding site: second-generation CK2 inhibitors

Compounds 3i and 3j showed the highest binding affinity and the best LE for the αD pocket over the fragments synthesised in the Spring group during the fragment elaboration phase. These molecules were not able to inhibit the catalytic activity of CK2 as they did not flip the Met163 and hence displace the ATP; therefore, they were used as the bottom part of the second-generation inhibitors. The αD fragments were merged with a benzimidazole fragment that binds at the ATP mouth without interacting with conserved residues within the ATP binding site to achieve inhibition.\textsuperscript{206} Optimisation of the benzimidazole fragment was carried out by Dr Claudia De Fusco in the Spring group. Unlike CAM4066, it was envisioned that CK2 inhibition could be achieved without the need of a fragment binding at the ATP pocket considering the affinity of the αD fragment was significantly improved respect to fragment used in CAM4066 (F5 $K_d = 267$ μM vs $K_d = 6.5$ and 12 μM for 3j and 3i respectively).\textsuperscript{198} It was decided to merge compounds 3i and 3j with the benzimidazole motif as an attempt to achieve CK2 inhibition.

Synthesis of 3k followed the synthetic route reported in Scheme 13. We were gratified to find that when 1c was reacted with phenylboronic acid under thermal heating using PdCl$_2$(dppf) $\text{CH}_2\text{Cl}_2$, K$_3$PO$_4$, DME, EtOH, and H$_2$O compound 2k was obtained in a superior yield of 54% compared to the 24% obtained when the corresponding nitrile (1b) was used (Scheme 11).
Intermediate 2k underwent a reductive amination in the presence of commercially-available 2-benzimidazolyl-ethylamine and NaBH(OAc)$_3$, followed by acidification, to afford compound 3k.$^{214}$

Compound 3l was synthesised in a similar way to compound 3k with the exception that isolation of the biaryl aldehyde intermediate 2j was not possible, as observed in the synthesis of 3j. The crude was therefore carried onto the reductive amination step, and the resulting amine purified via preparative HPLC to afford 3l as TFA salt with 13% overall yield (Scheme 14).

The crystallographic structure of 3k and 3l confirmed the expected binding mode with Met163, whose position is thought to be essential for the inhibition, flipped into the ‘out’ conformation to displace the ATP. As hoped, the benzimidazole motif binds at the mouth of the ATP binding site without interacting with residues in the ATP binding site (Figure 32).
Compound 3k proved to be insoluble under ITC assays condition and in the cell growth media, and therefore its biological evaluation was not possible.

Compound 3l showed improved solubility compared to 3k, but it was still not soluble enough to perform ITC direct binding experiments which require a high concentration of the compound. Therefore, ITC competition studies were performed (by Dr Paul Brear) to confirm the binding mode and to estimate the affinity of 3l for the αD site. Probe molecules that have well characterised binding modes and affinities were titrated into CK2α in the presence of 3l. In particular, the following experiments were performed, and the following observations were made:

-20 µM 3l inhibited the binding of CAM4066 (first-generation inhibitor) to CK2α.

-Compound 3j was titrated into CK2α in the presence of 20 µM 3l. This showed that 3l inhibited the binding of 3j to CK2α confirming that the binding site of 3l is the αD pocket.

-2-hydroxyl-5-methyl benzoic acid is a fragment that binds to the conserved Lys68 in the ATP site and occupies the right-hand side of the pocket. The binding of this compound was not inhibited by 3l confirming that the benzimidazole ring does not interact with the right-hand side of the ATP pocket and validates the binding mode derived from the crystal structure.

-CX4945, which from the analyses of crystal structures would clash with 3l in the hinge region, was titrated into CK2α in the presence of 3l. 3l was able to inhibit the binding of CX4945 to CK2α. This confirmed that the benzimidazole ring flips the Met163 channel and blocks access to the ATP site inhibiting the binding of CX4945.

These competition experiments suggested that the $K_d$ of 3l towards CK2α is approximately 4 µM and confirmed that the binding mode of 3l in the αD pocket and mouth of the ATP site corresponds to that seen in the crystal structure.
Compound 3l was able to inhibit the CK2 catalytic activity with an IC$_{50}$ of 7 μM (in vitro phosphorylation assay) and a GI$_{50}$ of 10.0 ± 3.6 μM (cellular assay using HCT116 cells). Pleasingly, 3l was able to inhibit the phosphorylation of specific CK2 substrates in a cellular context as shown in Figure 33.

Figure 33 – Bioactivity of 3l in cancer cells. a) Dose-response curve for the inhibition of growth of HCT116 cells by 3l, pro-CAM4066 and CX4945. Compounds concentrations on the x-axis are shown as natural logarithm. All graphs show the mean ± SEM of not less than three independent experiments with each in triplicate. b) Western blot analysis showing the specific CK2 phosphorylation targets: AKT1 Ser129 and G6DPH. HCT116 cells were treated with 2 × GI$_{50}$ of CX4945 (20 μM), 3l (20 μM) or pro-CAM4066 (20 μM) for 72 hours. Adapted from Iegre et al., ChemSci, 2018.

3l was subjected to a kinase selectivity panel (Dundee Kinase panel) at 30 μM concentration (4 x IC$_{50}$) to prove that CK2 inhibition from the αD pocket can lead to a more selective inhibitor than ATP-competitive compounds. Pleasingly, despite the relatively high concentration used, 3l showed good selectivity against 20 closely related kinases (CMGC family, Figure 34a). Even though for 4 kinases – CAMK1, SmMLCK, EF2K, SGK1 – out of 140 3l causes more than 50% inhibition, the compound showed a more selective profile compared to known CK2 ATP-competitive inhibitors (Figure 34b).
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The ultimate goal of this project was to develop second-generation CK2 inhibitor with improved properties in respect to CAM4066. Compound 3l was successful in that as it overcame the limitation of the first-generation inhibitor as shown in Table 12.

Table 12 - Properties and structural features of compound 3l compared to CAM4066.

<table>
<thead>
<tr>
<th>Property</th>
<th>Ideal range</th>
<th>CAM4066</th>
<th>3l</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW</td>
<td>&lt;500</td>
<td>494</td>
<td>453</td>
</tr>
<tr>
<td>TPSA</td>
<td>&lt;140</td>
<td>101</td>
<td>41</td>
</tr>
<tr>
<td>NRB</td>
<td>&lt;10</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>HBA</td>
<td>&lt;10</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>HBD</td>
<td>&lt;5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Conserved interaction</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Need of a pro-drug</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Amide bonds</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Drop in potency*</td>
<td>No</td>
<td>~10 fold</td>
<td>No</td>
</tr>
</tbody>
</table>

FW = formula weight in g/mol; TPSA = topological polar surface area in Å² NRB = number of rotatable bonds; HBA = number of hydrogen bond acceptors; HBD = number of hydrogen bond donors. Drop in potency between enzymatic and cellular assays.
3.5. Conclusions and future work

The work described in this chapter led to the development of 3l: the first CK2 inhibitor that targets the allosteric αD pocket and that does not interact with highly conserved residues located in the ATP site hence providing selectivity.

3l was developed using a FBDD approach starting from F5, a fragment with 267 μM affinity for the αD pocket. Through iterative cycles of design, synthesis, X-ray crystallography and biophysical evaluation the initial fragment was first elaborated into 3j (Kd 6.5 μM) and subsequently linked to a benzimidazole-like motif to reach the mouth of the ATP binding site and flip the Met163 - a residue seen as important for the catalytic activity. The merging strategy adopted in this work led to 3l which was able to bind to CK2α with a Kd of 4 μM, inhibit CK2 in vitro (IC50 7 μM), inhibit growth of colorectal cancer cells (GI50 10 μM) and showed promising selectivity for CK2 versus 140 other kinases.

This work showcases the successful application of FBDD to overcome the limitation of the first-generation inhibitor CAM4066. Compared to CAM4066, 3l presents a reduced number of rotatable bonds, HBDs, HBAs, does not contain amide bonds, and it maintains its activity in cells without the need of being formulated as a pro-drug.

In conclusion, 3l present itself as an ideal chemical tool that can be further elaborated into a drug molecule. Pleasingly, APOLLO therapeutics became interested in the project and are now running a drug-discovery programme to pursue this project further.

In addition, during the fragment optimisation carried out in this study, a few fragments were found to bind at the protein interface predominantly. One of them, 3g was found to bind deep in the Phe pocket – the only pocket present at the shallow interface – and it proved to be able to displace the CK2β-like probe with an IC50 of 44 μM. 3g is, therefore, the most promising fragment binding at the interface with structural information available reported in the literature. Due to its fragment-like properties, 3g is an ideal starting point to develop small molecule modulators of the CK2 interface. Considering the high number of attempts in the Spring group prior to this work to develop high-affinity fragments binding at the interface, it was decided not to optimise 3g further.

Instead, as a continuation of this project, Chapter 4 describes the development of conformationally-constrained peptides binding at the α/β interface.

Furthermore, additional fragment screenings of Diversity-Oriented-Synthesis (DOS) libraries are underway in the Spring group to identify novel sp3 reach molecules to disrupt the CK2 PPI.
CHAPTER 4:
Conformationally-Constrained Peptides
Targeting the PPI of the Anti-Apoptotic Protein CK2
4.1. Summary

The development of molecules able to disrupt the protein-protein interaction (PPI) between the α and the β subunits of the protein kinase CK2 has received increasing interest in the last few decades. However, the discovery of a selective, tissue permeable, and stable chemical tool that could act at the protein interface and that could be used in vivo is still missing.

This chapter describes the approach used to efficiently develop peptides acting at the CK2α/β interface that are highly functionalised, cell-permeable, stable in serum, and with structural information available (Figure 35). The lead probe developed in this work, P7-F1C5, should be of great help in understanding the regulatory mechanisms behind CK2 and their implication in oncogenesis.

Figure 35 – Functionalised stapled peptide P7-F1C5 described in this chapter.

Whilst the approach described herein is specific to CK2, a similar strategy could be applied to any target needing the fast development of highly functionalised peptide modulators of PPIs.
4.2. Project background

A limited number of small molecules have been developed before this work - namely DRB, W16, and azonaphthalene derivatives (structures shown below)\textsuperscript{160, 162, 201} - with the aim of inhibiting CK2 by acting at the protein interface.

![DRB](image1)

![W16](image2)

![azonaphthalene derivative](image3)

However, due to the nature of these small molecules and the characteristics of the interface, none of the compounds developed to date is selective for the interface. In some cases, structural evidence of the binding at the protein interface is also missing.

In the Spring group efforts have focused on the development of inhibitors of the CK2 α/β interface using a Fragment-Based Drug Discovery approach (FBDD) approach. However, the best fragment 3g, whose development is described in Chapter 3, needs to be elaborated further in order to identify a higher affinity molecule to act as a useful chemical tool.

On the other hand, Cochet et al. have used the sequence of the tail of the regulatory CK2β domain to design a cyclic peptide that could displace the native CK2β subunit and prevent the assembly of the holoenzyme.\textsuperscript{161} The disulfide-bridged peptide, hereafter known as Pc, showed a sub-micromolar affinity for CK2α and displaced CK2β \textit{in vitro}. However, due to the inherent low stability of disulfide bonds under reducing conditions, its cellular activity could not be investigated.\textsuperscript{161} Fast forward ten years and the same research group developed a lactam head-to-tail cyclised variant of Pc that was conjugated to a TAT peptide to gain cell-entry (IC\textsubscript{50} = 5 μM).\textsuperscript{164} Although cell permeable, no structural information on the new macrocyclic peptide or data on the stability of the latter molecule in physiological fluids were reported, and therefore, such molecule could not serve as a chemical probe without further validation.
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

4.3. Project aim and overview

The aim of this project was to develop conformationally-constrained peptide CK2 inhibitors targeting the protein interface and that have the potential to act as valuable chemical probes in vivo.

Initially, molecular modelling was carried out using the structural information available on P2152 and on the portion of CK2β interacting with the catalytic domain to identify a suitable position to place the covalent constraint. It was envisioned that a constraint was necessary to improve the peptide affinity for the target by locking it into the binding conformation and simultaneously enhance the stability to proteases. Consequently, a series of constrained peptides were synthesised and tested in vitro to assess their ability to engage CK2α. The most promising peptide was then modified to incorporate fragment-like amino acids to boost the potency. Successively, X-ray crystallography revealed insights into the peptide binding mode and guided the design further. Lastly, the higher affinity peptide, P7-C5 was efficiently functionalised employing a novel multi-functional constraint.

In total, twenty-two macrocyclic peptides were synthesised; the most promising one, P7-F1C5 engaged CK2α with sub-micromolar affinity, it is stable in serum, cell-permeable, and active in cancer cells. An overview of the workflow is shown in Figure 36.

![Figure 36 - Workflow of the project described in Chapter 4.](image-url)
4.4. Results and discussion

Unless otherwise stated, the peptides described in this chapter were synthesised using standard procedures for solid-phase peptide synthesis (SPPS) and peptide macrcyclisation was achieved by following standard procedures for two-component copper-catalysed azido-alkyne cycloaddition peptide stapling (2C CuAAC PS).

Biophysics experiments such as fluorescent polarisation (FP), isothermal titration calorimetry (ITC), kinase assays, and X-ray crystallography were performed under the supervision of Dr Paul Brear (Hyvönen group, Biochemistry, University of Cambridge). Cellular experiments such as cell culture, confocal microscopy, anti-proliferation assay, and cell-viability assay were performed under the supervision of Dr David J. Baker (Discovery Sciences, AstraZeneca, Cambridge). Molecular dynamics simulations were conducted by our collaborator Dr Yaw Sing Tan (A*STAR, Singapore).

4.4.1. Design of CK2β-like peptides

Analysis of the portion of CK2β interacting with CK2α has suggested that only a cluster of 8 amino acid (186-193) contribute to the binding significantly: RLYGFKIH. All of the amino acids mentioned above are included in the sequence of the disulfide-bridged Pc peptide developed by Cochet et al. Therefore, the X-ray crystallographic structure of Pc bound to CK2α (PDB code: 4I5B) was used to understand which of those residues could be replaced by amino acids amenable to side-chain cross-linking to form stable cyclic peptides. Computational alanine scanning (CAS) and energetic decomposition suggested that any of the terminal residues introduced in Pc – namely G1, C2, G11, C12, G13 – could be replaced to introduce the constraint as they did not contribute to the binding (Figure 37).

Figure 37 – Molecular modelling done on Pc peptide. a) CAS (top) and energetic decomposition (bottom) of the amino acids of Pc. Residues whose mutation to Ala negatively affected the binding significantly are shown in red, residues non-

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* The work described in chapter has been published in Iegre et al. ChemSci, 2019. Part of the text and figures have been adapted from this publication.
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

contributing to the binding are shown in green; b) Structure of Pc peptide (from PDB: 4I5B). Residues coloured in purple (C2 and G11) are the ones chosen to place the constraint in this study.

It was decided to employ the 2C CuAAC peptide macrocyclisation chemistry to constrain the peptides as this cross-linking technology has been successfully used in the past to constrain peptides into their binding conformation, improve PK properties and simultaneously allow for functionalisation via the constraint rather than via the linear sequence.79,83,84,86,87,216

C2 and G11 were positioned at a suitable distance from each other to accommodate a 2C double-click constraint (Figure 37). Consequently, the 13-mer linear peptide would feature the sequence Ac-GXRLYGFKHG-G-NH₂ where X indicates the azido amino acids incorporated for constraining. The peptide sequence mentioned above will be referred to as P1 hereinafter. It was envisioned that the constraint should be rather rigid to minimise entropic penalties upon binding that may occur as the result of excessive flexibility. Therefore, the azido amino acid selected for cross-linking was azido-alanine (5) which, once reacted with the appropriate dialkyne linkage, would generate a cyclic peptide of a generic structure P1-Cn shown in Scheme 15.

![Scheme 15 - Generic structure of the linear and cyclic peptides that are the subject of this work.](image)

4.4.1.1. Synthesis of Fmoc-azido-alanine 5

The synthesis of Fmoc-azido-alanine 5 was performed following literature precedent (Scheme 16).217 The synthetic route commenced from the commercially available Fmoc-Asn-OH that underwent a Hofmann rearrangement in the presence of [bis(trifluoroacetoxy)iodo]benzene (PIFA) to afford Fmoc-Dap-OH (4) in 81% yield. The amino acid was then treated with the azido transfer reagent imidazole-1-sulfonyl-azide hydrogen sulfate 6 to obtain 5 in 77% yield.
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

Scheme 16 - Synthesis of Fmoc-azido alanine 5 from commercially available Fmoc-Asn-OH. Fmoc = Fluorenylmethyloxycarbonyl

The azido transfer reagent 6 was synthesised in a one-pot procedure from the cheaply available starting materials sulfuryl chloride, imidazole, and NaN₃ (Scheme 17).

Scheme 17 - One-pot synthesis of azido transfer reagent 6.

The Fmoc protected azido amino acid 5 was used to synthesise the linear peptide P1 by SPPS.

4.4.2. Screening of constrained peptides

Once the linear sequence was identified and synthesised, the next step aimed to constrain the peptide into a conformation that resembles the portion of CK2β binding to CK2α.

The ultimate goal of this project was to develop highly functionalised peptides in which the functionalisation feature was on the constraint rather than the linear sequence itself. This strategy was considered advantageous as it requires the synthesis of one linear bioactive peptide only, whereas peptidic functionalities such as CPPs and fluorescent tags are added onto the constraint via amide coupling. To this end, a functional handle on the constraint would be required. However, it was decided to commence the screening of the constraints from commercially available materials or easily synthesised plain dialkynes. The plain cyclised peptides would then be tested to investigate their ability to displace a CK2β-like probe in an FP assay and the most promising ones re-synthesised using constraints bearing a functional handle.
4.4.2.1. Plain constraints: C1-C4

Molecular modelling performed on the X-ray structures of Pc suggested that four possible constraints could be used to constrain the peptide into a CK2β-like conformation (Scheme 18).

Constraints C1 and C2 were commercially available and were used as such in the CuAAC 2C macrocyclisation reaction. Initially, it was decided to use a shorter version of the linear peptide P1 by removing the three terminal glycine residues (P0, Ac-XRLYGFKIHX-NH2). Molecular dynamic simulations (MDs, performed by Dr Yaw Sing Tan, A*STAR, Singapore) showed that the terminal glycine residues did not contribute to the binding significantly (calculated ΔH -50.6 ± 1.7 and -56.6 ± 1.2 Kcal·mol⁻¹ for Pc and shorter Pc respectively). In addition, by removing the terminal residues, three coupling and deprotection steps could be avoided. Whilst constraining of P0 with C1 successfully provided P0-C1 (82% conversion), cyclisation with C2 was not achieved, with only the addition of two equivalents of the linker C2 per equivalent of the linear sequence P0, as observed by LCMS of the crude mixture. By changing the linear sequence to P1, the desired product P1-C2 was obtained (65% conversion) suggesting that the terminal glycine residues may help cyclisation.

Constraints C3 and C4 were not commercially available, and their synthesis is discussed below.

Constraints C3 could be obtained by TMS deprotection of the commercially available TMS-mono-protected analogue by treatment with TBAF for 14-16 h. However, due to the high volatility of the product, its isolation was not achieved. It was decided to perform a first CuAAC reaction in the presence of the commercially available trimethyl(penta-1,4-diyn-1-yl)silane and P0, followed by addition of CsF to remove the TMS group and a second CuAAC reaction (Scheme 19). Unfortunately, only the addition of two deprotected constraints was detected in the crude LCMS conversions are given for peptide macrocyclisation reactions instead of yield due to loss of material during the purification on preparative HPLC.
mixture. Pleasingly, the same procedure repeated with P1 afforded the desired product P1-C3 (62% conversion) as shown in Scheme 19.

Scheme 19 - Attempts of cyclising P0 into P0-C3 and P1 into P1-C3 using CuAAC peptide chemistry.

Considering that the terminal residues were required to obtain the desired cyclisation products with C2 and C3, it was decided to use the sequence of P1 for all the successive reactions described hereafter.

Constraint C4 was obtained from the commercially available 1,2-dibromobenzene. The latter molecule underwent a Sonogashira coupling in the presence of trimethylsilylacetylene, Pd(PPh3)2Cl2, CuI, PPh3, and DIPEA followed by TMS deprotection in the presence of KOH and MeOH to afford C4 as a 42% w/v solution in PE 40-60 (Scheme 20).

Scheme 20 - Synthesis of C4 from commercially available 1,2 dibromo-benzene.

2C CuAAC peptide macrocyclisation between C4 and P1 afforded P1-C4 in 79% conversion.
With the four cyclic peptides in hand, their biological activity was investigated using an FP assay. In this study, FP was used to determine the ability of the peptide to displace a commercially available fluorescent probe meant to mimic the CK2β subunit. The assay looked at the anisotropy resulting from the interaction of CK2α and the fluorescent probe in the presence of 15 μM peptide concentration. The results included Pc, its shorter analogue, and the linear Pc peptides for comparison (synthesised according to previous reports). The results are expressed as % inhibition and indicate the inhibition of the assembly of CK2α with the fluorescent probe (Table 13). The corresponding calculated binding energies are also shown in the table.

**Table 13 - % inhibition of the assembly between CK2α and the FP probe at 15 μM peptide concentration.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Peptide</th>
<th>Structure</th>
<th>% inhibition (at 15 μM)</th>
<th>Calculated ΔH (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pc</td>
<td>GCRLYGFKIHGC (S-S)</td>
<td>79.0 ± 7.0</td>
<td>-50.6 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>Short Pc</td>
<td>GCRLYGFKIHGC (S-S)</td>
<td>73.8 ± 2.5</td>
<td>-56.6 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>Linear Pc</td>
<td>GCRLYGFKIHGC</td>
<td>13.1 ± 2.3</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>P0-C1</td>
<td>Xc1LYGFKIHXc1</td>
<td>0.6 ± 0.1</td>
<td>-50.3 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>P1</td>
<td>GXLYGFKIHXGG</td>
<td>12.6 ± 1.7</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>P1-C2</td>
<td>GXc2LYGFKIHXc2GG</td>
<td>NA</td>
<td>-57.1 ± 2.1</td>
</tr>
<tr>
<td>7</td>
<td>P1-C3</td>
<td>GXc3LYGFKIHXc3GG</td>
<td>58.4 ± 4.0</td>
<td>-54.1 ± 2.1</td>
</tr>
<tr>
<td>8</td>
<td>P1-C4</td>
<td>GXc4LYGFKIHXc4GG</td>
<td>21.1 ± 1.9</td>
<td>NA</td>
</tr>
</tbody>
</table>

All the peptides feature an amide at the C-terminus and an acetyl capping at the N-terminus. X = Fmoc-Aza-OH (5).

The results confirmed that the terminal Gly residues did not contribute significantly to the binding as predicted by the molecular modelling (entries 1 and 2, Table 13). P1-C3 appeared to be the most promising of the cyclised peptides synthesised so far, and therefore, it was the one brought forward. Unfortunately, P1-C2 proved to be insoluble in the assay conditions and its evaluation was not possible at that stage.

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* Fluorescent probe of sequence FITC-βGly-RLYGFKIHPMAYQLQ.
4.4.2.2. Constraints with a functional handle: C5 and C6

Although the ability of P1-C2 to displace the CK2β-like probe from CK2α could not be studied due to solubility issues, MDs suggested that the peptides P1-C2 and P1-C3 may have a more favourable enthalpy of binding than Pc (-57.1 ± 2.1 Kcal·mol⁻¹ for P1-C2, -54.1 ± 2.1 Kcal·mol⁻¹ for P1-C3 and -50.6 ± 1.7 Kcal·mol⁻¹ for Pc, Table 13) and hence it was decided to consider them both for further evaluation.

It was envisioned that by introducing a suitable functional group – such as carboxylic acid or amine - the solubility of peptide P1-C2 in aqueous buffers could be improved and would simultaneously set up for the introduction of peptidic functionalities at a later stage.

Consequently, C2 was modified into the corresponding carboxylic acid arylalkyne C5. The synthesis followed previous reports²¹⁹,²²⁰ and commenced from a Sonogashira reaction between the commercially available methyl 3,5-dibromobenzoate and trimethylsilacetylene in the presence of Pd₂(dba)₃, PPh₃, Cul in TEA to afford C5-1 in 68% yield (Scheme 21). The intermediate was then subjected to ester hydrolysis and TMS deprotection in the presence of MeOH and KOH to afford the constraint C5 in 98% yield.

Functionalisation of constraint C3 presented some difficulties. Initially, it was envisioned that the desired one-carbon atom linker could be accessed from the cheap methyl formate (Scheme 22). It was proposed that the treatment of this ester with TMS-acetylene in the presence of nBuLi could afford C6-1. However, the reaction did not proceed as expected, and no desired product could be isolated. It was thus decided to perform the same reaction starting from 3-(trimethylsilyl)propioaldehyde. Pleasingly, the addition onto the aldehyde afforded C6-1 in quantitative yield. It was envisioned that the hydroxyl group in C6-1 could be converted into a good leaving group that would then allow for the introduction of a carboxylic acid or an amine. However, attempts to convert C6-1 into tosyl (C6-2) or into a phthalimide viaaza-Mitsunobu reaction (C6-3) failed, probably due to polymerisation as suggested by the formation of black, insoluble products (Scheme 22). A final attempt started from 3-(trimethylsilyl)propioaldehyde and p-bromo-aniline followed by addition of TMS-acetylene to obtain C6-4 (Scheme 22). As observed for C6-2 and C6-3 only black and insoluble products were obtained. If this reaction
had been successful, 4-aminobenzoic acid would have been used instead of \( p \)-bromo-aniline. The latter was used only to test the outcome of the reaction on a simplified substrate.

![Scheme 22 - Attempts to synthesise a one-carbon atom linker bearing a functional handle.](image)

It was proposed to change strategy, and a double Negishi cross-coupling reaction was attempted starting from the commercially available 4-(dibromomethyl)benzoic acid and ethynylmagnesium bromide to obtain linker C6-5 (Scheme 23). Unfortunately, only unidentified by-products were observed.

![Scheme 23 - Double Negishi reaction attempted to synthesise C6-5.](image)

Finally, it was decided to follow an alternative route using a substrate without a functional group that may complicate the synthesis. In particular, it was envisioned to start the synthesis from methyl 4-bromobenzoate (Scheme 24). The starting material was reacted with TMS-acetylene in the presence of \( \text{^nBuLi} \) to afford C6-6 in 93% yield. The TMS protecting groups on the intermediate were then removed in the presence of KOH and MeOH to afford C6-7 in 54% yield. An attempt to react methyl 4-bromobenzoate with ethynylmagnesium bromide to obtain C6-7 in one step failed. It was proposed that the tertiary alcohol in C6-7 could be removed via a deoxygenation reaction. However, only unidentified by-products were detected. This result, in combination with the failures encountered in the previous routes, suggested that the
intermediates deriving from the modifications of the tertiary alcohol group were extremely unstable.

To see whether the steric hindrance of constraint C6-7 would be tolerated in the 2C-CuAAC reaction, C6-7 was reacted with P1. Pleasingly, P1-C6-7 was obtained with 52% conversion. It was decided to repeat the reactions that led to C6-7 with a substrate carrying a carboxylic acid that could be used as a functional handle.

The synthetic route proceeded from the commercially available 4-(methoxycarbonyl)benzoic acid that underwent a double TMS-acetylene addition onto the ester to afford C6-9 in 46% yield (Scheme 25). The yield for this reaction was considerably lower than the analogue that led to C6-6 (93%) potentially due to the different electronics of the aromatic rings. The TMS protecting groups of C6-9 was removed in the presence of KOH and MeOH to yield C6 in 62% yield.

Constraints C5 and C6 were used in the 2C-CuAAC peptide macrocyclisation in the presence of P1 to afford P1-C5 and P1-C6 with 92% and 85% conversion respectively.

The affinity of the newly synthesised peptides for CK2α was measured using an ITC assay. ITC provides a more accurate measurement of the binding affinity than FP as it measures the direct binding to the protein of interest. However, ITC is not high-throughput and, consequently, was not used for the lower affinity peptides. P1-C5 and P1-C6 were soluble in the assay conditions and showed a Kd for CK2α of 460 nM and 58 μM respectively. Whilst P1-C6 showed a decreased
affinity for the protein compared to the reference Pc peptide \( (K_d 1 \mu M) \), **P1-C5** showed higher affinity than Pc (Figure 38).

![Figure 38 - ITC binding curves of P1-C5, P1-C6, and Pc binding to CK2α. The peptides shown feature an acetyl capping at the N-terminus and an amide at the C-terminus.](image)

With this promising result in hand, the ability of **P1-C5** to disrupt the interaction between the α and the β subunits of CK2 was investigated using an ITC competition assay. The affinity of the CK2β domain for the α was measured using a direct ITC assay and was found to be 9 nM (literature value 4 nM). Pleasingly, no binding of CK2β to α was detected under these conditions. Therefore, constraint **C5** proved to be the most effective at constraining the peptide in its binding conformation, and the resulting cyclic peptide **P1-C5** was able to disrupt the PPI of CK2.

### 4.4.3. Attempts to develop Conformationally-Constrained Hybrid Peptides (CCHPs)

Chapter 3 described the FBDD approach used in the Spring group to develop CK2 inhibitors binding in the cryptic αD pocket and at α/β interface. Fragment optimisation of the molecules binding at the interface did not result in significant improvement of the binding affinity.

* Concentration in the ITC titrant and ITC syringes.
compared to the initial hit fragment **NMR154L**, except for 3g. Considering that the initial hit fragment overlaps with the Phe7 hotspot residue of the CK2α (Figure 39a), it was decided to merge fragment **NMR154L** with peptide **P1-C5** (CCHPs) with the aim of increasing the binding affinity of the overall peptides and impart selectivity to the fragments. Fragments of bigger size, such as the more active 3g, were not considered at this stage as their introduction could lead to clashes with other residues within the peptide.

### 4.4.3.1. Chloro-phenylalanine containing peptides: **P2’-C5**, **P2”-C5**, **P3-C5**

Molecular modelling to design the CCHPs was performed using the X-ray structure of Pc (PDB: 4IB5)\(^{215}\) and **NMR154L** (PDB: 5CLP)\(^{198}\) due to the absence of X-ray crystal structures for the newly developed **P1-C5** peptide (Figure 39). Chlorobenzene probes in ligand-mapping simulations indicated that a chlorine atom in the 3 position of Phe7 residue (peptide **P2-C5**) could be introduced without compromising the binding affinity of the peptide (ΔH \(-58.07 ± 2.92\) Kcal·mol\(^{-1}\)) for **P2-C5** vs \(-57.1 ± 2.4\) Kcal·mol\(^{-1}\) for **P1-C5**). The introduction of a chlorine atom in the 4 position of Phe7 was also proposed (**P3-C5**) as the newly inserted atom should not affect the binding affinity significantly (ΔH \(-55.6 ± 2.2\) Kcal·mol\(^{-1}\)).

![Figure 39 – Structure-based design of CCHPs. a) Overlay of the X-ray structures of **NMR154L** (purple, PDB code: 5CLP)\(^{198}\) and Pc peptide (green, PDB: 4IB5)\(^{215}\) binding at the CK2 interface. b) Chlorobenzene probe used in MD shows the chlorine occupying a region (green mesh) corresponding to the 3-position of the Phe7 of Pc (orange).](image)

The Fmoc-3-chloro-Phe-OH amino acid (7) required for the synthesis of **P2-C5** was synthesised from the racemic, unprotected amino acid 3-chloro phenylalanine for availability reasons. The free amine was protected using N-(9-fluorenylmethoxycarbonyloxy) succinimide and NaHCO\(_3\) (Scheme 26).
As expected, incorporation of 7 in the peptide afforded two diastereomers \( \text{P2'} \) and \( \text{P2''} \) which were separated by preparative HPLC and cyclised with \( \text{C5} \) to afford \( \text{P2'-C5} \) and \( \text{P2''-C5} \) respectively. It was envisioned that X-ray crystallography of the peptides in complex with the protein would elucidate the stereochemistry.

The synthesis of \( \text{P3} \) required the amino acid L-Fmoc-4-chloro-Phe-OH that was commercially available. Cyclisation of linear peptide \( \text{P3} \) with \( \text{C5} \) afforded \( \text{P3-C5} \) in 53% conversion (Scheme 27).

ITC was performed to measure the binding affinities of \( \text{P2'-C5} \), \( \text{P2''-C5} \) and \( \text{P3-C5} \) for CK2α. Unfortunately, none of the CCHPs showed improved binding affinity compared to \( \text{P1-C5} \) (\( K_d \) of 460 nM): peptide \( \text{P2'-C5} \) was found to have a \( K_d \) of 2 \( \mu \)M, \( \text{P2''-C5} \) did not show binding to the protein at a concentration below 125 \( \mu \)M whilst \( \text{P3-C5} \) was found to have a \( K_d \) of 56 \( \mu \)M.
4.4.3.2. Chloro-phenylglycine containing peptides: P4-C5, P5'-C5, P5''-C5

Considering the lack of structural information available for the first set of CCHPs, it was hypothesised that the chloro-Phe residues in the peptides might bind deeper than expected making the interactions of the peptide with CK2α less optimal. Consequently, it was decided to shorten the Phe side chain of the amino acid residue by one carbon atom, introducing chloro-phenylglycine derivatives as the Phe7 replacements.

As for P2-C5, the synthesis of the peptides containing 3-chloro-phenylglycine (contained in peptide P4) and 4-chloro-phenylglycine (included in peptide P5) required the protection of the commercially available corresponding amines to Fmoc-protected amino acids 8 and 9 respectively. The 3-chloro-phenylglycine was provided enantiopure whereas the 4-chloro analogue was available as a racemic mixture. For both amino acids, Fmoc protection was performed using N-(9-fluorenylmethoxycarbonyloxy)succinimide and NaHCO₃ (Scheme 28). Fmoc protection of 3-chloro-phenylglycine afforded 8 in 86% yield, and 9 was obtained from 4-chloro-phenylglycine quantitatively.

Scheme 28 - Fmoc protection of 3- and 4- chloro-phenylglycine amino acids 8 and 9.

The linear peptides P4, P5' and P5'' were clicked with C5 to obtain the corresponding cyclic peptides P4-C5 (60% conversion), P5'-C5 (53% conversion) and P5''-C5 (47% conversion).
Unfortunately, all the chloro-phenylglycine containing peptides showed no detectable binding to CK2α on ITC. The lack of structural information at that stage made it impossible to build any SAR. If X-ray structures of the hybrid peptides bound to CK2α can be obtained, the design of further CCHPs could be adjusted.

4.4.4. X-ray crystal structures of the constrained peptides

Due to the crucial importance of structural information to guide the design further, priority was given to X-ray crystallography.

After several attempts of soaking and co-crystallisation with different commercially available and custom-made screens, as well as different constructs of the protein, we were pleased to find conditions that yielded crystals of P1-C5 bound to CK2α. The X-ray structure showed P1-C5 binding in a conformation that resembles the portions of CK2β and Pc binding to CK2α with the hotspot residues occupying their respective pockets. The backbone residues of P1-C5 are all slightly shifted compared to Pc especially closer to the constraint. This difference can be explained considering that C5 is larger and more rigid than the disulfide bridge in Pc and therefore, it holds the two ends of the peptide further apart (12.6 Å vs 9.8 Å).

Figure 40 - X-ray crystal structure of P1-C5 (PDB: 6Q38). a) Overlay of P1-C5 (green) and CK2β (yellow). b) Overlay of P1-C5 (green) and Pc (PDB: 4IB5, cyan). c) Comparison of binding modes of P1-C5 (green) and Pc (cyan). d) Differences between the triazole constraint and the disulfide bridge.

Details of the crystallisation attempts can be found in the Appendix A.5.
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

Using similar conditions as for P1-C5 and matrix seeding from the co-crystals grown of the peptide, P2'-C5 was crystallised with CK2α. The X-ray structure showed that P2'-C5 contained the L-enantiomer of the 3-chloro-phenylalanine residue 7 (Figure 41a) and that the modified Phe7 did not overlay with the fragments that the modifications and MDs were based upon. Indeed, the binding mode of P2'-C5 forces the 3-Cl Phe to sit deeper in the pocket than the fragment which potentially leads to a clash with Tyr5 and accounts for the reduction in affinity. The structure shows that whilst chlorine in the 3-position could partially be accommodated in the Phe pocket (P2'-C5 Kd 2 μM), a chloro atom in the 4-position would disrupt the binding of the peptide as confirmed by the ITC experiments (P3-C5 Kd 56 μM). Even though the CCHP strategy did not result in an improvement in binding affinity of the peptides, the X-ray crystal structure of P2'-C5 with CK2α revealed the protein in complex with a peptide substrate (Figure 41b).

![Figure 41 - X-ray crystal structure of P2'-C5. a) Overlay of NMR154L (purple) and P2'-C5 (green) showing how the 3-chlorophenylalanine residue in P2'-C5 binds deeper than NMR154L resulting in no space to accommodate a chlorine in the 4-position. b) CK2 substrate (purple) occupying the substrate channel (highlighted by black dashed lines).](image)

This unprecedented result is of crucial importance to the CK2 kinase community as the structural information obtained opens opportunities to develop a novel class of substrate-competitive CK2 inhibitors and will help to elucidate the phosphorylation mechanism of the protein.

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*Peptide substrate mimicked by the N-terminal extension of CK2α (GSMD1EFDDADDGSGSGSGSGS) in the protein construct used CK2α_FP10.*
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

4.4.5. Further sequence modification: P6-C5, P7-C5, and P8-C5

Molecular modelling and rational design based on the available crystal structures of P1-C5 and P2'-C5, suggested that further modification of the sequence may result in enhancement of the binding affinity of the peptides.

The first variation of interest involves the substitution of Tyr5 residue (Table 14). This residue is located on the portion of the peptide that forms the β-turn in Pc, and it makes hydrophobic interactions with the so-called Tyr pocket. Therefore, it was decided to vary the Tyr5 to Pro, a residue known to help β-turn formation which could make the overall peptide more rigid, reducing the entropic penalty upon binding. With this in mind, peptide P6 was synthesised and constrained into the cyclic peptide P6-C5 with 88% conversion.

Table 14 – Sequences modification of peptides P6-, P7-, P8-C5 respect to P1-C5.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-C5</td>
<td>Z = Y; X = F; J = I</td>
</tr>
<tr>
<td>P6-C5</td>
<td>Z = P; X = F; J = I</td>
</tr>
<tr>
<td>P7-C5</td>
<td>Z = Y; X = F; J = W</td>
</tr>
<tr>
<td>P8-C5</td>
<td>Z = Y; X = W; J = I</td>
</tr>
</tbody>
</table>

The second sequence modification concerned the Ile9 residue (Table 14). The X-ray crystal structure of P1-C5 (Figure 42a) shows that the Ile9 could be replaced by an amino acid capable of π-π stacking with the aromatic ring of C5. Likewise, chlorobenzene probes in ligand-mapping simulations indicated an unfilled hydrophobic region around Ile9 (Figure 42b) suggesting that a larger, nonpolar side chain could be introduced (ΔH = -59.7 ± 1.2 Kcal·mol⁻¹ for P7-C5 compared to -57.0 ± 2.1 Kcal·mol⁻¹ for P1-C5, as calculated from MD simulations).
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Figure 42 – Peptide design guided by the X-ray structure of P1-C5 bound to CK2α. a) X-ray structure of P1-C5 showing Ile9 oriented in the right direction to stack with the constraint C5. b) Chlorobenzene mapping on Pc showing a high occupancy region by the aromatic carbon atoms of the probe around Ile9. Areas shown as black mash represent regions around the CK2α protein (blue) occupied by the hydrophobic chlorobenzene probe during the MD simulation. Peptide P1-C5 is shown in yellow to show which residues of the peptide correspond to the regions occupied by the probe during the MD. Ile9 of P1-C5, which is mutated to Trp in P7-C5, is shown in orange.

The sequence of P1 was modified to P7 to accommodate the largest aromatic residue Trp. Automated MW-assisted SPPS of P7 using standard Fmoc protected amino acids led to on-resin aggregation after the attachment of the first five amino acids as suggested by poor Fmoc deprotection of the amino acids. On-resin aggregation was successfully prevented by replacing standard Fmoc-Gly-OH with backbone protected Fmoc-(Dmb)Gly-OH residues at the 2- and 8-position of the peptide. The resulting peptide P7 was macrocyclised with C5 as the linker to afford P7-C5 (83% conversion).

Finally, the last modification was performed to generate a peptide that could be used as negative control in the functional and cellular assays. Such peptide (P8) featured a Trp residue as replacement of the Phe7 (Table 14). The Trp was chosen as it is the biggest natural amino acid and therefore should not be able to fit in the Phe pocket.

Disappointedly, peptide P6-C5 (Y5P) showed no binding to CK2α as measured by ITC. On the other hand, P7-C5 (I9W) appeared to be the highest affinity peptide developed thus far with a Kd of 150 nM. In favour of the hypothesis that a suitable constraint may enhance the binding of the peptide significantly, the constrained peptide showed a 300-fold improvement compared to the linear variant (Kd 44 μM, Figure 43). As for P1-C5, the ability of the peptide to prevent CK2β binding to CK2α was investigated using an ITC competition assay. No binding of the regulatory β subunit to the catalytic subunit was observed in the presence of 100 μM of the peptide.

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Fmoc deprotection in automated MW-assisted SPPS is monitored by UV absorbance of the dibenzofulvene (DBF) intermediate formed during DIPEA-mediated Fmoc removal. Peptide concentration in the ITC titrant and syringe.
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

Figure 43 - ITC binding curves of P7-C5 and P7 binding to CK2α.

X-ray crystal structure of P7-C5 allowed rationalisation of the improved binding affinity of the peptide compared to P1-C5. The structure shows the introduced Trp residue π-π stacking with the benzene ring of the constraint. No other additional interactions with CK2α were observed. Therefore, the increased binding affinity of P7-C5 for CK2 may be rationalised with a reduced entropic penalty upon binding due to the rigidifying interaction occurring between the constraint and the Trp residue (Figure 44).

Figure 44 - Comparison of P1-C5 and P7-C5 bound to CK2α. a) X-ray crystal structure of P7-C5 bound to CK2α (PDB: 6Q4Q). b) A comparison of the stacking with C5 in P1-C5 (green) and P7-C5 (green).

As expected, no binding of the negative peptide P8-C5 was detected in the ITC direct binding assay.

The effect of PPI inhibition on substrate phosphorylation was also studied using CK2β dependent and independent substrates. It was shown that P7-C5 was able to inhibit the
phosphorylation of a β-dependent substrate\textsuperscript{aa} with an IC\textsubscript{50} of 206 ± 29 nM. Pleasingly, the negative peptide P8-C5 did not cause inhibition of the phosphorylation of the β-dependent substrate (Figure 45). The peptide did not affect the phosphorylation of a β-independent substrate peptide (RRRADDSDDDD) suggesting that the binding of the peptide at the interface site does not displace the ATP.

\textbf{4.4.6. Multi-functionalisation of the constraint C5}

Preliminary confocal microscopy experiments carried out with FITC-labelled peptide P1-C5 (FITC-P1-C5)\textsuperscript{bb} showed that the peptide was not able to permeate the membrane of human colorectal cancer cells (HCT116). Z-stacks of fixed cells showed no green emission (corresponding to the FITC signal) whilst only the blue signal corresponding to the nuclei stain was observed (Figure 46).

\textsuperscript{aa} β-dependent substrates rely on the CK2β subunit to be bound to CK2α to be phosphorylated. Whilst CK2β does not phosphorylate the substrate itself, it acts as a docking station, and it is therefore crucial for substrate recognition. The CK2β-dependent substrate used in this work is the transcription factor eIF2β.

\textsuperscript{bb} Sequence FITC-Ahx-P1 cyclised with C5 to obtain FITC-Ahx-P1-C5 in 56% conversion.
Therefore, since both cell-penetrating motifs and fluorescent tags were necessary for the cellular assays, it was decided to develop a novel multi-functional constraint that would simultaneously constrain the peptide in its binding conformation, enhance stability to proteases, provide cell-permeability to the CK2 peptide and act as a fluorophore (Figure 47).

In addition, it was proposed that the functionalised constraints could be entirely synthesised in an automated manner using SPPS and independently from the CK2 peptide. The benzoic acid derivative linker was attached on-resin to a cell-penetrating peptide (CPP) via two molecules of aminohexanoic acid to avoid steric clashes with the CK2 peptide and the CK2α domain. Several reports make use of a poly-arginine tripeptide as a useful CPP to carry peptide cargos into cells. The previously used L-arginine was replaced with the D-arginine isomer to confer cell-permeability to the CK2 peptide and provide a proteolysis resistant alternative (F1C5, 81,193).
CHAPTER 4: Conformationally-constrained peptides targeting the protein-protein interaction of the anti-apoptotic proteins CK2

Figure 47. The CPP was in turn attached to the fluorescent tag FITC via an orthogonally-protected Lys – namely Lys(ddve) - to monitor peptide uptake into the cells (F2C5). FITC was also directly attached to the spacer via the Lys to provide a cell-impermeable fluorescent tag (F3C5) to use as a negative control. The functionalised linkers were then reacted with the linear peptides to obtain highly functionalised peptides and study their biological activity in cells was subsequently studied.

The ability of the functionalised constraint to improve the overall stability of the peptide to proteases was investigated using human serum. Importantly, the cell-permeable lead peptide (P7-F1C5) displayed significant stability in human serum (47% intact peptide after 24-hour incubation, Figure 48) whilst the linear analogue P7 was degraded entirely after 8-hour incubation.

![Serum stability test of P7 (black line) and P7-F1C5 (dashed-black line) in the presence of 20% serum. The results shown are the average of two independent repeats.](image)

4.4.7. Cellular work on P7-C5 derivatives

The ultimate goal of this project was to investigate the activity of the peptide in cancer cells. Therefore, further cellular experiments were carried out using two cell-lines: HCT116 and U2OS. The HCT116 cell line was chosen to study the activity of the peptides in cells due to its documented high expression levels of CK2. U2OS, on the other hand, was chosen to perform intracellular localisation experiments due to its great cytosol/nucleus ratio.

4.4.7.1. Intracellular localisation experiments

The ability of the functionalised constraint F2C5 to impart cell-permeability to the peptide was initially investigated using the FITC-labelled peptide P1-C5 which was previously found to be cell-impermeable in preliminary experiments (Figure 46). To this end, P1 was constrained with the FITC-labelled penetrating constraint F2C5 and with the FITC-labelled impermeable constraint F3C5 (Figure 49).
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The results showed that F2C5 was effective at transporting peptide P1 into cells whilst F3C5 was not. Consequently, F2C5 was used to constrain the lead peptide P7 and investigate its intracellular localisation. Confocal microscopy of U2OS osteosarcoma cancer cells treated with P7-F2C5 showed the peptide localising to the cell nucleus and also accumulating in proximity to the nuclear membrane at different time points (5 minutes to 6 hours) as shown in Figure 50. Thus, further studies were undertaken to elucidate the exact intracellular localisation of the peptide.

There are several reports of positively charged peptides being unable to escape the endosomes and ending up in the lysosomes, and therefore, imaging experiments were carried out to look at co-localisation with endosomal and lysosomal markers. Surprisingly, no co-localisation with endosomal markers was detected at any time-point (first and second row, Figure 51). On the other hand, partial co-localisation was detected with the lysosome stain (third row, Figure 51).

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1.25 μM concentration was chosen for its optimal signal/background ratio compared to the other concentrations screened (between 20 μM and 0.2 μM).
Despite partial co-localisation of the peptide with the lysosome stain, there was still a significant portion of the FITC signal that did not co-localise with any of the markers used thus far. It was decided to look at the co-localisation with Trans and Cis Golgi stains and markers for the endoplasmic reticulum (ER). These organelles are in proximity to the nuclear membranes and are involved with the trafficking of vesicles going in and out the lysosomes. It was envisioned that the peptide might co-localise with some of these organelles. Whilst minimal co-localisation was observed with the trans-Golgi (TNG46) at the early time-points, the peptide was found to co-localise with Cis-Golgi (ZFLP1) and ER markers (Calnexin) as shown in Figure 52.
This is an unexpected and unprecedented result that would need further investigation to understand ways of preventing the peptide from becoming trapped in the Golgi and ER. Despite this event, the peptide was detected in the nuclei at all time points. CK2 is a protein that shuttles between the cytosol and the nucleus; therefore, we proposed that the peptide present in the nucleus would be able to interact with CK2 and exert its bioactivity.

4.4.7.2. Anti-proliferative activity on colorectal cancer cells treated with P7-F1C5

Disruption of the PPI of CK2 has been reported to arrest cell cycle progression, induce apoptosis and slow cell proliferation in a variety of cell lines including human colorectal cancer cells (HCT116).\(^\text{144}\) To this end, the bioactivity of P7-F1C5 in HCT116 was investigated.

The effect of the peptide on cell proliferation was studied in two ways: (1) monitoring the rate of growth of the cells in the presence of increasing concentration of the peptide over 4 days; (2) using a Sytox Green™ assay to quantify the cells after 4-day incubation with the peptides. In the first case, the cells were treated with the peptide, and their rate of growth monitored at 37 °C. Scans of the cell confluency were recorded every 2 hours to give a percentage of the cell confluency at each scan (Figure 53).

The scans showed that P7-F1C5 slows down the proliferation of the cells at a concentration of 32 μM with 80% inhibition at 100 μM. For this experiment, two types of controls were used: CX4945 (positive control), P7-C5 (cell impermeable constrained peptide) and the functionalised cell-penetrating constraint F1C5 (cell-penetrating constraint without the peptide). CX4945 is a clinical candidate CK2α inhibitor inhibiting CK2 in an ATP-competitive manner.\(^\text{139}\) It is known to inhibit proliferation in HCT116 cells, and it was therefore used as positive control. Due to the mechanistic differences between CX4945 and P7-F1C5 a difference in the outcome of the assay was expected; whilst inhibition of the CK2α catalytic subunit is known to cause drastic effects in cells, inhibition of the holoenzyme assembly has been reported to have less severe consequences due to the fact that the activity of the catalytic α subunit is not affected.\(^\text{164}\) In addition, CX4945 is known to inhibit other kinases, and therefore, its more pronounced cytotoxic activity could be attributed to off-target effects.\(^\text{198}\) The constraint F1C5 was used as negative control to ensure that the positive charges on it would not cause any unspecific cytotoxicity while P7-C5 was used as negative control considering its limited cell-permeability.
Once it was verified that P7-F1C5 slowed down the proliferation of HCT116 cells, it was decided to determine its GI\textsubscript{50} using the Sytox Green™ assay. Sytox Green™ is a reagent that would generally stain nucleic acids of dead cells. If the detergent Saponin is added to the assay, cellular membranes become permeable, and the stain permeates both viable and dead cells and therefore gives an indication of the total number of cells. Consequently, the reagents were added straight after the peptide addition and after 4-day incubation to provide the number of cells in
the plate. In this assay, CX4945 was used as a positive control whereas F1C5, the cell impermeable P7-C5 and the cell-permeable negative peptide P8-F1C5 were used as negative controls. The results are shown in Figure 54.

**Figure 54** – Percentage of the proliferation of HCT116 cells after 70-hour incubation with the compounds. a) GI_{50} curves for CX4945 and CX4945. b) Percentage proliferation of HCT116 cells treated with P7-F1C5 and negative peptides at the top concentrations (100, 75, 50, 32 μM).

P7-F1C5 was able to inhibit cell proliferation with a GI_{50} of 76 ± 4.1 μM whilst the negative peptides did not show inhibition. However, the activity drop-off in the cellular assay was significant with respect to the enzymatic assays (K_{d} 170 nM, IC_{50} 370 nM). This result could be speculatively rationalised with the fact that the organelle trapping causes a drop-off in the concentration of peptide able to interact with CK2 intracellularly. It is also possible that the effect of the inhibition of the phosphorylation of CK2β-dependent substrates in outbalanced by the phosphorylation of CK2β-independent substrates.

### 4.4.7.3. Cell viability of colorectal cancer cells treated with P7-F1C5

We went on to investigate the ability of P7-F1C5 to induce apoptosis in cancer cells using the CytoTox-Glo™ Cytotoxicity Assay (Promega). As for the proliferation assays, several peptides were used as negative controls. Unlike the proliferation assay, the outcome of the cell viability assay was determined after 5-hour incubation with the peptides. The results are shown in Figure 55.
Once again, the EC$_{50}$ (87.5 ± 6.8 μM) of P7-F1C5 was considerably higher than expected. Pleasingly, no cytotoxic effect was detected for P7-C5 and F1C5 whilst 60% viability was observed when the top concentration of the negative peptide P8-F1C5 was used. It should be noted that no inhibition of the proliferation was observed when the same concentration was used in the anti-proliferation assay. Whilst the outcome of the viability assay is determined after 5-hour incubation, the anti-proliferative effect is measured after 70-hour incubation. Therefore, the initial unspecific cytotoxic effect of P8-F1C5 is somehow overcome by the viable cells whose long-term proliferation is not affected.
4.5. Conclusions and future work

In this work, conformationally-constrained peptides that act as CK2 α/β PPI inhibitors were developed. The lead peptide, P7-C5, presents an enhanced binding affinity for CK2α with respect to the previously developed Pc (Kd 150 vs 1000 nM) and it is stable under conditions mimicking physiological fluids. Due to the lack of intrinsic cell-permeability of the peptides, an easily-synthesised multi-functional constraint was developed giving P7-F1C5 which allowed the investigation of the intracellular activity. P7-F1C5 was found to arrest cancer cell proliferation and induce apoptosis in a dose-dependent manner. Importantly, the multi-functional constraint developed herein could be used to lock other peptides into their binding conformation and simultaneously functionalise them.

P7-F1C5 is the first inhibitory peptide of the CK2α/β PPI that is stable in serum, cell-permeable, active in cells, able to engage the target and with structural information available. Such a peptide could act as a chemical probe allowing study of the CK2 PPI using endogenous levels of proteins and could, therefore, be a powerful tool for validating and dissecting biological processes associated with CK2.

The downside of P7-F1C5 is its accumulation in some cytosolic organelles – namely lysosomes, Golgi and ER. Future work will focus on understanding the causes of this effect by introducing mutations in the peptide sequence or by changing the cell-penetrating tag. In addition, further attempts to investigate the ability of P7-F1C5 to engage CK2 intracellularly will be carried out.

Shorter peptides with improved ligand efficiency will be developed using the X-ray structures obtained in this work as a starting point. The ultimate goal is to develop peptides that are cell-permeable without the introduction of cell-penetrating motifs. Considering that the peptide hotspots are known, the shorter peptides will have a sequence based around them (RLYGFK), and new constraints will be screened to lock the shorter peptides in their binding conformation. At a later stage, the peptides could be merged with fragments found to bind in the Tyr pocket in FBDD screening campaigns carried out by collaborators in the Hyvönen group (Biochemistry, University of Cambridge). Whilst the CCHP strategy around the Phe7 residue failed in this study, shorter peptides should allow more flexibility around the Tyr pocket, and therefore, it is envisioned that the CCHP strategy would work with the shorter peptides.

CK2 is overexpressed in cancer cells but it is also present in healthy cells. Selective cancer-cell targeting would improve the therapeutic window of CK2 inhibitors therefore reducing side effects. Selective cancer-cell targeting may be achieved with the use of homing peptides – short amino acid sequences that recognise the receptor exposed on the membrane of cancer cells.
iRGD, for instance, is a homing peptide that targets colorectal cancer cells, and it could be attached to the CK2 peptides for CK2 targeting in selected cells. Stapling of the homing peptide will be considered to avoid its degradation in the bloodstream.

Finally, in this work P2'-C5 provided the first X-ray structure of the CK2 substrate binding in the substrate channel. Such information will be used to develop high-affinity peptides that could displace the native CK2α substrates, and therefore the structural information obtained in this work opens up the possibility to develop a novel class of CK2 inhibitors and to fully understand the phosphorylation mechanisms of CK2.
CHAPTER 5:
Targeting of Anti-Apoptotic Bcl-2 Proteins in Platelets Using Stapled Peptides
CHAPTER 5: Targeting of anti-apoptotic Bcl-2 proteins in platelets using stapled peptide

5.1. Summary

Platelets are blood cells with numerous crucial pathophysiological roles in haemostasis, inflammation, cardiovascular thrombotic events, and cancer metastasis.\textsuperscript{180-182} Platelet activation and survival require the engagement of intracellular signalling pathways that involve protein-protein interactions (PPIs), and a better understanding of these pathways is crucial for the development of selective antiplatelet drugs. Conventional platelet research methods present several limitations. For example, small molecule inhibitors can lack selectivity and are often challenging to design and synthesise; the development of transgenic animal models is costly and time-consuming; and conventional recombinant techniques are ineffective due to the lack of a nucleus in platelets.\textsuperscript{193}

In order to overcome the limitations of conventional research methods, this chapter describes the first application of stapled peptides in human platelets to study PPIs of the Bcl-2 protein family (Figure 56).

![Diagram of platelet activation](image)

\textit{Figure 56} - First application of stapled peptides in platelets to study PPIs.

This work shows that stapled peptides have the potential to unlock the number of PPIs that can be studied in human platelets, and they could help in the development of safer platelet-targeting drugs for treatment of a variety of diseases.
5.2. Project background

Of interest is the investigation of the Bcl-2 proteins, in particular, the BH3-only proteins which play a crucial role in regulating intrinsic apoptosis and the lifetime of platelets.\textsuperscript{167,178,186,187} Whilst all-hydrocarbon stapled peptides corresponding to the BH3 domains of the BH3-only protein family members have previously been used in haematological cancer cells, the efficacy of these molecules for use in platelets has yet to be examined. On the other hand, the small molecule Bad-BH3 mimetic ABT-737\textsuperscript{21} has been employed in platelets where it was found to cause platelet apoptosis but no platelet activation.\textsuperscript{188,224} The pathways that lead to intrinsic apoptosis in platelets overlap to some degree with those leading to platelet activation and clot formation as described previously (Figure 20), and it is believed that the different BH3-only proteins may trigger activation or apoptosis selectively (Figure 57). The elucidation of these pathways could have a remarkable impact on the development of selective platelet-targeting therapy.

\textbf{Figure 57 – Schematic representation of the Bcl-2 family members and their relationship with each other. Pro-apoptotic BH3-only proteins are shown in green (light green for sensitisers and dark green for activators), anti-apoptotic proteins in pink and effectors of apoptosis in blue. BH3-only proteins can inhibit one class or both classes of anti-apoptotic proteins, and the effect of inhibiting one class or another on platelet activation and apoptosis is not known.}
5.3. Project aim and overview

This project aimed to develop stapled peptides able to mimic the BH3-only proteins of the Bcl-2 family and utilise these to dissect their role in platelet apoptosis and activation.

Initially, uptake experiments were carried out using model peptides with different tags on the staple. This showed how the nature of the tags impacted on the ability of the stapled peptides to permeate the membrane of the platelets. The model peptides were based on the p53 sequence and did not affect platelet activation or apoptosis: platelets are anucleated cells, and p53 is a nuclear protein. Successively, the tags that allowed the p53-based model peptides to enter the cytosol of platelets were used to staple Bim-BH3-based peptides at an $i,i+4$ position, and their effect on platelet activation and apoptosis studied. At a later stage, the ability of the peptides to engage with their target was investigated in vitro. Mechanistic differences between the Bim-BH3 peptide and the small molecule Bad-BH3 mimetic ABT-737 utilised as positive control inspired us to investigate the effects of all the other BH3-only proteins on platelet activation and apoptosis. Initially, we intended to employ an $i,i+11$ stapling strategy to constrain the BH3 peptides; however, due to problematic functionalisation of the linker, an $i,i+7$ stapling chemistry was applied instead. The workflow described in this chapter is summarised in Figure 58.

![Figure 58 - Overview of the work described in this chapter.](image)
CHAPTER 5: Targeting of anti-apoptotic Bcl-2 proteins in platelets using stapled peptides

5.4. Results and discussion

Unless otherwise stated, the peptides described in this chapter were synthesised using standard procedures for solid phase peptide synthesis (SPPS) and peptide macrocyclisation was achieved by following previously reported procedures for two-component copper-catalysed azido-alkyne cycloaddition peptide stapling (2C CuAAC PS).

The cellular experiments reported in this chapter were performed at Anglia Ruskin University, Cambridge, under the supervision of Dr Nicholas Pugh. The Surface Plasmon Resonance (SPR) assay was performed at AstraZeneca, Cambridge, under the supervision of Dr Kara Herilhy.

5.4.1. Uptake and bioactivity of model stapled peptides in human platelets

Since no reports existed in the literature on using stapled peptides in platelets, we started investigating cell permeability. To this end, a panel of sixteen model stapled peptides (synthesised by Dr Yuteng Wu) was used. The model peptides were TAMRA labelled to allow for fluorescent visualisation on flow cytometry, and their sequence was based on the p53 protein (a nuclear effector) and should have no effect on anucleated platelets. In addition, the model peptides were stapled with functionalised peptidic and non-peptidic groups providing enough variety to understand which groups are more effective at carrying the cargo peptides into platelets (Figure 59). Functionalised groups included: polycationic peptidic chains, the nuclear localisation sequences PKKRKV (NLS) and a polyguanidine small molecule carrier (SMoC). Moreover, a panel of anionic and polar peptidic chains was also screened.

Figure 59 - TAMRA-labelled functionalised stapled peptides synthesised by Dr Yuteng Wu used to analyse platelet uptake of peptides. Schematics of the different peptidic (blue), non-peptidic functionalised staples (green) and a SMoC staple (yellow). * indicates unlabelled peptides (N-terminus capped as acetyl). Adapted from Iegre et al., ChemSci, 2018.

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ad The work described in paragraphs 5.4.1 and 5.4.2 have been published in a scientific journal prior to publication of this PhD thesis and some text and figures reported herein have been adapted from the publication Iegre et al., ChemSci, 2018.
Flow cytometry was employed to assess the uptake of the peptides into platelets at different time points: internalisation of the TAMRA labelled peptides resulted in fluorescent platelets with emission at 550 nm (Figure 60a). Treatment with M13-M15 (carrying NLS) or M16 (bearing the SmoC motif) showed the highest platelet fluorescence after a 3-hour incubation (96.1 ± 0.8%, 98.1 ± 1.1%, 97.5 ± 1.4% and 96.1 ± 2.2% respectively) indicating a substantial association of the platelets with the stapled peptide. Similarly, M2 (ethyl ester) and the poly-arginine stapled peptides M4 and M5 showed significant uptake (88.8 ± 5.6%, 73.0 ± 13.3 % and 87.0 ± 7.3% respectively) whereas only 34.9 ± 7.8% of platelets were fluorescent following treatment with M8 (poly-lysine). No changes in fluorescence were observed with the other peptides, indicating that the other tags were ineffective as cell-penetrating peptides (CPPs).

For the peptides that showed maximum fluorescence after 3-hour incubation, the uptake was monitored after 15, 30, 45, and 60 minutes (Figure 60b). More than 50% of platelets became fluorescent during the first 15 minutes of incubation with M13-M16 with a further increase after the first 45 minutes. Some 40-50% of platelets were fluorescent after 15 minutes following treatment with M2, M5 or M8, and no further increase was detected thereafter. Continuous

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**Figure 60 – Uptake experiments using model peptides M1-M16.** a) Platelets fluorescence after 1 h (white), 2 h (grey) and 3 h incubation (black). b) Peptide uptake of model peptides monitored during the first 60 minutes of treatment. ◆: vehicle (DMSO). ●: M16, ■: M15, ▲: M14, ▼: M13, ◆: M8, □: M5, ○: M2. c) Five-minute real-time measurement of peptide uptake. Unlabelled platelets were recorded for 30 sec prior to addition of the given peptide. d) Confocal microscopy images of live platelets incubated with the TAMRA-model peptides. Adapted from Iegre et al., ChemSci 2018.

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*In all the flow cytometry experiments described in this chapter, the fluorescence associated with untreated platelets (no compounds, no fluorescently-labelled markers) was recorded and used to normalise the results. In particular, the peak corresponding to the untreated platelet signal read on the laser of interest was considered as zero. Shift of such peak towards the right was considered as positive platelet and quantify accordingly (vide 9.2.2, Figure 72 for more details).*
assessment of platelet fluorescence in the first 5 minutes of treatment demonstrated that peptides M5 and M16 were instantly internalised by a fraction of the cells (Figure 60c).

Peptide uptake experiments of the most permeable model peptides M5, M13-M16 were repeated in the presence of platelet-rich plasma (PRP) to ensure realistic conditions. The results showed only a small reduction in peptide uptake between 10 and 20% compared to buffer, indicating that the uptake is not prevented by the interaction of the peptides with the other components of the plasma.

Whilst flow cytometry quantifies the fluorescence of the platelet population, it is not indicative of whether the fluorescence is a result of peptide internalisation or association with the membranes. Consequently, confocal microscopy of live platelets was carried out to elucidate the peptide localisation. It should be noted that platelets are small-sized (Φ ~ 2 μm); therefore, confocal microscopy cannot assign pixel localisation unequivocally but can, nonetheless, indicate whether the fluorescence comes from the platelet membrane or cytosol. Live platelets were imaged after 1-hour incubation with the TAMRA-labelled model peptides, and the results suggested that peptides M5 and M13 permeated into the platelet cytosol. On the contrary, M2, M8, and M16 localised predominantly on the platelet membrane (Figure 60d).

Conventional platelet apoptosis and activation responses were assessed to investigate potential off-target effects following treatment with the platelet-permeable model peptides. As a response to external stimuli, platelets expose phosphatidylserine (PS) on the plasma-oriented surface of their membrane. It is widely recognised that plasma-exposed PS plays a regulatory part in blood coagulation acting as a secondary messenger and is an index of apoptosis (in both platelets and nucleated cells). When exposed on the platelet surface, PS binds the marker Annexin V in the presence of Ca²⁺ ions. If the marker is fluorescently labelled (i.e. with a FITC group), its signal can be used in flow cytometry to detected PS exposure (Figure 61). Similarly, other platelet activation markers (dense and α granules release, integrin αIIbβ3) can be detected using flow cytometry when specific fluorescently-labelled antibodies are used. For instance, exposure of integrin αIIbβ3 on the platelet surface can be detected by the antibody PAC-1 (Figure 61). When platelets become activated, they expose integrin αIIbβ3 to bind substrates that once complexed, promote platelet aggregation and clot formation.

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8 Different excitation/emission wavelengths for TAMRA (Ex-max 546 nm/Em-max 579 nm) and FITC (Ex-max 494 nm/520 nm Em-max) allowed selective detection of a fluorescent signal over the other.
CHAPTER 5: Targeting of anti-apoptotic Bcl-2 proteins in platelets using stapled peptide

Since the model peptides used were based on the sequence of the nuclear effector p53, and platelets have no nucleus, no biological effect was expected after treatment with the peptides. As expected, only low activation changes were observed following treatment with NLS-bearing peptides M13-M15 and SmoC peptide M16 (Figure 62a and b). No change in platelet activation markers was found for the other peptides, indicating that the treatment of platelets with the model peptides did not cause unspecific responses. Undesired platelet aggregation - as noted by analysis on the aggregometer - was detected in response to treatment with peptide M16, but not for the other peptides (Figure 62c). Thus, peptide M16 was excluded from further studies.

This data set provides evidence that functionalisation of stapled peptides is required for the uptake of peptides into platelets, with the NLS sequence (M13-M15) and poly-arginine staple (M5) being the most effective. In addition, these preliminary experiments showed that the staples themselves do not exert any unspecific activity in platelets.
5.4.2. Investigation of the Bim/Bcl-xL PPI in platelets using i,i+4 stapled peptides

Once it was confirmed that platelets were able to uptake stapled peptides, further experiments were performed to investigate whether peptides based on biologically relevant sequences could elicit a relevant intracellular response in platelets.

The role of BH3-only proteins in the development of a pro-coagulant platelet phenotype has been investigated using the Bad-BH3 mimetic small molecule ABT-737. This induces mitochondrial membrane depolarisation, activation of caspases -9, -8 and -3, PS exposure, and causes inhibition of the platelet activation process by interacting with the pro-survival Bcl-2 proteins. However, the role of the other BH3-only proteins (Bid, Bim, Bmf, Noxa, Puma) in platelet activation and apoptosis is not fully understood. Previous work has demonstrated that an all-hydrocarbon (AH) Bim sequence-based peptide (denoted Bim SAHBa, Table 15) induces Bax mediated apoptotic responses in nucleated cancerous cells. To the best of our knowledge, the implication of the interaction of Bim-BH3 mimetics with the anti-apoptotic Bcl-2 proteins in platelets is unknown. Hence, CuAAC 2C stapled peptides based on the Bim-BH3 sequence were synthesised and utilised to investigate their effects on platelet processes. The CuAAC 2C peptide stapling methodology was chosen as it allows to access multiple stapled peptides from one linear sequence in a combinatorial manner. This results in a more efficient access to libraries of functionalised peptides.

5.4.2.1. Design and synthesis of i,i+4 Bim-BH3 stapled peptides

Inspired by the work of Walensky et al., we decided to synthesise i,i+4 stapled peptides based on the SAHBa sequence. Molecular dynamic simulations (MDs) were performed by Dr Yaw Sing Tan on the complex form by the Bcl-xL protein and the peptide mimicking the BH3-only domain of the protein Bim to suggest amino acid and staple combination to use to retain the binding affinity of the native Bim peptide. MDs simulations suggested employing the azido ornithine amino acid (12) as the unnatural amino acid to perform the 2C CuAAC stapling. This would facilitate the synthesis of different stapled peptides from one linear precursor (P9, Figure 63a). 3,5 dialkyne benzoic acid (C5) was used as the linker for stapling. MDs simulations of the peptide complexed with Bcl-xL suggested that the stapled peptide P9-C5 (Figure 63b) would retain an α-helical conformation upon binding and that its binding affinity would be comparable to that of the unstapled wild-type (WT) peptide (P10) (ΔH -107.6 ± 4.9 Kcal·mol⁻¹ vs -108.3 ± 1.5 Kcal·mol⁻¹, Figure 63).

**An anti-apoptotic Bcl-2 protein which is known to be expressed in platelets.**
Synthesis of peptide P9 required Fmoc-azido ornithine amino acid 12 which was synthesised according to literature precedent. The synthesis commenced from the commercially available Fmoc-Ornithine(Boc)-OH that underwent Boc deprotection in the presence of 4 M HCl in 1,4-dioxane to afford Fmoc-Ornithine-OH 11. The crude product was then subjected to treatment with azido-transfer reagent imidazole-1-sulfonyl azide hydrogen sulfate (6) in the presence of CuSO₄·5H₂O to yield the desired product 12 in 78% yield (Scheme 29).

**Scheme 29** - Synthesis of azido amino acid 12 starting from commercially available Fmoc-Ornithine(N-Boc)-OH.
Synthesis of the azido-transfer reagent 6 and constraint C5 are described in Chapter 4.

Synthesis of staples F4C5 and F5C5 was achieved using the standard procedure for SPPS (Figure 64). The \( N \)-terminus of the peptides were capped with C5 before peptide cleavage from the resin, trituration with \( \text{Et}_2\text{O} \) and purification.

![Structures of constraints C5, F4C5 and F5C5.](image)

Based on the platelet permeability results of the model peptides, P9 was then stapled with C7 (non-functionalised staple to be used as negative control), F4C5 (poly-arginine staple) and F5C5 (NLS staple) where F4C5 and F5C5 are the same functionalised staples as the model peptides with the highest cytosolic platelet uptake M5 and M14. A list of all the peptides used in this study and conversions of the CuAAC 2C-PS reactions can be found in Table 15.

Additionally, a negative cell-permeable peptide was synthesised and used as a negative control. The negative peptide (P11-F4C5) presented an R153D mutation in its sequence which was found not to exert the Bim-BH3 biological effect in nucleated cells. FITC-labelled analogues of all the stapled peptides were also synthesised to monitor their uptake into platelets. In this study, FITC was attached to the N-terminus of the peptides by treating the on-resin Fmoc-deprotected peptide with FITC and DIPEA in the dark for 14-16 hours. An aminohexanoic acid spacer (Ahx) was placed between the N-terminal peptide residue and the FITC group to avoid steric clashes. The SAHBa peptide, which inspired this work, and its FITC-labelled analogue, were also synthesised for comparison.

Table 15 - Names and structures of the peptides used in this study, conversions of the 2C-PS stapling and schematic representation of the 2C CuAAC reaction.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Staple</th>
<th>Conversions</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>Ac-IWIAQELRXIGDXFNAYYARR</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Peptide</td>
<td>Structure</td>
<td>Conversion of stapling reactions</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>---------------------------------</td>
<td></td>
</tr>
<tr>
<td>P9-F4C5</td>
<td><img src="image" alt="Structure" /></td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-P9-F4C5</td>
<td><img src="image" alt="Structure" /></td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>P9-F5C5</td>
<td><img src="image" alt="Structure" /></td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-P9-F5C5</td>
<td><img src="image" alt="Structure" /></td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>P9-C7</td>
<td><img src="image" alt="Structure" /></td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-P9-C7</td>
<td><img src="image" alt="Structure" /></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td><img src="image" alt="Structure" /></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-P10</td>
<td><img src="image" alt="Structure" /></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>P11-F4C5</td>
<td><img src="image" alt="Structure" /></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-P11-F4C5</td>
<td><img src="image" alt="Structure" /></td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>SAHBa</td>
<td><img src="image" alt="Structure" /></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FITC-Ahx-SAHBa</td>
<td><img src="image" alt="Structure" /></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

All the peptides have an amide at the C-terminus. X = azido ornithine; The schematic structure showed above the table does not represent one of the SAHBa peptides which were cyclised using a RCM reaction. Z = (S)-2-(pent-4-enyl) alanine and the AH staple is shown in the column "Staple". Conversion of stapling reactions as determined by LCMS of the crude reaction mixture. NA = not available due to complicated LCMS chromatogram.
CHAPTER 5: Targeting of anti-apoptotic Bcl-2 proteins in platelets using stapled peptide

Synthesis of peptide P9 proved challenging. Standard microwave-assisted SPPS procedure using MBHA Rink Amide (0.35 meq/g resin loading) resulted in some impurities with a similar retention time to the desired product hence preventing its purification. The use of lower loading resin (0.19 meq/g) did not resolve the issue, and neither did room temperature peptide synthesis. Pleasingly, a double coupling for each of the amino acids afforded the desired product P9. The latter crude peptide presented solubility issues in the solvents used for HPLC purification (MeCN, H2O, TFA) and in DMSO. High dilution of the crude material followed by several filtrations using 0.45 µm PTFE syringe filters resulted in a clear solution that underwent purification successfully. Similarly, synthesis of P10 was achieved on a microwave-assisted peptide synthesiser using double coupling for all the amino acid. Unlike P9, P10 did not present solubility issues

5.4.2.2. The uptake of Bim peptides into platelets

As with the model peptides, flow cytometry was used to detect fluorescent platelets in the presence of the FITC-labelled Bim-BH3 peptides. The PRP was incubated with the FITC-labelled peptides, and the uptake monitored after 15, 30 and 60-minute incubation (Figure 65a).

The uptake experiment showed that all the peptides tested associated rapidly with platelets except for the WT peptide FITC-Ahx-P10.

Successively, confocal microscopy was used to understand whether the fluorescence observed by flow cytometry was cytosolic or associated with the platelet membranes (Figure 65b). Consistently with the behaviour of the non-functionalised model peptide M1, the images showed poor membrane permeability of peptide FITC-Ahx-P10. Even though flow cytometry of FITC-
Ahx-P9-C7 suggested high platelet fluorescence in plasma, confocal imaging showed the peptide forming aggregates outside the platelet cytosol and hence being unable to reach the target protein. Peptide FITC-Ahx-P9-F4C5 was the most effective at localising into the platelet cytosol: FITC-Ahx-P9-F5C5, in fact, aggregates partially with the platelet membrane. Both the positive and negative control peptides (SAHBa and FITC-Ahx-P11-F4C5 respectively) were successfully internalised in the platelet cytosol.

5.4.2.3. Phosphatidylserine exposure induced by Bim-BH3 peptides: a sign of apoptosis or platelet activation

Once it was confirmed that Bim-BH3 peptide uptake is dependent on a functionalised staple, the influence of these peptides on activating processes in washed platelet suspensions was studied. The activity of the peptides was compared with ABT-737, the previously reported Bad-BH3 small molecule mimetic.21,188 Initially, the ability of the peptides to cause PS exposure was investigated on flow cytometry using FITC-labelled Annexin V.

Treatment of platelets with P9-F4C5 or P9-F5C5 resulted in PS exposure (with 78 ± 1.3% and 42.9 ± 6.0% Annexin V binding after 3-hour incubation), consistent with the generation of a pro-coagulant phenotype. Of note, P9-F4C5 showed a similar profile to ABT-737 and appeared to be more potent than the AH stapled Bim peptide SAHBa (Figure 66). No Annexin-V binding was observed following treatment with the WT peptide P10 nor with the non-functionalised stapled peptide P9-C7, consistent with their cell-impermeable nature. Pleasingly, the negative peptide P11-F4C5 did not result in PS exposure in platelets despite being platelet-permeable, suggesting that the effects of the BH3 stapled peptide are specific to biologically relevant processes.

![Figure 66](image-url) - Functionalised, stapled Bim-BH3 peptides induce PS exposure in platelets. Platelets were treated with 10 μM compounds, and changes in Annexin-V binding were quantified using flow cytometry at different time points. ●: P9-F4C5, □: P9-F5C5, ▲: P9-C7, ○: P10, ■: ABT-737, ◆: vehicle (DMSO), ●: P11-F4C5, ○: SAHBa.
5.4.2.4. Activation marker binding induced by Bim-BH3 peptides: a sign of platelet activation

We then assessed the effect of permeable stapled peptides P9-F4C5 and P9-F5C5 on platelet activation markers since previous work has indicated that the small molecule Bad-mimetic ABT-737 induces apoptosis without causing platelet activation.\textsuperscript{236-238} As in the previous experiments, P11-F4C5 peptide was employed as the negative control.

In addition to the previously described PAC-1 (\textit{vide supra} 5.4.1), two other platelet activation markers were used in this experiment: CD62P and CD63. CD62P indicates secretion of α granules while CD63 is an index of secretion of dense granules.\textsuperscript{236,238} α granules are secreted by activated platelets into the plasma and contain several growth factors - such as IGF-1, PDGFs, TGFβ, CXCL4 - and clotting proteins - including thrombospondin, fibronectin, factor V, and the VWF - which trigger clot formation. On the other hand, dense granules contain ADP, ATP, calcium ions and serotonin; all cofactors required to prompt the coagulation cascade. α and dense granules express the adhesion molecules CD62P and CD63 respectively and, when exposed on the platelet surface, can be recognised by the corresponding fluorescently labelled-antibodies.

Initially, it was decided to use the three antibodies (anti-PAC1, anti-CD63 and anti-CD62P) simultaneously as the markers carried different fluorophores that could be detected at three different wavelengths by the flow cytometer.\textsuperscript{hh} However, assessment of the three activation markers together was not possible as the readings generated inconclusive results with the DMSO control giving high response for all the markers. Therefore, the activation markers were assessed separately.

As shown in Figure 67, after 1-hour incubation both Bim-BH3 peptides induced integrin α\textsubscript{IIb}β\textsubscript{3} activation (as measured by PAC-1 binding), whilst ABT-737 was ineffective. In particular, treatment with P9-F4C5 or P9-F5C5 resulted in 24.1 ± 2.4% and 50.8 ± 0.3% PAC1-positive platelets, respectively.\textsuperscript{ii} Treatment with both peptides resulted in similar levels of CD62P expression (53.7 ± 2.5% and 62.1 ± 7.8%, respectively), indicating peptide-induced α granule secretion. Additionally, both peptides increased CD63 expression (correlated with dense granule release), with peptide P9-F4C5 being more effective than P9-F5C5 (72.9 ± 5.3% vs 34.4 ± 6.7% respectively). Pleasingly, unspecific activation was not observed when the mutant P11-F4C5 was used.

\textsuperscript{hh} FITC-labelled PAC-1 (Ex-max 482 nm/Em-max 520 nm); PE-labelled CD62P (Ex-max 496 nm/Em-max 478 nm); APC-labelled CD63 (Ex-max 650 nm/Em-max 660).

\textsuperscript{ii} Positive platelets are ones that showed greater fluorescence than any platelets in the untreated control sample. \textit{Vide} Figure 72, section 9.2.2 for more details.
CHAPTER 5: Targeting of anti-apoptotic Bcl-2 proteins in platelets using stapled peptide

In conclusion, despite having similar effects on PS exposure with respect to the clinical candidate ABT-737, our Bim BH3 peptides showed a pronounced increase in activation markers. These results highlight that Bim and Bad have different effects on platelet activation, thus pointing to a difference in the roles and mechanisms of these two proteins. Further investigation dissecting the roles of each BH3-only protein in platelets would help to understand these mechanistic differences.

5.4.2.5. In vitro target engagement using an SPR assay

Surface Plasmon Resonance (SPR) assay was used to understand whether the peptides were able to bind to the anti-apoptotic proteins of the Bcl-2 family (Bcl-xL, Mcl-1, A1, Bcl-2, Bcl-w). In particular, the ability of P10, P9-F4C5, P11-F4C5 and SAHBa to bind to Bcl-xL was investigated. Bcl-xL was chosen among the other members of the anti-apoptotic family for its well-documented presence in platelets.

In the SPR assay performed in this study, the Bcl-xL protein was immobilised on a flexible dextran matrix, and the peptides were allowed to flow across that surface. The SPR assay was configured to examine the direct binding of peptides to Bcl-xL derivatised sensor surfaces and generate apparent affinity values \((K_{dapp})\) to provide ranking data for the series of peptides under examination.

The results confirm that the WT peptide P10 and platelet-permeable stapled peptide P9-F4C5 were able to engage with Bcl-xL protein \((K_{dapp} 7.3 \pm 0.2 \text{ and } 26 \pm 0.3 \text{ nM respectively})\) supporting the hypothesis that P10 does not exert a biological response in platelet due to the lack of cell-permeability. Interestingly, the AH stapled Bim-BH3 peptide SAHBa showed a reduced binding.
affinity compared to the WT and peptide P9-F4C5 ($K_{dapp} > 33$ nM).\textsuperscript{ii} No binding was observed for the negative peptide P11-F4C5.

### 5.4.2.6. Effect of stapling on the peptide secondary structure and stability to serum proteases

Circular dichroism (CD)\textsuperscript{ik} was used to elucidate the effect of the $i,i+4$ stapling on the secondary structure of the Bim peptides. To this end, the functionalities that P9-F4C5 and P9-F5C5 carry on the staples were removed to avoid interference, and P9 was stapled with C5 (3,5-dialkyne benzoic acid, 58% conversion). The secondary structure of P9-C5 was measured in a 50:50 mixture of MeCN and H$_2$O at a concentration of 50 μM. For comparison, the structure of the WT Bim-BH3 peptide P10 in solution was determined using the same conditions (Figure 68).

![Figure 68](image.png)

**Figure 68** - Mean Residue Ellipticity of the Bim-BH3 peptides P9-C5 (green) and P10 (red).

The spectra showed that P9-C5 was able to maintain a helical structure although its % helicity (at 222 nm) was reduced compared to the WT peptide P10 (10% vs 20%, respectively).

In order to understand whether stapling via 2C CuAAC enhanced the proteolytic stability of the peptides to proteases, serum stability tests were undertaken for the stapled peptide P9-C5 and the WT linear peptide P10.

---

\textsuperscript{ii} Exact quantification of the $K_{dapp}$ for SAHBa was not possible due to solubility issues encountered when concentrations higher than 33 nM were used.

\textsuperscript{ik} Circular dichroism (CD) is a technique used to investigate the peptide secondary structure in solution. For α-helical peptides, a typical CD spectrum shows a characteristic double minimum at 208 and 222 nm. CD provides a simple method for estimating α-helical content of peptides and proteins but should not be considered definitive.
The staple was effective at improving the stability of the peptide to the serum proteases: the WT peptide P10 was fully degraded after 4 hours while 20% of the stapled peptide was still detected after 26-hour incubation.

5.4.2.7. Considerations on the use of i,i+4 Bim-BH3 peptide in platelets

The results obtained thus far showed that platelets are amenable to treatment with stapled peptides. In particular, a functionalised staple was required to translocate p53 and Bim-based 2C CuAAC stapled peptides into the platelet cytosol. The i,i+4 stapled peptides developed in this work proved to be more stable than the WT peptide to serum proteases. Besides, the peptides engaged the Bcl-xL target with nM affinity and were able to cause platelet activation.

5.4.3. Dissecting the role of Bim, Bad, and Bid protein in platelets using stapled peptides

Intrigued by the results obtained when comparing Bim to Bad mimetics, we decided to investigate the effect of mimics of each member of the pro-apoptotic proteins of the Bcl-2 family on platelet activation and apoptosis. In nucleated cells, Noxa-BH3 antagonises exclusively the anti-apoptotic proteins Mcl-1 and A1. Bim, Puma, and Bid inhibit all of the anti-apoptotic proteins whereas Bad, and Bmf engage Bcl-2, Bcl-xL, and Bcl-w (Figure 57). To this aim, Bim, Bad and Bid-BH3 mimetics were synthesised, and their characterisation is described in this work; Noxa, Puma, and Bmf-BH3 peptides were synthesised by Josephine Gaynord, PhD student in the Spring group.

5.4.3.1. Looking at i,i+11 stapling

The i,i+4 stapling of the Bim-BH3 peptides synthesised in the first part of this study did not enhance the helicity of the WT peptide (Figure 68). It is highly debatable whether the
enhancement of helicity results in an improved binding affinity for the BH3 peptides; however, the majority of reports indicate that greater helicity results in greater binding.\cite{176,186,239}

Consequently, alternative stapling positions to the $i,i+4$ were considered to impart greater helicity on the peptides. The use of $i,i+11$ stapling in 2C CuAAC is highly underexplored with only one study done on one model peptide sequence: a 3,6-diethynyl-9H-carbazole used in combination with azido-ornithine amino acids was the most effective, among other stapling combinations explored, at inducing $\alpha$-helical structures.\cite{240} In addition, the proteolytic stability as a result of constraining 11 residues instead of 4 may be improved further. Encouraged by this evidence, we decided to synthesise $i,i+11$ Bim, Bad, and Bid BH3 peptides using a carbazole linker as the staple.

5.4.3.1.1. Design and synthesis of $i,i+11$ stapled peptides

The $i,i+11$ stapled peptides were designed from the X-ray structures available. The staple was placed on those residues that did not contribute to the binding and that are solvent exposed to avoid clashes with other residues on the peptide or the target proteins. A summary of the $i,i+11$ stapled peptides proposed, their corresponding WT peptides, a schematic representation of the stapling reaction, and the reference PDB codes can be found in Table 16.

### Table 16 – WT and $i,i+11$ Bim, Bad, Bid stapled peptides subject of this work.

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB</th>
<th>WT peptide</th>
<th>Azido-linear peptide</th>
<th>Stapled peptide</th>
<th>Conversions$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bim</td>
<td>2YQ7\cite{211}</td>
<td>IAQELRRGDEFNAYYARR (P10)</td>
<td>IAXELRRGDEFNXYYA (P14)</td>
<td>P14-C8</td>
<td>NA</td>
</tr>
<tr>
<td>Bad</td>
<td>1G5J\cite{242}</td>
<td>RYGRELRRMSDFVDSF (P12)</td>
<td>XYGRELRRMSDFPVDSF (P15)</td>
<td>P15-C8</td>
<td>90%</td>
</tr>
<tr>
<td>Bid</td>
<td>5C3F\cite{243}</td>
<td>IIIRNIARLAQVGDSMDRS (P13)</td>
<td>IIIRNIAXLAQVGDSMDXS (P16)</td>
<td>P16-C8</td>
<td>60%</td>
</tr>
</tbody>
</table>

$^a$All the peptides present an amide at the C-terminus and are capped with an acetyl group at the N-terminus. X in the linear peptides refers to the azido-ornithine (12). *Conversion of stapling reactions as determined by LCMS of the crude reaction mixture. NA = conversions of stapling reaction not available due to complicated LCMS chromatogram.
Synthesis of the a 3,6-diethynyl-9H-carbazole staple (C8) required for peptide stapling was carried out according to literature reports.\textsuperscript{244} The synthetic route commenced from the bromination of the commercially available carbazole in the presence of NBS (Scheme 30). The reaction afforded the desired intermediate C8-1 which was then subjected to a Sonogashira coupling in the presence of TMS-acetylene, Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}, PPh\textsubscript{3}, and CuI to afford the TMS-protected analogue of C8. The latter compound underwent basic treatment in the presence of K\textsubscript{2}CO\textsubscript{3} and MeOH to afford the desired staple C8 in 81\% yield.

Scheme 30 - Synthetic route leading to the carbazole staple C8.

The synthesis of 12 was described in section 5.4.2.1.

Peptides were assembled using microwave-assisted SPPS performing double coupling for all amino acids with the exception of azido amino acid (12), which was coupled using longer, single coupling under MW.

The crude mixture of Bim-derived peptide P14 presented solubility issues prior to purification as observed for its analogue P9. High dilution and multiple filtrations resolved the issue with loss of crude material.

Assessment of the i,i+4 Bim-BH3 stapled peptides in platelets showed that the peptides required a functionalised staple (preferably a tri-arginine tag) to gain cytosolic entry. It was decided to functionalise the staple C8 using SPPS, so as to make it easily accessible. To achieve that, a chemical handle in the form of carboxylic acid was introduced. It was decided to employ 6-bromo hexanoic acid so that it would act as a functionalisable spacer. Reaction of C8 with KOH under ultrasonic waves at 35 °C for 2 hours afforded the title staple C9 bearing a carboxylic acid as a functional handle (Scheme 31).
In a similar method for functionalising the CK2 peptides described in Chapter 4, a tri-D-arginine tag was chosen as the tag to attach to C9 to improve cell permeability and stability to proteases of the overall construct. However, C9 appeared to be unstable under the acidic conditions used to cleave the peptide from the resin. After capping the tri-peptide with C9, the TFA cleavage cocktail was added to the resin either at room temperature or 42 °C: the resin instantly turned black, and LCMS could detect no desired product once the crude reaction mixture was concentrated and triturated with Et2O. To confirm whether the instability could be attributed to the staple itself, C9 was subjected to treatment with TFA in solution leading to the same problem: formation of a black product that was insoluble in any NMR solvents preventing its characterisation. An alternative, milder cleavage cocktail was used to cleave the peptide from the resin. However, the TFA-free cocktail (containing 0.1 M HCl in hexafluoroisopropanol) led to the same issue. Before attempting the time-consuming synthesis of the functionalised staple in solution, it was decided to investigate the secondary structure of the i,i+11 stapled peptides using CD.

**5.4.3.1.2. Helicity of i,i+11 stapled peptides**

CD spectra for all the i,i+11 stapled peptides and their corresponding linear WT peptides were recorded in a 50:50 mixture of H2O/MeCN at a concentration of 100 μM and the results are shown in Figure 70.
The staple significantly disrupted the helicity of the Bad peptide (7% compared to 54% of the WT) whilst it did not affect the helicity of the Bid-BH3 peptide (17% compared to 18% of the WT). The $i,i+11$ stapling negatively affected the structure of the Bim-BH3 peptide even more than the previously used $i,i+4$ stapling (20% helicity for the WT, 10% for the $i,i+4$ and 7% for the $i,i+11$).

Considering the difficulties associated with the staple functionalisation and the fact that the $i,i+11$ stapling did not enhance the helicity of the peptides, it was decided not to pursue this strategy further.

### 5.4.3.2. Looking at $i,i+7$ stapling

The use of the $i,i+7$ stapling is well-established, and the combination of azido-ornithine amino acids (12) and 3,5-dialkyne benzene derivatives has been used on several occasions to induce $\alpha$-helical structures to peptides with helical propensity.\(^{81,86,87}\) In addition, the 3,5-dialkyne benzoic acid staple (C5) has been straightforwardly functionalised on-resin in a variety of different ways.\(^{81,84,86,87}\) Taking into account the problems encountered with the $i,i+11$ stapling, it was decided to staple the BH3 peptides using the $i,i+7$ stapling technique to cross-link the side chains of azido-ornithine amino acids with the staple C5.

#### 5.4.3.2.1. Design and synthesis of $i,i+7$ BH3 stapled peptides

In a similar method described for the $i,i+11$ stapled peptides, the design of the new peptides was based on the available X-ray structures of the proteins of interest. The staple was placed in a way...
to avoid clashes with other residues on the peptide or the target proteins. A list of the \(i,i+7\) stapled peptides synthesised, their corresponding linear peptides, the reference PDB codes, and a schematic representation of the stapling reaction can be found in Table 17.

The synthesis of the peptides presented in the table was achieved using double coupling for all amino acids. Solubility problems were encountered with the crude P17-C5 peptide which, as previously, required high dilution and filtrations before purification. Percentage conversion of the stapling reactions are reported in Table 17.

*Table 17 – \(i,i+7\) stapled and linear Bim, Bad, Bid peptides subject of this work.*

All the peptides present an amide at the C-terminus and are capped with an acetyl group at the N-terminus. X in the azido-linear peptides refers to azido-ornithine as shown in the reaction scheme. R on the 3, 5 dialkyne benzene ring is a functional group as specified in the column “Staple”.+ Conversion of stapling reactions as determined by LCMS of the crude reaction mixture.

5.4.3.2.2. *Helicity of \(i,i+7\) stapled peptides*

As for the other peptides, the secondary structure of the \(i,i+7\) stapled BH3 peptides was investigated using CD spectrometry. Only the structure of the peptides without a functional tag was analysed as it was envisioned that the tag would interfere with the analysis. The results are shown in Figure 71.
The results showed that the *i,i*+7 stapling was effective at enhancing the helicity of the Bim-BH3 peptide (47% helicity for the stapled peptide and 20% for the WT) whilst did not affect the structures of the Bad (35% vs 28%) and Bid peptides (22% vs 17%) significantly.

With these peptides in hand, their effect on platelet activation and apoptosis will be investigated in a similar manner to that reported for the *i,i*+4 Bim-BH3 peptides (*vide supra*, 5.4.2.3, 5.4.2.4).
5.5. Conclusions and future work

This work is the first to describe the use of functionalised stapled peptides to perturb and investigate signalling pathways in human platelets. Using a panel of model stapled peptides, it was found that the nature of the motif incorporated onto the staple impacts the ability of the peptides to enter the platelet cytosol and modulate activity. Utilising the functionalised staples that allowed the model peptide to permeate the platelet membranes, stapled \( i,i+4 \) Bim peptides were synthesised to investigate PS exposure in platelets. The most promising peptide, **P9-F4C5**, showed a binding affinity of 26 nM for Bcl-xL, and its bioactivity in platelets was comparable to that of the small molecule Bad mimetic ABT-737. Importantly, unlike ABT-737, stapled peptide Bim-BH3 mimetics caused platelet activation, demonstrating a differential activation of signalling pathways.

In order to dissect the role of each BH3-only protein in platelet apoptosis and activation, \( i,i+7 \) Bad, Bim and Bad peptides have been synthesised, and their bioactivity in platelets will be studied.

Perturbation of the Bcl-2 pathway demonstrates the considerable potential of functionalised stapled peptides to investigate other PPIs in human platelets and for future development of new antiplatelet drugs. One of the advantages of this technology over small molecule use is the ease of design and synthesis of the peptides, as they are a mimetic of the natural components. Moreover, limitations such as the lack of membrane permeability and poor serum stability are overcome by the presence of the staple. The double click approach only requires one linear peptide to enable the generation of a variety of functionalised stapled peptides, which facilitates the exploration of various functionalities on the linker and thus the properties of the overall peptide. Most importantly, this methodology allows testing in human platelets directly, avoiding the inter-species difference, cost and time limitations of transgenic animal models.

Further work on the peptides developed in this project would involve co-immunoprecipitation assays to elucidate intraplatelet target engagement and rationalise the biological results obtained. In addition, Bcl-2 proteins are present in nucleated cells and therefore a way of targeting platelets selectively or under specific pathological conditions would be investigated to develop novel therapeutics. During this project, it was observed that the peptides require a cell-penetrating tag to gain platelet entrance. Consequently, a homing-peptide that recognises membrane receptors expressed on the membrane of platelets but not on the membrane of nucleated cells could be incorporated on the staple to gain selectivity for the desired cell type. An alternative approach could see the use of a thrombin-sensitive tag attached to a cell-impermeable sequence. When the thrombin levels increase, such as during cardiovascular
thrombotic event, the thrombin-sensitive tag is recognised by the thrombin protease, which will cleave the cell-impermeable tag off. This will reveal the stapled cell-permeable peptide, which would be able to exert its bioactivity under pathological conditions only.
SECTION III
Experimental
CHAPTER 6:
Chemistry Experimental
All experiments were carried out in oven-dried glassware under an atmosphere of N₂ using distilled solvents unless otherwise stated.

Reagents: Chemicals were purchased from commercial sources and used without further purification.

Yield: refer to chromatographically and spectroscopically pure compounds unless otherwise stated and are reported as follows: mass, moles, percentage.

Temperature: Reaction temperatures of 0 °C were maintained using an ice-water bath and those of -78 °C using dry-ice and acetone; room temperature (rt) refers to 20-25 °C.

Flash chromatography: Analytical thin layer chromatography was carried out on SiO₂ Merck Kieselgel 60 F254 plates with visualisation either by ultraviolet light or staining with potassium permanganate or ninhydrin dips made using standard procedures. Retention factors (Rf) are quoted to 0.01. Flash column chromatography was performed using silica gel 60 (230-400 mesh), or standardised aluminium oxide 90 (150 mesh), under a positive pressure of N₂. Eluent systems are expressed in % v/v. NH₃ used in flash chromatography is a 7 N solution in MeOH.

Nuclear Magnetic Resonance (NMR): ¹H, ¹³C and ¹⁹F NMR spectra were recorded using an internal deuterium lock at ambient probe temperatures on the following instruments: Bruker Avance III 400 MHz HD Smart Probe Spectrometer, Bruker Avance III 400 MHz HD Spectrometer, Bruker 400 MHz QNP Cryoprobe Spectrometer, Bruker 500 MHz DCH Cryoprobe Spectrometer, Bruker Avance III 500 MHz HD Smart Probe Spectrometer. The following deuterated solvents were used: chloroform (CDCl₃), dimethylsulfoxide (DMSO-d₆) and methanol (CD₃OD). ¹H-NMR chemical shifts (δ) are quoted in ppm to the nearest 0.01 ppm, relative to the residual non-deuterated solvent peak and coupling constants (J) are quoted to the nearest 0.1 Hertz (Hz). ¹³C-NMR chemical shifts are quoted to the nearest 0.1 ppm, relative to the solvent peak and coupling constants are quoted to the nearest 0.1 Hz. ¹⁹F-NMR chemical shifts are quoted to the nearest 0.1 ppm. Spectral data is reported as follows: chemical shift, integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; sept, septet; m, multiplet; br, broad; or as a combination of these e.g. br s, dd, dt), coupling constant(s) and assignment. The numbering system used in the assignments does not necessarily follow the IUPAC convention. Assignment of all spectra is supported by DEPT, COSY, HSQC and HMBC or done by analogy to fully assigned spectra of closely related compounds.

Infra-red spectroscopy (IR): Infra-red spectra were recorded neat on a Perkin Elmer Spectrum One FT-IR spectrometer fitted with an Attenuated Total Reflectance (ATR) sampling accessory. Selected absorption maxima (νmax) are quoted in wavenumbers (cm⁻¹) with the following abbreviations: w, weak; m, medium; s, strong; vs, br, broad.
**Liquid chromatography-mass spectrometry (LCMS):** LCMS was carried out using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; ESI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH₄OAc in H₂O/MeCN (95:5); solvent B: MeCN; solvent C: 2% formic acid; column: ACQUITY UPLC® CSH C18 (2.1 mm × 50 mm, 1.7 μm, 130 Å) at 40 °C; gradient: 5 – 95% B with constant 5% C over 1 min at flow rate of 0.6 mL/min; Injection volume: 5 μL. Chromatographs were monitored by absorbance using diode array detection at a wavelength range of 190-600 nm, interval 1.2 nm.

**High resolution mass spectrometry (HRMS):** HRMS was carried out using a Waters LCT Premier® Time of Flight (ToF) mass spectrometer or the ThermoFinnigan Orbitrap Classic mass spectrometer. Reported mass values are within the error limits of ± 5 ppm mass units. ESI refers to the electrospray ionisation technique. ASAP refers to the atmospheric solids analysis probe ionisation technique.

**Analytical HPLC:** Chromatographs were obtained on an Agilent 1260 Infinity® using a reversed-phase Supelcosil ABZ+PLUS column (150 mm x 4.6 mm, 3 μm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 15 min, unless otherwise stated, at a flow rate of 1 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

**Preparative HPLC:** Preparative HPLC was carried out on an Agilent 1260 Infinity® using a reversed-phase Supelcosil ABZ+PLUS column (250 mm x 21.2 mm, 5 μm) eluting with a linear gradient system (solvent A: 0.1% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 20 min at a flow rate of 20 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

**Automated Solid Phase Peptide Synthesis (SPPS):** automated SPPS was carried out on solid-phase using a Fmoc-protecting group strategy on a CEM Liberty Blue® automated microwave peptide synthesiser.

**Microwave irradiation:** Microwave irradiation was performed in a Biotage® microwave reactor.

**Melting points:** Melting points were measured using a Büchi melting point B545 apparatus and are uncorrected.
6.1. Experimental synthetic details

6.1.1. Small molecules

General method 1: Phenol triflation.\textsuperscript{204}

To a solution of phenol (1.0 equiv) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (reaction molarity 3.00 mM) was added anhydrous pyridine (1.6 equiv). The solution was cooled to 0 °C and trifluoromethanesulfonic anhydride (1.4 equiv) was added dropwise over 30 minutes. The reaction was allowed to warm to rt and stirred for 16 hours. CH\textsubscript{2}Cl\textsubscript{2} was removed under reduced pressure, the residue was diluted with H\textsubscript{2}O and extracted with EtOAc. The organic layer was washed successively with 10% aqueous HCl, 5% aqueous NaHCO\textsubscript{3}, brine, dried (MgSO\textsubscript{4}), and concentrated under reduced pressure. The crude residue was purified by column chromatography to yield the desired product.

General method 2a: Suzuki-Miyaura coupling.\textsuperscript{203}

To a solution of aryl triflate (1.6 equiv) and the appropriate boronic acid (1.0 equiv) in anhydrous DME (0.16 M) was added 2 M aqueous Na\textsubscript{2}CO\textsubscript{3} solution (1.6 equiv). The reaction mixture was degassed by bubbling N\textsubscript{2} for 15 minutes before the addition of Pd(PPh\textsubscript{3})\textsubscript{4} (2.0-2.5 mol%). The solution was refluxed for 3-7 hours. The reaction was allowed to cool to room temperature and then diluted with EtOAc and H\textsubscript{2}O. The aqueous phase was extracted with EtOAc (3 ×). The combined organic extracts were washed with brine, dried (MgSO\textsubscript{4}), concentrated under reduced pressure, and purified by column chromatography to yield the desired product.

General method 2b: Suzuki-Miyaura coupling.\textsuperscript{210}

To a solution of the appropriate boronic acid (1.5 equiv) and the appropriate aryl bromide (1.0 equiv) in 1,4-dioxane was added 1.75 M aqueous K\textsubscript{3}PO\textsubscript{4} solution (1.7 equiv). The mixture was degassed by bubbling N\textsubscript{2} for 10 minutes before PCy\textsubscript{3} (0.5 mol%) and Pd\textsubscript{2}(dba)\textsubscript{3} (0.25 mol%) were added. The mixture was refluxed for 4-8 hours. The crude mixture was diluted with EtOAc and filtered through Celite. The organic phase was washed with H\textsubscript{2}O and dried (MgSO\textsubscript{4}). The solvent was evaporated, and the product purified by column chromatography to yield the desired product.

General method 2c: Suzuki-Miyaura coupling.\textsuperscript{213}

A mixture of the aryl triflate (1.0 equiv), appropriate boronic acid (1.2 equiv), PdCl\textsubscript{2} (dppf)-CH\textsubscript{2}Cl\textsubscript{2} (1 mol%) and K\textsubscript{3}PO\textsubscript{4} (2.0 equiv) were solvated with DME (0.2 M), EtOH (1.7 M) and
H$_2$O (2.5 M). The reaction mixture was degassed by bubbling N$_2$ through the solution for 15 minutes and then heated in the MW to 110 °C (2-3 hours) or refluxed (1-6 hours). The reaction was allowed to cool to room temperature, filtered through Celite™, washed with Et$_2$O and the solvent removed under reduced pressure. The residue was dissolved in Et$_2$O and H$_2$O, the phases were separated, and the organic layer was extracted with Et$_2$O (3 ×). The combined organic extracts were washed with brine, dried (MgSO$_4$), filtered, and concentrated under reduced pressure. The crude product was then purified by flash column chromatography or preparative TLC to yield the desired product.

**General method 3: Nitrile reduction using LiAlH$_4$.**

To a suspension of LiAlH$_4$ (2.0 equiv) in Et$_2$O (0.27 M) at 0°C was added AlCl$_3$ (1.0 equiv) and the mixture stirred for 10 minutes. The appropriate benzonitrile (1.0 equiv) was added and the reaction mixture stirred at rt for 30 minutes and then refluxed for 16 hours. The mixture was cooled to 0 °C, diluted with EtOAc and a saturated aqueous solution of potassium sodium tartrate tetrahydrate was added. The suspension was stirred for 1 hour and then poured into a 2 M aqueous solution of Na$_2$CO$_3$. The aqueous phase was extracted with EtOAc (3 ×). The combined organic phases were washed with brine and dried (MgSO$_4$). The product was purified by column chromatography to yield the desired product.

**General method 4: Nitrile reduction using Raney Nickel and H$_2$.**

To a solution of the appropriate nitrile in NH$_3$ (8% in MeOH, 0.15 M) was added a spatula of Raney Nickel (slurry solution). An atmosphere of H$_2$ was applied and the reaction mixture was vigorously stirred at rt for 16 hours. After this time, EtOAc was added and the suspension filtered under gravity. The filtrate was concentrated under reduced pressure, and the crude material purified by column chromatography to yield the desired product.

**General method 5: Formation of hydrochloric amine salts.**

The appropriate amine (1.0 equiv) was dissolved in the minimum amount of anhydrous CH$_2$Cl$_2$ and HCl (2 M in 1,4-dioxane, 10 equiv) was added dropwise. The reaction mixture was stirred at rt for 1 hour. The resulting precipitate was filtered and washed with cold Et$_2$O to afford the desired salt.

**General method 6: Reductive amination.**

Anhydrous DCE (0.33 M) was added to a flask charged with the appropriate aldehyde (1.0 equiv), the amine (1.5 equiv) and molecular sieves (4 Å). The mixture was stirred at rt for 2.5 hours. After this time NaBH(OAc)$_3$ (1.4 equiv) was added in three portions over 15 minutes and the mixture stirred at rt for 16 hours. The reaction was quenched with a NaHCO$_3$ aqueous
solution and the product extracted with EtOAc (3 ×). The combined organic phases were dried (MgSO₄), and the solvent evaporated under reduced pressure. The residue was purified by column chromatography to afford the free amine.

**General method 7: Fmoc protection of primary amine.**

Solid NaHCO₃ (4.0 equiv) was dissolved in H₂O (0.2 M) and the solution was diluted with MeCN (0.2 M). To the solution were added the amine (1.0 equiv) and N-(9-fluorenylmethoxycarbonyloxy)succinimide (1.5 equiv). The mixture was stirred at rt for 24-48 hours. The organic solvent was removed under reduced pressure, and the aqueous phase was washed with CH₂Cl₂ (3 ×). The aqueous phase was adjusted to pH 2 with concentrated HCl to form a white precipitate. The precipitate was filtered, and the aqueous phase extracted with EtOAc (3 ×). The organic phases were combined with the precipitate, dried (MgSO₄), and the solvent evaporated under reduced pressure. The crude material was purified by column chromatography to yield the desired product.

**General method 8: TMS deprotection.**

Aqueous 6 M KOH (10 equiv) was added to a stirred solution of the TMS-protected alkyne (1 equiv) in MeOH (0.45 M). The mixture was stirred at rt for 18 hours. MeOH was removed under a stream of N₂ and the aqueous phase was acidified to pH 4 with HCl (6 N) and extracted with EtOAc (3 ×). The combined organic phases were dried (MgSO₄), and the solvent evaporated under reduced pressure to yield the desired compound.

**General method 9: Addition of TMS-alkyne to esters and anhydride.**

Trimethylsilylacetylene (1.5-3 equiv) in anhydrous THF (1.2 M) was cooled down to -78 °C. n-BuLi 1.6 M in hexane (3 equiv) was added dropwise and the reaction mixture was stirred at -78 °C for 1 hour. After this time, the reaction was warmed to 0 °C for 10 minutes and then cooled down to -78°C. The appropriate benzoate or aldehyde (1 equiv) in anhydrous THF (1.1 M) was added over 10 minutes and the mixture stirred at rt for 2 hours. After this time, the reaction was quenched with NH₄Cl (saturated aqueous) solution, THF removed under a stream of N₂ and the product extracted with Et₂O (3 ×). The combined organic phases were dried (MgSO₄), and the residue purified by flash chromatography to give the desired product.
6.1.1.1. Fragment-based drug design

2-Chloro-4-cyanophenyl trifluoromethanesulfonate (1a)

Prepared following general method 1 using 3-chloro-4-hydroxybenzonitrile (2.00 g, 13.0 mmol), CH₂Cl₂ (40.0 mL), pyridine (3.20 mL, 39.6 mmol) and trifluoromethanesulfonic anhydride (2.84 mL, 16.9 mmol). After work-up, the crude residue was purified by column chromatography on silica gel (5% EtOAc/hexane) to yield 1a as a white crystalline solid (3.37 g, 11.8 mmol, 91%).

R_f = 0.68 (20% EtOAc/hexane); Mp = 61-62 °C; δ_H (400 MHz, CDCl₃): 7.88 (1H, d, J = 2.0 Hz, H5), 7.70 (1H, dd, J = 8.6, 2.0 Hz, H1), 7.53 (1H, d, J = 8.6 Hz, H2); δ_C (101 MHz, CDCl₃): 148.5 (C3), 134.9 (C5), 132.2 (C1), 128.9 (C4), 124.2 (C2), 118.5 (q, J = 227.3 Hz, C8), 116.0 (C7), 114.9 (C6); δ_F (376 MHz, CDCl₃): -74.1 ν_max: 2245 (C≡N, m), 1575 (C=C, w), 1478 (C=C, m); LCMS: Rt = 1.56 min, [M-H] - 284.0.

Characterisation data in accordance with literature.¹⁹⁸

2,6-Dichloro-4-cyanophenyl trifluoromethanesulfonate (1b)

Prepared following general method 1 using 3,5-dichloro-4-hydroxybenzonitrile (500 mg, 2.66 mmol), CH₂Cl₂ (2.40 mL), pyridine (300 μL, 4.26 mmol) and trifluoromethanesulfonic anhydride (600 μL, 3.46 mmol). After work-up, the crude residue was purified by column chromatography on silica gel (10% EtOAc/hexane) to yield 1b as white solid (630 mg, 1.97 mmol, 74%).

R_f = 0.40 (10% EtOAc/hexane); Mp = 93-96 °C; δ_H (400 MHz, DMSO-d₆): 8.50 (2H, s, H1); δ_C (101 MHz, DMSO-d₆): 144.7 (C4), 134.4 (C1), 129.2 (C3), 117.3 (q, J = 225.3 Hz, C5), 115.6 (C6), 113.9
2,6-Dichloro-4-formylphenyl trifluoromethansulfonate (1c)

Prepared following general method 1 using 3,5-dichloro-4-hydroxybenzaldehyde (200 mg, 1.05 mmol), trifluoromethanesulfonic anhydride (260 μL, 1.52 mmol), pyridine (150 μL, 1.88 mmol) and CH₂Cl₂ (1.50 mL). After work-up, the crude material was purified by column chromatography on silica gel (10% EtOAc/hexane) to yield 1c as a colourless oil (191 mg, 600 μmol, 57%).

R_f = 0.19 (10% EtOAc/hexane); δ_H (500 MHz, CDCl₃): 9.95 (1H, s, H6), 7.95 (2H, s, H1); δ_C (126 MHz, CDCl₃): 187.8 (C6), 146.2 (C4), 136.1 (C2), 130.8 (C3), 130.2 (C1), 117.0 (q, J = 320 Hz, C5); δ_F (376 MHz, CDCl₃): -71.3; ν_max: 1706 (C=O, s), 1429 (H-C=O, s), 1210 (C-F), 1129 (SO₂, s); HRMS (ESI+): m/z found [M+H]+ 322.9153, C₈H₄O₄35Cl₂F₃S required 322.9159 (Δ-1.9 ppm).

2-Chloro-2'-isopropyl-[1,1'-biphenyl]-4-carbonitrile (2a)

Prepared following general method 2a using (2-isopropylphenyl)-boronic acid (50.0 mg, 230 μmol), 2 M aqueous Na₂CO₃ solution (300 μL, 530 μmol), DME (5.00 mL) and Pd(PPh₃)₄ (10.5 mg, 10.0 μmol). The reaction was refluxed for 3 hours. After work-up, the crude material was purified by column chromatography on silica gel (20% EtOAc/hexane) to yield 2a as a colourless oil (40.0 mg, 150 μmol, 85%).

R_f = 0.25 (20% EtOAc/hexane); δ_H (400 MHz, CD₃OD): 7.94 (1H, dd, J = 1.6, 0.3 Hz, H5), 7.74 (1H, dd, J = 7.9, 1.6 Hz, H1), 7.45-7.40 (3H, m, H2, H11, H12), 7.27-7.25 (1H, m, H13), 7.03 (1H, ddd, J = 7.2, 1.8, 0.7 Hz, H10), 2.58 (1H, sept, J = 6.7 Hz, H14), 1.20 (3H, d, J = 6.7 Hz, H15), 1.07 (3H, d, J
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= 6.7 Hz, H15); δc (101 MHz, CD3OD): 147.5 (C3 or C8), 147.3 (C3 or C8), 138.0 (C9), 135.9 (C4), 133.9 (C5), 133.7 (C2), 131.6 (C1), 130.2 (C11 or 12), 130.0 (C10), 126.8 (C13), 126.6 (C11 or 12), 118.4 (C7), 113.9 (C6), 31.7 (C14), 24.8 (C15), 23.4 (C15); vmax: 2962 (=C-H, m), 2326 (C≡N, m), 1473 (CH3, m), 1383 (CH3, m); HRMS (ESI+): m/z found [M]+: 256.0893, C16H14N35Cl required 256.0893 (Δ 0.0 ppm).

2-Chloro-2'-ethyl-[1,1'-biphenyl]-4-carbonitrile (2b)

Prepared following general method 2a using 3-chloro-4-bromobenzonitrile (300 mg, 1.39 mmol), 2-ethylphenylboronic acid (208 mg, 1.39 mmol), Pd(PPh3)4 (80 mg, 0.07 mmol), K2CO3 (383 mg, 2.77 mmol), DME (3.0 mL) and H2O (1.0 mL). The reaction was refluxed for 4 hours. After work-up, the crude product was purified by flash column chromatography (10% EtOAc/PE 40-60) to yield 2b as a clear oil (206 mg, 0.85 mmol, 61%).

Rf = 0.40 (10% EtOAc/PE 40-60); δh (400 MHz, CDCl3): 7.79 (1H, d, J = 1.5 Hz, H5), 7.62 (1H, dd, J = 7.9, 1.5 Hz, H1), 7.45-7.36 (3H, m, H2, H10, H11), 7.30 (1H, app. td, J = 7.5, 1.7 Hz, H12), 7.10 (1H, dd, J = 7.5, 0.9 Hz, H9), 2.56-2.33 (2H, m, H13), 1.10 (3H, t, J = 7.6 Hz, H14); δc (101 MHz, CDCl3): 145.8 (C8), 141.7 (C3), 137.1 (C4), 134.8 (C7), 132.8 (C5), 132.2 (C1), 130.2 (C2), 129.1 (C9), 129.0 (C10), 128.6 (C11), 125.9 (C12), 117.6 (C15), 112.7 (C6), 26.2 (C13), 15.1 (C14); vmax: 2967 (=C-H, m), 2232 (C≡N, m), 1472 (CH3, m), 1445 (CH3, m); HRMS (ESI+): m/z found [M+H]+ 242.0735, C15H13N35Cl required 242.0737 (Δ -0.8 ppm).

3-Chloro-4-(1H-indole-4-yl)benzonitrile (2c)

Prepared following general method 2a using 4-(1H-indole)-boronic acid pinacol ester (150 mg,
620 μmol), \textbf{1a} (125 mg, 440 μmol), 2 M aqueous Na₂CO₃ solution (700 μL, 1.32 mmol), DME (15.0 mL) and Pd(PPh₃)₄ (25.4 mg, 20.0 μmol). The mixture was refluxed for 3 hours. After work-up, the crude material was purified by column chromatography on silica gel (20% EtOAc/hexane) to yield \textbf{2c} as a dark orange oil (104 mg, 410 μmol, 67%).

Rᵣ = 0.44 (20% EtOAc/hexane); δₜ (400 MHz, CD₃OD): 7.94 (1H, d, J = 1.6 Hz, H13), 7.73 (1H, dd, J = 8.0, 1.6 Hz, H11), 7.61 (1H, d, J = 8.0 Hz, H10), 7.46 (1H, dd, J = 7.3, 0.8 Hz, H8), 7.27 (1H, d, J = 3.2 Hz, H3), 7.20 (1H, t, J = 7.3 Hz, H7), 6.99 (1H, dd, J = 7.3, 0.8 Hz, H6), 6.15 (1H, dd, J = 3.2, 0.8 Hz, H4); δₗ (101 MHz, CD₃OD): 146.8 (C2), 137.9 (C5), 135.4 (C14), 134.3 (C13), 134.0 (C10), 131.4 (C11), 130.9 (C3), 128.0 (C1), 126.5 (C12), 121.9 (C7), 121.0 (C6), 118.8 (C15), 113.4 (C8), 112.8 (C9), 101.5 (C4); νₘₐₓ: 3402 (N-H, br), 2231 (C≡N, m); HRMS (ESI+): m/z found [M+H]+ 253.0545, C₁₅H₉N₂Cl required 253.0533 (Δ 4.7 ppm).

2-Chloro-4'-fluoro-2'-methyl-[1,1'-biphenyl]-4-carbonitrile (2d)

Prepared following general method 2a using compound \textbf{1a} (66.0 mg, 230 μmol), 4-fluoro-2-methylphenyl boronic acid (50.0 mg, 320 μmol), Pd(PPh₃)₄ (13.0 mg, 10.0 μmol), 2 M aqueous Na₂CO₃ solution (300 μL) and DME (5.00 mL). The reaction mixture was refluxed for 4 hours. After work-up, the crude product was purified by flash column chromatography on silica gel (0 to 5% EtOAc/hexane) to yield \textbf{2d} as a colourless oil (55.0 mg, 220 μmol, 97%).

Rᵣ = 0.37 (20% EtOAc/hexane); δₜ (500 MHz, CDCl₃): 7.80 (1H, d, J = 1.4 Hz, H5), 7.64 (1H, dd, J = 7.8, 1.4 Hz, H1), 7.36 (1H, d, J = 7.8 Hz, H2), 7.11-6.97 (3H, m, H10, H12, H13), 2.12 (3H, s, H14); δₗ (126 MHz, CDCl₃): 163.8 (d, J = 242.5 Hz, C11), 144.9 (C3), 138.4 (d, J = 8.1 Hz, C9), 134.8 (C4), 133.5 (d, J = 3.3 Hz, C8), 132.9 (C5), 132.1 (C2), 130.5 (d, J = 8.6 Hz, C13), 130.3 (C1), 117.4 (C7), 116.9 (d, J = 21.3 Hz, C10), 112.9 (C6), 112.8 (d, J = 21.4 Hz, C12), 19.8 (d, J = 1.8 Hz, C14); δᵣ (375 MHz, CDCl₃): 114.3; νₘₐₓ: 2233 (C≡N, w), 1383 (CH₃, s); HRMS (ESI+): m/z found [M+H]+ 246.0486, C₁₄H₁₀N₃ClF required 246.0493 (Δ 0.8 ppm).
2-Chloro-[2'-(hydroxymethyl)-1,1'-biphenyl]-4-carbonitrile (2e)

Prepared following *general method 2a* using 2-hydroxymethyl-benzenboronic acid (432 mg, 2.84 mmol), compound 1a (600 mg, 2.20 mmol), Pd(PPh$_3$)$_4$ (128 mg, 110 μmol), 2 M aqueous Na$_2$CO$_3$ solution (2.80 mL) and DME (73.0 mL). The reaction was refluxed for 7 hours. After work-up, the crude material was purified by column chromatography on silica gel (20 to 30% EtOAc/hexane) to yield 2e as a colourless oil (490 mg, 1.99 mmol, 90%).

R$_f$ = 0.25 (20% EtOAc/hexane); δ$_H$ (400 MHz, CDCl$_3$): 7.78 (1H, d, $J = 1.6$ Hz, H9), 7.63-7.60 (2H, m, H3 or H4, H11), 7.51 (1H, app. td, $J = 7.8$, 1.6 Hz, H3 or H4), 7.42 (1H, d, $J = 7.8$ Hz, H12), 7.39 (1H, app. td, $J = 7.8$, 1.0 Hz, H2 or H5), 7.14 (1H, dd, $J = 7.8$, 1.0 Hz, H2 or H5), 4.51 (1H, d, $J = 12.9$ Hz, H13), 4.38 (1H, d, $J = 12.9$ Hz, H13); δ$_C$ (101 MHz, CDCl$_3$): 144.6 (C7), 138.3 (C1), 136.5 (C8), 134.5 (C6), 132.8 (C9), 132.1 (C12), 130.2 (C11), 129.3 (C3 or 4), 129.2 (C2 or 5), 128.2 (C3 or 4), 127.8 (C2 or 5), 117.4 (C14), 113.1 (C10), 62.9 (C13); $\nu$$_{max}$: 2988 (OH, br), 2226 (C≡N, w); HRMS (ESI+): m/z found [M+H]$^+$ 244.0537, C$_{14}$H$_{11}$NO$_3$Cl required 244.0529 (Δ -3.3 ppm).

tert-Butyl ((2'-chboro-4'-cyano-[1,1'-biphenyl]2-yl)methyl)carbamate (2f)

Prepared following *general method 2a* using 1a (131 mg, 460 μmol), 2-(((tert-butoxycarbonyl)amino)methyl)phenyl]boronic acid (150 mg, 590 μmol), 2 M aqueous Na$_2$CO$_3$ solution (700 μL, 1.38 mmol), DME (15.0 mL) and Pd(PPh$_3$)$_4$ (26.5 mg, 20.0 μmol). The reaction was refluxed for 3 hours. After work-up, the crude material was purified by column chromatography on silica gel (30% Et$_2$O/hexane) to yield 2f as a colourless oil (140 mg, 410 μmol, 89%).

R$_f$ = 0.22 (30% Et$_2$O/hexane); δ$_H$ (400 MHz, CDCl$_3$): 7.77 (1H, d, $J = 1.2$ Hz, H9), 7.61 (1H, dd, $J =
7.9, 1.2 Hz, H11), 7.44-7.43 (2H, m, H2, H3), 7.39-7.36 (2H, m, H4, H12), 7.11 (1H, d, J = 7.3 Hz, H5), 4.60 (1H, br s, NH), 4.10-4.08 (2H, m, H14), 1.39 (9H, s, H17); δC (101 MHz, CDCl3): 155.6 (C15), 144.6 (C7), 136.9 (C6 or C8), 136.5 (C6 or C8), 134.5 (C10), 132.9 (C9), 131.9 (C12), 130.3 (C11), 129.3 (C2), 129.2 (C3), 128.2 (C4), 127.5 (C5), 117.3 (C13), 113.0 (C1), 80.0 (C16), 42.3 (C14), 28.4 (C17); νmax: 3369 (N-H, br), 2978 (C-H, m), 2233 (C≡N, m), 1696 (C=O, s), 1247 (C-O, s); HRMS (ESI+): m/z found [M+H]+ 265.0493, C14H1135ClN2Na required 265.0503 (Δ -3.6 ppm).

2'-(Aminomethyl)-2-chloro-[1,1'-biphenyl]-4-carbonitrile (2f')

Prepared following a literature procedure. Compound 2f (60.0 mg, 180 μmol) was dissolved in CH2Cl2 (600 μL) and treated with HCl (2 M in Et2O, 1.80 mL, 3.70 mmol). The reaction was refluxed for 16 hours. The solvent was removed under reduced pressure, the residue diluted with cold Et2O and the white solid collected by filtration. The salt was suspended in EtOAc and the free base obtained by addition of 2 M aqueous Na2CO3 solution (2 mL). The solvent was evaporated and the residue purified by column chromatography on silica gel (1:9:90 NH3:MeOH:CH2Cl2) to afford the product as an orange oil (45.0 mg, 170 μmol, 98%).

Rf = 0.12 (1:9:90 NH3:MeOH:CH2Cl2); δH (400 MHz, CDCl3): 7.80 (1H, d, J = 1.5 Hz, H9), 7.64 (1H, dd, J = 7.9, 1.5 Hz, H11), 7.56 (1H, d, J = 7.5 Hz, H5), 7.47 (1H, td, J = 7.5, 1.0 Hz, H4), 7.40 (1H, d, J = 7.9 Hz, H12), 7.36 (1H, td, J = 7.5, 1.0 Hz, H3), 7.12 (1H, d, J = 7.5 Hz, H2), 3.69 (1H, d, J = 14.6 Hz, H14), 3.58 (1H, d, J = 14.6 Hz, H14); δC (101 MHz, CDCl3): 145.1 (C7), 141.2 (C10), 136.5 (C8), 134.6 (C6), 132.9 (C9), 132.1 (C12), 130.2 (C11), 129.2 (C4), 129.2 (C5), 127.8 (C2), 126.9 (C3), 117.4 (C13), 112.9 (C1), 43.9 (C14); νmax: 3373 (N-H, w), 2232 (C≡N, m); HRMS (ESI+): m/z found [M+Na]+ 265.0493, C14H1135ClN2Na required 265.0503 (Δ -3.6 ppm).

2'-(1H-Pyrrol-1-yl)methyl)-2-chloro-[1,1'-biphenyl]-4-carbonitrile (2f'')
Prepared following a literature procedure.\textsuperscript{209} NaO\textsubscript{t}Bu (47.0 mg, 490 \textmu mol) was added slowly to a solution of pyrrole (28.0 \textmu L, 410 \textmu mol) in MeCN (5.00 mL). The reaction mixture was stirred for 5 minutes before compound 2\textsuperscript{f}''' (150 mg, 490 \textmu mol) was added dropwise. The reaction was stirred at rt 16 hours. The solvent was then removed under reduced pressure, the reaction mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} and washed with water (2 x 10 mL). The combined organic phases were washed with brine and dried (MgSO\textsubscript{4}). The crude material was purified by column chromatography on silica gel (0 to 2\% EtOAc/hexane) to yield 2\textsuperscript{f}'' as a white film (132 mg, 453 \textmu mol, 93\%).

\[ R_F = 0.39 \ (10\% \text{ EtOAc/hexane}) \]
\[ \delta_H (500 \text{ MHz, CDCl}_3): 7.78 \ (1H, d, J = 1.5 \text{ Hz, H1}), 7.55 \ (1H, dd, J = 7.9, 1.5 \text{ Hz, H5}), 7.47-7.38 \ (2H, m, H8, H10), 7.18 \ (1H, d, J = 7.9 \text{ Hz, H4}), 7.18-7.12 \ (2H, m, H9, H11), 6.38 \ (2H, app. t, J = 2.1 \text{ Hz, H14}), 6.07 \ (2H, app. t, J = 2.1 \text{ Hz, H15}), 4.88 \ (1H, d, J = 15.0 \text{ Hz, H13}), 4.76 \ (1H, d, J = 15.0 \text{ Hz, H13}) \]

\[ \delta_C (126 \text{ MHz, CDCl}_3): 144.1 \ (C7), 136.8 \ (C12), 135.8 \ (C3), 134.3 \ (C2), 132.7 \ (C1), 131.9 \ (C4), 130.4 \ (C5), 129.4 \ (C8), 129.3 \ (C9), 128.4 \ (C10), 128.0 \ (C11), 120.9 \ (C14), 117.3 \ (C16), 113.1 \ (C6), 108.5 \ (C15), 51.2 \ (C13) \]
\[ \nu_{\text{max}}: 2359 (\text{C}=\text{N}, \text{ w}) \]

HRMS (ESI\textsuperscript{+}): m/z found [M+H\textsuperscript{+}] 293.0835, C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}Cl required 293.0846 (Δ -3.8 ppm).

Prepared following a literature procedure.\textsuperscript{208} A solution of compound 2\textsuperscript{e} (100 mg, 410 \textmu mol) in anhydrous THF (2.50 mL) was cooled down to 0 °C. N-bromosuccinimide (153 mg, 860 \textmu mol) was added followed by PPh\textsubscript{3} (215 mg, 820 \textmu mol). The mixture was allowed to stir at room temperature for 5 hours. The reaction was quenched with saturated aqueous NaHCO\textsubscript{3} and the solvent removed under a stream of N\textsubscript{2}. The residue was diluted with Et\textsubscript{2}O and washed with water (2 x 10 mL). The combined organic phase was dried (MgSO\textsubscript{4}), filtered and the solvent removed under reduced pressure. The crude material was purified by column chromatography on silica gel (20\% EtOAc/hexane) to yield 2\textsuperscript{f}''' as a white solid (110 mg, 360 \textmu mol, 88\%).

\[ R_F = 0.30 \ (20\% \text{ EtOAc/hexane}) \]
\[ Mp = 73-76 °C; \delta_H (400 \text{ MHz, CDCl}_3): 7.80 \ (1H, d, J = 1.5 \text{ Hz, H11}), 7.66 \ (1H, dd, J = 7.9, 1.5 \text{ Hz, H9}), 7.53 \ (1H, dd, J = 7.6, 1.4 \text{ Hz, H1}), 7.53 \ (1H, dd, J = 7.9 \text{ Hz, H8}), 7.46 \ (1H, td, J = 7.5, 1.4 \text{ Hz, H3}), 7.41 \ (1H, app. td, J = 7.5, 1.4 \text{ Hz, H4}), 7.15-7.13 \ (1H, m, H2), 4.39 \ (1H, d, J = 10.4 \text{ Hz, H13}), 4.13 \ (1H, d, J = 10.4 \text{ Hz, H13}) \]

\[ \delta_C (101 \text{ MHz, CDCl}_3): 143.7 \ (C7), 137.5 \ (C5), 135.4 \ (C12), 134.5 \ (C6), 132.9 \ (C11), 132.2 \ (C9), 130.7 \ (C3), 130.2 \ (C8), 129.8 \ (C1), \]
129.5 (C4), 128.8 (C2), 117.3 (C14), 113.4 (C10), 30.9 (C13); ν\text{max}: 2232 (C≡N, m), 1447 (C=C, m), 1384 (C-N, m);

**HRMS** (ESI+): m/z found [M+H]+ 305.9683, C_{14}H_{10}N_{35}Cl_{79}Br required 305.9685 (Δ -0.7 ppm).

3-Chloro-5-((1H-indol-4-yl)benzonitrile (2g)

![Chemical structure of 3-Chloro-5-((1H-indol-4-yl)benzonitrile](image)

Prepared following *general method 2b* using 1-H-indole boronic acid (250 mg, 1.55 mmol), 3-bromo-5-chloro-benzonitrile (167 mg, 1.03 mmol), Pd$_2$(dba)$_3$ (9.50 mg, 10.0 μmol), PCy$_3$ (7.00 mg, 20.0 μmol), 1.5 M aqueous K$_3$PO$_4$ solution (1.00 mL) and dioxane (3.50 mL). The reaction was refluxed for 6 hours. After work-up, the crude material was purified by column chromatography on aluminium oxide gel (0 to 20% EtOAc/hexane) to yield 2g as white solid (155 mg, 570 μmol, 55%).

R$_f$ = 0.24 (20% EtOAc/hexane); Mp = 134-137 °C;  δ$_H$ (500 MHz, CDCl$_3$): 8.41 (1H, br s, NH), 7.92 (1H, t, J = 1.6 Hz, H3), 7.88 (1H, t, J = 1.6 Hz, H5), 7.63 (1H, t, J = 1.6 Hz, H1), 7.48 (1H, d, J = 8.2 Hz, H15), 7.33-7.29 (2H, m, H11, H14), 7.15 (1H, d, J = 8.2 Hz, H13), 6.65-6.64 (1H, m, H10);  δ$_C$ (126 MHz, CDCl$_3$): 144.4 (C2), 136.3 (C12), 135.3 (C4), 133.2 (C3), 130.5 (C9), 130.4 (C5), 129.9 (C1), 125.7 (C8), 125.5 (C14), 122.4 (C11), 120.0 (C15), 117.8 (C7), 114.0 (C6), 111.9 (C13), 101.2 (C10);  ν\text{max}: 3429 (N-H, m), 2236 (C≡N, w), 1566 (C=C, m); **HRMS** (ESI+): m/z found [M+H]+ 253.0524, C$_{15}$H$_{10}$N$_3$Cl required 253.0527 (Δ -1.0 ppm).

3-((1H-indol-4-yl)-5-(trifluoromethyl)benzonitrile (2h)

![Chemical structure of 3-((1H-indol-4-yl)-5-(trifluoromethyl)benzonitrile](image)

Prepared following *general method 2b* using 3-bromo-5-trifluoride benzonitrile (257 mg, 1.03 mmol), 1H-indole-4-boronic acid (250 mg, 1.55 mmol), Pd$_2$(dba)$_3$ (9.50 mg, 10.0 μmol), PCy$_3$ (7.00 mg, 20.0 μmol), 1.75 M aqueous K$_3$PO$_4$ solution (1.00 mL) and 1,4-dioxane (3.50 mL). The mixture was refluxed for 5 hours. After work-up, the residue was purified by column chromatography on aluminium oxide gel (0 to 20% EtOAc/hexane) to yield 2h as white solid (153 mg, 484 μmol, 58%).

R$_f$ = 0.19 (20% EtOAc/hexane); Mp = 152-155 °C;  δ$_H$ (500 MHz, CDCl$_3$): 8.54 (1H, br s, NH), 7.91 (1H, t, J = 1.8 Hz, H3), 7.89 (1H, t, J = 1.8 Hz, H5), 7.65 (1H, t, J = 1.8 Hz, H1), 7.47 (1H, d, J = 8.2 Hz, H15), 7.31-7.25 (2H, m, H11, H14), 7.14 (1H, d, J = 8.2 Hz, H13), 6.67-6.66 (1H, m, H10);  δ$_C$ (126 MHz, CDCl$_3$): 161.1 (C2), 144.4 (C12), 135.2 (C4), 133.3 (C3), 130.5 (C9), 130.4 (C5), 129.9 (C1), 125.7 (C8), 125.5 (C14), 122.4 (C11), 120.0 (C15), 117.8 (C7), 114.0 (C6), 111.8 (C13), 101.4 (C10);  ν\text{max}: 3419 (N-H, m), 2236 (C≡N, w), 1566 (C=C, m); **HRMS** (ESI+): m/z found [M+H]+ 252.0433, C$_{15}$H$_{10}$N$_3$Cl required 252.0411 (Δ -0.4 ppm).
chromatography on silica gel (0 to 10% EtOAc/hexane) to yield 2h as a sticky, clear oil (110 mg, 360 μmol, 35%).

\( R_f = 0.24 \) (10% EtOAc/hexane); \( \delta_H \) (500 MHz, DMSO-\( d_6 \)): 11.42 (1H, br s, NH), 8.39 (1H, s, H3), 8.32 (1H, app. s, H5), 8.24 (1H, app. s, H1), 7.53-7.50 (1H, m, H12), 7.50-7.49 (1H, m, H10), 7.24-7.21 (2H, m, H11, H16), 6.75-6.54 (1H, m, H15); \( \delta_C \) (126 MHz, DMSO-\( d_6 \)): 143.8 (C2), 136.9 (C13), 136.0 (C1), 131.1 (q, J = 32.9 Hz, C4), 129.5 (C14), 129.4 (q, J = 3.6 Hz, C5), 127.9 (q, J = 3.6 Hz, C3), 127.4 (C10), 125.7 (C8), 124.0 (q, J = 273.0 Hz, C9), 121.9 (C11), 120.0 (C16), 118.1 (C7), 113.8 (C6), 113.0 (C12), 99.7 (C15); \( \delta_F \) (376 MHz, DMSO-\( d_6 \)): -63.0; \( \nu_{\text{max}} \): 3378 (N-H, m), 2235 (C≡N, w), 1105 (C-F, s); HRMS (ESI+): \( m/z \) found [M+H]+ 287.0789, \( C_{16}H_{10}N_2F_3 \) required 287.0796 (Δ -2.4 ppm).

2,6-Dichloro-[1,1'-biphenyl]-4-carbonitrile (2i)

\[
\begin{array}{c}
\text{N} \\
\text{Cl} \\
\text{Cl} \\
\text{N} \\
\text{Cl} \\
\end{array}
\]

Prepared following general method 2c using 1b (300 mg, 940 μmol), phenylboronic acid (160 mg, 1.31 mmol), K3PO4 (239 mg, 1.13 mmol), DME (800 μL), EtOH (600 μL), H2O (150 μL), PdCl2(dppf)-CH2Cl2 (48.0 mg, 60.0 μmol). The mixture was heated to 110 °C in the MW for 2 hours. After work-up, the crude material was purified by flash chromatography on silica gel (0 to 2% EtOAc/hexane) followed by preparative TLC (100% Hexane) to yield 2i as a white film (55.0 mg, 220 μmol, 24%).

\( R_f = 0.37 \) (100% hexane); \( \text{Mp} = 100-103 ^\circ C \); \( \delta_H \) (500 MHz, CDCl3): 7.71 (2H, s, H1), 7.52-7.47 (3H, m, H6, H8), 7.24-7.22 (2H, m, H7); \( \delta_C \) (126 MHz, CDCl3): 144.6 (C3), 136.2 (C4 or C5), 135.3 (C4 or C5), 131.2 (C1), 128.9 (C7), 128.9 (C6), 128.5 (C8), 116.3 (C9), 113.3 (C2); \( \nu_{\text{max}} \): 2236 (C≡N, m), 1532, 1444 (C=C, m), 1207 (C-H, s); HRMS (ESI+): \( m/z \) found [M+H]+ 248.0022, \( C_{13}H_{8}N_2Cl_2 \) required 248.0028 (Δ -2.4 ppm).
2,6-Dichloro-1[1,1′-biphenyl]-4-carbaldehyde (2k)

Prepared following general method 2c using compound 1c (162 mg, 50.0 μmol), phenylboronic acid (73.3 mg, 600 μmol), K3PO4 (213 mg, 1.00 mmol), DME (1.00 mL), EtOH (700 μL) and H2O (150 μL), PdCl2(dppf)-CH2Cl2 (20.5 mg, 30.0 μmol). The mixture was refluxed for 4 hours. After the workup, the crude material was purified by column chromatography on silica gel (0 to 1% EtOAc/hexane) to yield 2k as a white solid (68.0 mg, 270 μmol, 54%).

Rf = 0.30 (10% EtOAc/hexane); Mp = 95-97 °C; δH (500 MHz, CDCl3): 9.97 (1H, s, H8), 7.91 (2H, s, H1), 7.52-7.44 (3H, m, H10, H5 or H6), 7.28-7.25 (2H, m, H5 or H6); δC (126 MHz, CDCl3): 189.3 (C8), 145.2 (C3), 136.8 (C7), 136.3 (C2), 136.0 (C4), 129.0 (C1), 128.8 (C5 or C6 or C10), 128.7 (C5 or C6 or C10), 128.4 (C5 or C6 or C10); νmax: 1707 (C=O, s); HRMS (ESI+): m/z found [M+H]+ 251.0029, C13H9O3Cl2 required 251.0030 (Δ -0.4 ppm).

(2-Chloro-2'-isopropyl-[1,1'-biphenyl]-4-yl)methanaminium chloride (3a)

Prepared following general method 3 using compound 2a (40.0 mg, 150 μmol), LiAlH4 (11.4 mg, 300 μmol), AlCl3 (20.0 mg, 150 μmol) and Et2O (3.00 mL). The amine product (35.0 mg, 130 μmol, 87%) was subjected to general method 5 using 2 M HCl solution in 1,4-dioxane (0.700 mL) to yield 3a as a white solid (35.0 mg, 120 μmol, 77%).

Mp = 218-220°C; δH (500 MHz, CD3OD): 7.68 (1H, d, J = 1.5 Hz, H5), 7.50 (1H, dd, J = 7.8, 1.5 Hz, H1), 7.42-7.36 (2H, m, H10, H11), 7.35 (1H, d, J = 7.8 Hz, H2), 7.25 (1H, app. td, J = 6.9, 1.7 Hz, H12), 7.03 (1H, dd, J = 6.9, 1.7 Hz, H9), 4.21 (2H, s, H15), 2.65 (1H, sept, J = 7.4 Hz, H13), 1.20 (3H, d, J = 7.4 Hz, H14), 1.06 (3H, d, J = 7.4 Hz, H14); δC (126 MHz, CD3OD): 146.4 (C6), 141.5 (C3 or 7), 137.4 (C3 or 7), 134.1 (C4), 132.0 (C8), 131.9 (C2), 129.6 (C9), 129.0 (C5), 128.4 (C10 or 11), 127.1 (C1), 125.3 (C12), 125.0 (C10 or 11), 42.1 (C15), 30.1 (C13), 23.4 (C14), 22.1 (C14);
ν<sub>max</sub>: 2960 (N-H, br), 1478 (CH<sub>3</sub>, m), 1403 (CH<sub>3</sub>, m); HRMS (ASAP): m/z found [M]<sup>+</sup> 260.1196, C<sub>16</sub>H<sub>19</sub>N<sub>2</sub>Cl<sup>35</sup> Cl required 260.1206 (Δ -3.8 ppm).

(2-Chloro-2'-ethyl-[1,1'-biphenyl]-4-yl) methanaminium chloride (3b)

Prepared by general method 3 using LiAlH<sub>4</sub> (42 mg, 1.1 mmol), Et<sub>2</sub>O (5.0 mL), AlCl<sub>3</sub> (147 mg, 1.10 mmol) and 2b (132 mg, 0.550 mmol). The crude product was purified by flash column chromatography on silica gel (1:5:14 NH<sub>3</sub>:MeOH:CH<sub>2</sub>Cl<sub>2</sub>) to provide the free amine. The free amine was then subjected to general method 5 using 2 M HCl solution in 1,4-dioxane (2.75 mL) to yield 3b as a white solid (65.0 mg, 0.230 mmol, 43%).

R<sub>f</sub> (amine) = 0.18 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); Mp = 181-183 °C; δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.63 (3H, s, NH), 7.78 (1H, d, J = 1.6 Hz, H5), 7.55 (1H, dd, J = 7.8, 1.6 Hz, H1), 7.39-7.33 (3H, m, H10, H11, H12), 7.28-7.24 (1H, m, H2), 7.04 (1H, d, J = 7.3 Hz, H9), 4.09 (2H, s, H15), 2.47-2.24 (2H, m, H13), 0.98 (3H, t, J = 7.6 Hz, H14); δ<sub>C</sub> (126 MHz, DMSO-d<sub>6</sub>): 141.4 (C6), 139.7 (C3), 137.7 (C7), 135.4 (C4), 132.4 (C8), 131.5 (C2), 129.7 (C9), 129.4 (C5), 128.3 (br s, C10, C11), 127.8 (C1), 125.7 (C12), 41.3 (C15), 25.7 (C13), 15.1 (C14); ν<sub>max</sub>: 3300 (N-H, m), 1476 (CH<sub>3</sub>, m), 1401 (CH<sub>3</sub>, m); HRMS (ESI+): m/z found [M+H]<sup>+</sup> 246.1061, C<sub>15</sub>H<sub>17</sub>N<sup>35</sup>Cl required 246.1049 (Δ 4.8 ppm).

(3-Chloro-4-(1H-indol-4-yl) phenyl)methanamine (3c)

Prepared following general method 3 using compound 2c (95.0 mg, 380 μmol), LiAlH<sub>4</sub> (29.0 mg, 750 μmol), AlCl<sub>3</sub> (50.0 mg, 370 μmol) and Et<sub>2</sub>O (7.00 mL). After work-up, the crude material was purified by column chromatography on silica gel (0:2:98 to 1:4:95 NH<sub>3</sub>:MeOH:CH<sub>2</sub>Cl<sub>2</sub>) to yield 3c as a white solid (55.0 mg, 210 μmol, 55%).
**Chapter 6: Chemistry Experimental**

\( R_f = 0.20 \) (0.2:1.8:98 NH\(_3\):MeOH:CH\(_2\)Cl\(_2\)); \( \text{Mp} 150-152^\circ \text{C} \); \( \delta_n \) (400 MHz, CD\(_3\)OD): 7.55 (1H, d, \( J = 1.5 \) Hz, H11), 7.43-7.41 (2H, m, H5, H14), 7.34 (1H, dd, \( J = 7.8, 1.5 \) Hz, H13), 7.23 (1H, d, \( J = 3.2 \) Hz, H9), 7.17 (1H, t, \( J = 7.3 \) Hz, H6), 6.96 (1H, dd, \( J = 7.3, 0.7 \) Hz, H7), 6.16 (1H, dd, \( J = 3.2, 0.7 \) Hz, H8), 3.88 (2H, s, H15); \( \delta_c \) (101 MHz, CD\(_3\)OD): 142.3 (C4), 138.8 (C1), 136.3 (C12), 132.9 (C10), 131.7 (C14), 131.1 (C2), 128.4 (C11), 127.1 (C3), 125.4 (C13), 124.4 (C9), 120.5 (C6), 119.8 (C7), 110.4 (C8), 100.5 (C5), 44.4 (C15); \( \nu_{\text{max}} \): 2983 (N-H, br), 1373 (C-N, s); \( \text{HRMS (ESI+)} \): m/z found \([\text{M}+\text{H}]^+\) \( 257.0834 \), C\(_{15}\)H\(_{14}\)N\(_2\)Cl required \( 257.0840 \) (\( \Delta -2.5 \) ppm).

(2-Chloro-4′-fluoro-2′methyl-[1,1′-biphenyl]-4-yl)methanaminium chloride (3d)

Prepared following general method 3 using compound 2d (70.0 mg, 280 \( \mu \)mol), LiAlH\(_4\) (32.0 mg, 840 \( \mu \)mol), AlCl\(_3\) (56.0 mg, 480 \( \mu \)mol) and Et\(_2\)O (7.20 mL). After work-up, the crude material was purified by column chromatography on silica gel (1:4:95 NH\(_3\):MeOH:CH\(_2\)Cl\(_2\)) to afford the desired amine as a colourless oil (28.0 mg, 110 \( \mu \)mol, 39%). The amine (23.0 mg, 92.0 \( \mu \)mol) was converted to its hydrochloride salt via general method 5 with HCl (2 M solution in 1,4-dioxane, 500 \( \mu \)L) and CH\(_2\)Cl\(_2\) (100 \( \mu \)L) to yield 3d as a white solid (8.00 mg, 30.0 \( \mu \)mol, 32%).

\( R_f \) (amine) = 0.70 (2:8:90 NH\(_3\):MeOH:CH\(_2\)Cl\(_2\)); \( \text{Mp} = 189-193^\circ \text{C} \); \( \delta_n \) (500 MHz, CD\(_3\)OD) 7.52 (1H, d, \( J = 1.5 \) Hz, H5), 7.35 (1H, dd, \( J = 7.8, 1.5 \) Hz, H1), 7.19 (1H, d, \( J = 7.8 \) Hz, H2), 7.08-7.05 (1H, m, H10), 7.03-7.01 (1H, m, H13), 6.96 (1H, app. td, \( J = 8.5, 2.6 \) Hz, H12), 3.87 (2H, s, H7), 2.07 (3H, s, H14); \( \delta_c \) (126 MHz, CD\(_3\)OD): 163.2 (d, \( J = 244.5 \) Hz, C11), 144.1 (C6), 139.9 (d, \( J = 8.0 \) Hz, C9), 136.6 (C3), 134.7 (C4), 132.5 (C8), 132.2 (C5), 132.1 (d, \( J = 8.4 \) Hz, C13), 129.6 (C1), 127.3 (C2), 117.2 (d, \( J = 21.5 \) Hz, C10), 113.3 (d, \( J = 21.5 \) Hz, C12), 45.6 (C7) 20.0 (d, \( J = 1.6 \) Hz, C14); \( \nu_{\text{max}} \) (376 MHz, d\(_6\)-DMSO): -115.0; \( \text{HRMS (ASAP)} \): m/z found \([\text{M}]^+\) \( 250.079 \), C\(_{14}\)H\(_{14}\)F\(_3\)ClN required \( 250.0793 \) (\( \Delta -1.6 \) ppm).
(4’-(Aminomethyl)-2’-chloro-[1,1’-biphenyl]-2-yl)methanol (3e)

Prepared following general method 3 using compound 2e (50.0 mg, 200 μmol), LiAlH₄ (15.0 mg, 400 μmol), AlCl₃ (27.0 mg, 200 μmol) and Et₂O (3.70 mL). After work-up, the crude material was purified by preparative HPLC (5-95% B over 30 mins) to yield 3e as a white solid (27.0 mg, 110 μmol, 54%).

Rₚ (HPLC) = 8.76 mins (5-95% B over 15 mins); Mp = 205-211 °C; δₜ (500 MHz, CD₃OD): 7.66 (1H, dd, J = 7.0, 1.5 Hz, H12), 7.62 (1H, d, J = 1.5 Hz, H9), 7.47 (1H, app. td, J = 7.7, 1.2 Hz, H4), 7.40-7.38 (2H, m, H3, H11), 7.35 (1H, d, J = 7.7 Hz, H5), 7.12 (1H, dd, J = 7.7, 1.2 Hz, H2), 4.46 (1H, d, J = 13.6 Hz, H13), 4.32 (1H, d, J = 13.6 Hz, H13), 4.20 (2H, s, H14); δₜ (126 MHz, CD₃OD): 140.3 (C10), 139.0 (C1), 137.1 (C7), 134.4 (C8), 133.6 (C6), 131.8 (C12), 129.6 (C9), 129.1 (C5), 128.1 (C4), 127.1 (C11), 127.0 (C3), 126.8 (C2), 61.3 (C13), 42.1 (C14); δₕ (376 MHz, CD₃OD): -76.9; ν_{max}: 3380 (N-H, br), 2977 (O-H, br); HRMS (ESI+): m/z found [M+H]+ 248.0834, C_{14}H_{15}NO_{35}Cl required 248.0837 (Δ -1.0 ppm).

Prepared following general method 4 using compound 2f (30.0 mg, 100 μmol), Raney Ni and NH₃ (8% in MeOH, 2.00 mL). After the work-up, the crude product was purified by column chromatography on silica gel (0:5:95 to 0.5:4.5:95 NH₃:MeOH:CH₂Cl₂) to yield 3f as a colourless viscous oil (8.50 mg, 29.0 μmol, 28%).

Rₚ = 0.51 (2:18:80 NH₃:MeOH:CH₂Cl₂); δₜ (400 MHz, CD₃OD): 7.56 (1H, s, H1), 7.35-7.33 (3H, m, H5, H9, H10), 7.16 (1H, d, J = 7.5 Hz, H4), 7.14-7.13 (1H, m, H8), 7.03-7.01 (1H, m, H11), 6.48 (2H, app. t, J = 2.0 Hz, H14), 6.01 (2H, app. t, J = 2.0 Hz, H15), 4.90 (1H, d, J = 17.0 Hz, H13), 4.80 (1H, d, J = 17.0 Hz, H13), 3.86 (2H, s, H16); δₜ (101 MHz, CD₃OD): 142.2 (C6), 138.0 (C12), 138.0 (C3), 152
136.6 (C7), 133.0 (C2), 131.3 (C4), 129.5 (C8), 128.3 (C1), 128.1 (C5), 127.5 (C11), 127.1 (C9), 126.1 (C10), 120.6 (C14), 107.7 (C15), 50.4 (C13), 44.4 (C16); ν<sub>max</sub>: 2989 (N-H, br), 1264 (C-N, s); HRMS (ESI+): m/z found [M+H]<sup>+</sup> 297.1194, C<sub>18</sub>H<sub>18</sub>N<sub>2</sub>Cl required 297.1153 (Δ -1.2 ppm).

(3-Chloro-5-(1H-indol-4-yl)phenyl)methanamine (3g)

Prepared following general method 3 using compound 2g (77.5 mg, 280 μmol), LiAlH<sub>4</sub> (22.0 mg, 570 μmol), AlCl<sub>3</sub> (38.0 mg, 285 μmol) and Et<sub>2</sub>O (5.25 mL). After work-up, the crude mixture was purified by flash column chromatography on aluminium oxide (2:18:80 NH<sub>3</sub>:EtOAc:hexane) to yield 3g as a white film (57.0 mg, 220 μmol, 79%).

R<sub>f</sub> = 0.40 (0.2:8.2:7 NH<sub>3</sub>:EtOAc:Hexane); δ<sub>H</sub> (500 MHz, CD<sub>3</sub>OD): 7.56-7.54 (2H, m, H3, H5), 7.42 (1H, app. td, J = 8.1, 0.8 Hz, H15), 7.36 (1H, t, J = 1.7 Hz, H1), 7.30 (1H, d, J = 3.2 Hz, H11), 7.21-7.18 (1H, m, H14), 7.10 (1H, dd, J = 7.4, 0.8 Hz, H13), 6.60 (1H, dd, J = 3.2, 0.8 Hz, H10), 3.85 (2H, s, H7); δ<sub>C</sub> (126 MHz, CD<sub>3</sub>OD): 144.5 (C6), 143.9 (C2), 136.7 (C12), 134.0 (C4), 132.0 (C9), 126.6 (C3), 123.5 (C5), 125.7 (C1), 125.3 (C14), 125.0 (C11), 121.2 (C15), 118.6 (C13), 110.8 (C8), 99.9 (C10), 44.8 (C7); ν<sub>max</sub>: 3437 (N-H, m), 679 (=C-H, s); HRMS (ESI+): m/z found [M+H]<sup>+</sup> 257.0831, C<sub>15</sub>H<sub>13</sub>N<sub>2</sub>Cl required 257.0840 (Δ -3.4 ppm).

(3-(1H-Indol-4-yl)-5-(trifluoromethyl)phenyl)methanamine (3h)

Prepared following general method 4 using 2h (100 mg, 360 μmol), NH<sub>3</sub> (8% in MeOH, 2.00 mL) and Raney Nickel. The crude material was purified by column chromatography on silica gel (1:5:95 NH<sub>3</sub>:MeOH:CH<sub>2</sub>Cl<sub>2</sub>) to yield 3h as a sticky, clear oil (29.0 mg, 100 μmol, 28%).

R<sub>f</sub> = 0.09 (1:5:95 NH<sub>3</sub>:MeOH:CH<sub>2</sub>Cl<sub>2</sub>); δ<sub>H</sub> (400 MHz, CD<sub>3</sub>OD): 7.92 (1H, s, H2), 7.84 (1H, s, H4), 7.68 (1H, s, H6), 7.46 (1H, d, J = 8.0 Hz, H8), 7.34 (1H, d, J = 3.2 Hz, H13), 7.23 (1H, t, J = 8.0 Hz, H9), 7.15 (1H, dd, J = 8.0, 0.9 Hz, H10), 6.59 (1H, dd, J = 3.2, 0.7 Hz, H14), 3.97 (2H, s, H15); δ<sub>C</sub>
(101 MHz, CD$_3$OD): 143.8 (C1), 143.0 (C11), 136.8 (C3), 131.9 (C7), 130.9 (C2), 130.5 (q, J = 32.0 Hz, C5), 125.9 (C12), 125.1 (C13), 123.2 (q, J = 260.6 Hz, C16), 122.0 (q, J = 3.6 Hz, C4), 121.2 (C10), 118.7 (C9), 110.9 (C8), 99.7 (C14), 44.9 (C15); δ$_F$ (376 MHz, CD$_3$OD): -64.8; ν$_{\text{max}}$: 2921 (N-H, br), 1257 (C-F, m); HRMS (ESI+): m/z found [M+H]$^+$ 291.1101, C$_{16}$H$_{14}$F$_3$N$_2$ required 291.1104 (Δ -0.8 ppm).

(2,6-Dichloro-[1,1'-biphenyl]-4-yl)ethanaminium chloride (3i)

Prepared following general method 3 using nitrile 2i (22.0 mg, 90.0 μmol), LiAlH$_4$ (7.00 mg, 180 μmol), AlCl$_3$ (12.0 mg, 90.0 μmol) and Et$_2$O (1.70 mL). After work-up, the crude material was purified by flash column chromatography on silica gel (1:9:90 NH$_3$:MeOH:CH$_2$Cl$_2$) to afford the amine (20.0 mg, 80.0 μmol, 88%). The amine underwent general method 5, using HCl (2 M in 1,4-dioxane, 500 μL, 870 μmol) and CH$_2$Cl$_2$ (1.00 mL). Compound 3i was obtained as a white solid (12.0 mg, 40.0 μmol, 48%).

R$_f$(amine) = 0.44 (1:9.90 NH$_3$:MeOH:CH$_2$Cl$_2$); Mp = 246-250 °C; δ$_H$ (500 MHz, CD$_3$OD): 7.62 (2H, s, H1), 7.50-7.41 (3H, m, H6, H8), 7.22-7.19 (2H, m, H7), 4.17 (2H, s, H9); δ$_C$ (126 MHz, CD$_3$OD): 141.7 (C4), 137.7 (C2), 136.6 (C3), 136.4 (C5), 131.7 (C1), 130.7 (C7), 129.8 (C6), 129.5 (C8), 43.0 (C9); ν$_{\text{max}}$: 3332 (N-H, br), 892, 879 (=C-H, m); HRMS (ASAP): m/z found [M]$^+$ 252.0337, C$_{13}$H$_{12}$N$_{35}$Cl$_2$ required 252.0341 (Δ -1.7 ppm).

(2,6-Dichloro-2'-ethyl-[1,1'-biphenyl]-4-yl)methanamine trifluoroacetic salt (3j)

Prepared following general method 2c using 1c (771 mg, 2.40 mmol), 2-ethyl-phenylboronic acid (430 mg, 2.87 mmol), PdCl$_2$(dppf)-CH$_2$Cl$_2$ (98.0 mg, 0.12 mmol), K$_3$PO$_4$ (1.02 g, 4.80 mmol), DME (5.60 mL), EtOH (0.35 mL) and H$_2$O (0.06 mL) and refluxed for 3 hours. A solution of crude 2,6-dichloro-2'-ethyl-[1,1'-biphenyl]-4-carbaldehyde (200 mg), t-butylcarbamate (170 mg, 4.30
mmol), Et₃SiH (0.66 mL, 4.30 mmol) and TFA (0.21 mL, 2.72 mmol) in MeCN (3.20 mL) was stirred at room temperature for 14 h. The mixture was diluted with Et₂O and washed with an aqueous solution of NaHCO₃ and brine. The combined organic layers were dried (MgSO₄), filtered and concentrated under reduced pressure. TFA (4.5 mL) was added to the N-Boc derivative and the mixture stirred at room temperature for 15 minutes. The excess TFA was blown off under a stream of nitrogen to give 3j as a white solid (240 mg, 0.900 mmol, 37%).

\[ \text{R}_f(\text{amine}) = 0.05 \ (10\% \ \text{CH}_2\text{Cl}_2/\text{MeOH}); \text{Mp} = 218-222 \ ^\circ\text{C}; \delta_H (500 \text{ MHz, CD}_3\text{OD}): 7.62 \ (2\text{H, s, H1}), 7.39-7.35 \ (2\text{H, m, H5, H6}), 7.28-7.24 \ (1\text{H, m, H7}), 6.97 \ (1\text{H, d, J = 7.4 Hz, H8}), 4.17 \ (2\text{H, s, H12}), 2.33 \ (2\text{H, q, J = 7.6 Hz, H10}), 1.04 \ (3\text{H, t, J = 7.6 Hz, H11}); \delta_C (126 \text{ MHz, CD}_3\text{OD}): 141.7 \ (C13), 139.9 \ (C9), 135.6 \ (C2), 135.2 \ (C3), 128.9 \ (C4), 128.7 \ (C8), 128.4 \ (C6), 128.2 \ (C5), 125.7 \ (C1), 125.7 \ (C7), 41.8 \ (C12), 26.0 \ (C10), 13.8 \ (C11); \delta_F (376 \text{ MHz, CD}_3\text{OD}): -76.2; \nu_{\text{max}}: 3373 \ (\text{N-H, m}), 1674 \ (\text{N-H, m}), 1674 \ (\text{N-H, m}), 1118 \ (\text{C-N, m}); \text{HRMS} (\text{ESI}): m/z \text{ found } [M+H]^+ \ 280.0641, \text{C}_{15}\text{H}_{16}\text{Cl}_2\text{N req} \text{ required } 280.0654 \ (\Delta - 4.6 \text{ ppm}).

\text{N-(2-(1H-Benzo[d]imidazol-2-yl)ethyl)-2,6-dichloro-[1,1’-biphenyl]-4-aminium chloride (3k)}

Prepared following general method 6 using DCE (1.60 mL), 2k (54.0 mg, 210 \mu\text{mol}), 2-(1H-benzo[d]imidazol-2-yl)ethan-1-amine (50.8 mg, 320 \mu\text{mol}), 4Å molecular sieves (50 mg), NaBH(OAc)$_3$ (62.3 mg, 290 \mu\text{mol}). After work-up, the crude material was purified by column chromatography on aluminium oxide gel (5 to 10% MeOH/CH$_2$Cl$_2$) to yield the free amine (32.0 mg, 90.0 \mu\text{mol}, 42%). The amine underwent general method 5 using HCl (2 M in dioxane, 450 \mu\text{L}) to yield 3k as a white solid (14.0 mg, 32.0 \mu\text{mol}, 36%).

\[ \text{R}_f(\text{amine}) = 0.48 \ (10\% \ \text{MeOH/CH}_2\text{Cl}_2); \delta_H (500 \text{ MHz, DMSO-d$_6$): 7.87 \ (2\text{H, s, H1}), 7.75-7.73 \ (2\text{H, m, H5}), 7.52-7.43 \ (5\text{H, m, H6, H7, H14}), 7.24-7.22 \ (2\text{H, m, H15}), 4.32 \ (2\text{H, s, H9}), 3.62-3.59 \ (4\text{H, m, H10, H11}); \delta_C (126 \text{ MHz, CDCl}_3): 154.0 \ (C12), 141.4 \ (C13), 141.3 \ (C8), 138.4 \ (C4), 136.7 \ (C3), 135.1 \ (C2), 129.6 \ (C6), 128.2 \ (C5 or C7), 128.1 \ (C5 or C7), 127.7 \ (C14 or C15), 122.3 \ (C14 or
C15), 114.9 (C1), 52.8 (C9), 47.0 (C10), 28.9 (C11); $\nu_{\text{max}}$: 2720 (N-H, s); HRMS (ESI+): $m/z$ found [M+H]$^+$ 396.1035, C$_{22}$H$_{20}$N$_3$Cl$_2$ required 396.1029 ($\Delta$ 1.6 ppm).

2-(1H-Benzimidazol-2-yl)-N-((2,6-dichloro-2'-ethyl-[1,1'-biphenyl]-4-yl)methyl)ethan-1-amine (3l)

Prepared by general method 2c using 1c (250 mg, 0.770 mmol), 2-ethylphenylboronic acid (139 mg, 0.930 mmol), PdCl$_2$(dppf)-CH$_2$Cl$_2$ (32.0 mg, 0.390 mmol), K$_3$PO$_4$ (329 mg, 1.55 mmol), DME (2.00 mL), EtOH (0.300 mL) and H$_2$O (0.150 mL) and refluxed for 6 hours. The crude material (80.0 mg, 0.290 mmol) was then subjected to general method 6 using 2-(1H-benzimidazol-2-yl)ethylamine (69.0 mg, 0.430 mmol), 4Å molecular sieves (50 mg), DCE (1.00 mL) and sodium triacetoxyborohydride (86.0 mg, 0.410 mmol). The crude amine was purified by preparative HPLC (5-95% B over 20 mins) to yield 3l as a sticky film (26.0 mg, 0.0400 mmol, 13%).

$R_f$ (HPLC) = 9.43 mins (5-95% B over 15 mins); $\delta_H$ (500 MHz, CD$_3$OD): 7.76-7.71 (2H, m, H18), 7.70 (2H, s, H1), 7.53-7.49 (2H, m, H5, H6), 7.40-7.35 (2H, m, H19), 7.29-7.25 (1H, m, H7), 6.97 (1H, d, $J = 7.4$ Hz, H8), 3.75-3.63 (4H, m, H13, H14), 3.31-3.29 (2H, m, H15), 2.34 (2H, q, $J = 7.6$ Hz, H10), 1.05 (3H, t, $J = 7.6$ Hz, H11); $\delta_C$ (126 MHz, CD$_3$OD): $\delta$ 150.8 (C16), 143.0 (C9), 141.9 (C17), 137.1 (C12), 136.7 (C2), 134.4 (C3), 134.3 (C4), 130.7 (C8), 130.2 (C7), 130.1 (C6), 129.6 (C1), 127.1 (C5), 126.7 (C19), 115.2 (C18), 51.2 (C13), 45.6 (C14), 27.2 (C15), 25.1 (C10), 15.1 (C11); $\delta_F$ (376 MHz, CD$_3$OD): -76.9, -77.1; $\nu_{\text{max}}$: 2975 (N-H, s), 1667 (C-Cl, m); HRMS (ESI+): $m/z$ found [M+H]$^+$, 424.1346, C$_{24}$H$_{25}$N$_3$Cl$_2$ required 424.1347 ($\Delta$ -0.2 ppm).
6.1.1.2. Unnatural amino acids

Fmoc-Dap-OH (4)

Fmoc-Asn-OH (4.00 g, 11.3 mmol) was added to a solution of [bis(trifluoroacetoxy)iodo]benzene (5.40 g, 16.9 mmol) in DMF/H₂O (2:1 53.2 mL: 26.4 mL). After 15 minutes, pyridine (2.10 mL) was added and the mixture was stirred at rt for 16 hours. The solvent was removed under reduced pressure and the oily residue was dissolved in H₂O (60.0 mL). Concentrated HCl (2 mL) was added and the acidified solution was washed with Et₂O (3 x 40 mL). The aqueous phase was adjusted to pH 6 with 2 M NaOH solution and the resulting precipitate was filtered, washed with H₂O, ice-cold EtOH, Et₂O and the residual solvent removed under reduced pressure to yield Fmoc-Dap-OH as a beige powder (2.99 g, 9.16 mmol, 81%).

δ(H (400 MHz, DMSO-d₆)): 7.89 (2H, d, J = 7.5 Hz, H₃), 7.70 (2H, d, J = 6.3 Hz, H₄), 7.41 (2H, d, J = 7.4 Hz, H₂), 7.35 (2H, J = 7.3 Hz, H₁), 6.77 (1H, d, J = 6.1 Hz, CONH), 4.32-4.24 (3H, m, H₅, H₆), 3.69-3.44 (1H, m, H₈), 3.00 (1H, dd, J = 10.8, 4.8 Hz, H₉), 2.75 (1H, app t, J = 10.8 Hz, H₉); δ(C (101 MHz, DMSO-d₆)): 169.1 (C₁₀), 156.3 (C₇), 144.4 (C₁₁), 141.2 (C₁₂), 128.1 (C₂), 127.8 (C₁), 125.8 (C₃), 120.6 (C₄), 66.2 (C₆), 52.4 (C₈), 47.1 (C₉), 41.1 (C₅); HRMS (ESI⁺): m/z found [M+H]+ 327.1343, C₁₈H₁₉O₄N₂ required 327.1339 (Δ 1.2 ppm).

Characterisation data in accordance with literature.²³⁵

Fmoc-Aza-OH (5)

Compound 4 (4.04 g, 12.4 mmol) was added to a mixture of H₂O (60.0 mL), MeOH (180 mL), and CH₂Cl₂ (120 mL). CuSO₄·5H₂O (20.0 mg, 0.0800 mmol) and 6 (8.08 g, 29.9 mmol) were added, the mixture adjusted to pH 9 with saturated K₂CO₃ and stirred for 18 hours. The mixture was then diluted with CH₂Cl₂ (120 mL), and the aqueous phase isolated. The organic phase was extracted with saturated NaHCO₃ (2 x 200 mL). The aqueous extract was washed with Et₂O (2 x 200 mL), acidified to pH 2 with conc. HCl, and extracted with Et₂O (3 x 240 mL). The organic extracts were dried (MgSO₄) and the solvent removed under reduced pressure. The oily
residue was re-dissolved in EtOAc and the solvent removed under a stream of N₂ to yield 5 as a beige, amorphous solid (3.58 g, 9.53 mmol, 77%).

δ_H (400 MHz, DMSO-d₆): 7.94-7.89 (2H, m, H10), 7.74 (2H, d, J = 7.4 Hz, H7), 7.43 (2H, app t, J = 7.4 Hz, H9), 7.33 (2H, app t, J = 7.4 Hz, H8), 4.34-4.27 (2H, m, H5), 4.24-4.22 (2H, m, H1, H12), 3.64-3.62 (2H, m, H3);

δ_C (101 MHz, DMSO-d₆): 171.5 (C2), 156.5 (C4), 144.2 (C6), 141.2 (C11), 128.1 (C9), 127.5 (C8), 125.7 (C7), 120.6 (C10), 66.3 (C5), 54.2 (C1), 51.4 (C3), 47.1 (C12);

[α]_D²⁵ -9.9 (c = 1, DMF);

HRMS (ESI+): m/z found [M+H]⁺ 353.1248, C₁₈H₁₇O₄N₄ required 353.1244 (Δ 1.0 ppm).

Characterisation data in accordance with literature.

Imidazole-1-sulfonyl azide hydrogen Sulfate (6)

Sulfuryl chloride (16.1 mL, 200 mmol) was added dropwise to an ice-cold suspension of NaN₃ (13.0 g, 200 mmol) in MeCN (200 mL) and the mixture stirred for 16 hours. Imidazole (25.9 g, 380 mmol) was added and the pink mixture stirred at 0°C for 5 hours. The mixture was then diluted with EtOAc (400 mL) and H₂O (400 mL). The organic fraction was isolated and washed with H₂O (400 mL) and saturated NaHCO₃ (2 x 200 mL) and then dried (MgSO₄). The solvent was reduced to 200 mL under reduced pressure. A solution of conc. H₂SO₄ (11.0 mL) in EtOAc (100 mL) was added to the ice-cold reaction mixture over 30 min. The reaction mixture was warmed to room temperature and stirred for 16 hours. The precipitate was filtered, washed with EtOAc (3 x 60 mL), and dried under reduced pressure to yield 6 as a white powder (34.9 g, 129 mmol, 64%).

δ_H (500 MHz, DMSO-d₆): 8.64 (1H, s, H3), 7.97 (1H, app t, J = 1.6 Hz, H1), 7.35 (1H, app q, J = 0.8 Hz, H2); δ_C (101 MHz, DMSO-d₆): 138.2 (C3), 130.9 (C1), 119.4 (C2); HRMS (ESI+): m/z found [M+H]⁺ 174.0073, C₁₈H₁ₙO₄N₂ required 174.0080 (Δ 4.2 ppm).

Characterisation data in accordance with literature.
Fmoc-3-Chloro-phenylalanine (7)

Prepared following general method 7 using NaHCO$_3$ (1.59 g, 19.0 mmol), H$_2$O (109 mL), MeCN (109 mL), 3-chloro-DL-phenylalanine (1.00 g, 5.00 mmol) and N-(9-fluorenylmethoxycarbonyloxy)succinimide (1.86 g, 5.50 mmol). The mixture was stirred at rt for 24 hours. After work-up, the crude mixture was purified by flash chromatography on silica gel (0 to 20% MeOH in CH$_2$Cl$_2$) to yield 7 as a white solid (650g, 1.96 mmol, 39%).

R$_f$ = 0.1 (20 % MeOH/ CH$_2$Cl$_2$); Mp = 78.6-87.5°C; $\delta_H$ (400 MHz, DMSO-d$_6$): 7.87 (2H, d, $J = 7.5$, 14H), 7.64-7.61 (2H ,m, H17), 7.42-7.19 (8H, m, H5, H7, H8, H9, H15, H16), 4.24-4.10 (4H, m, H1, H11, H12), 3.13 (1H, app dd, $J = 13.6$, 3.9 Hz, H3), 2.91-2.85 (1H, m, H3); $\delta_C$ (101 MHz, DMSO-d$_6$): 174.1 (C2), 156.1 (C10), 144.4 (C18), 141.7 (C13), 141.3 (C4), 133.2 (C6), 130.4 (C8), 129.6 (C5), 128.6 (C15), 128.2 (C16), 127.6 (C9), 126.7 (C7), 125.7 (C14), 120.5 (C17), 66.0 (C11), 56.4 (C1), 47.1 (C12), 36.9 (C3); $\nu_{max}$: 1707 (C=O, s), 757 (C=C, s), 737 (C=C, s); HRMS (ESI+): m/z found [M+Na]$^+$ 444.0964, C$_{24}$H$_{20}$O$_4$N$_3$ClNa$^+$ required 444.0973 (Δ -1.9 ppm).

(S)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-2-(3-chlorophenyl)acetate (B)

Prepared following general method 7 using NaHCO$_3$ (903 mg, 10.8 mmol), H$_2$O (60 mL), MeCN (60 mL), (S)-2-(3-chlorophenyl)glycine (500 mg, 2.69 mmol) and N-(9-fluorenylmethoxycarbonyloxy)succinimide (998 mg, 2.96 mmol). The mixture was stirred at rt for 48 hours. After the work-up, the crude product was purified by flash chromatography on silica gel (0 to 15% MeOH in CH$_2$Cl$_2$) to yield 8 as a white solid (940 mg, 2.31 mmol, 86%).

R$_f$ = 0.54 (10% MeOH/CH$_2$Cl$_2$); Mp = 241.0-242.4 °C; $\delta_H$ (400 MHz, DMSO-d$_6$): 7.89 (2H, d, $J = 7.1$ Hz, H16), 7.71 (2H, d, $J = 7.1$ Hz, H13), 7.44-7.39 (3H, m, H5, H15), 7.35-7.28 (4H, m, H1, H2, H3, H14), 4.87 (1H, d, $J = 7.1$ Hz, H7), 4.29-4.22 (3H, m, H10, H11); $\delta_C$ (101 MHz, DMSO-d$_6$): 171.4 (C8), 155.6 (C9), 144.4 (C12), 144.2 (C17), 141.2 (C6), 133.0 (C4), 130.1 (C2), 128.1 (C5), 127.5
(C1 or C3), 127.3 (C1 or C3), 127.0 (C15), 125.7 (C14), 120.6 (C13), 66.1 (C10), 49.1 (C7), 47.1 (C11); \( \nu_{\text{max}} \): 3387 (OH, br), 1666 (C=O, m); \([\alpha]_{D}^{20} + 79.00\) (c = 0.22, MeOH); HRMS (ESI+): \(m/z\) found [M+H]\(^+\) 408.0997, C\(_{23}\)H\(_{19}\)O\(_4\)N\(_3\)Cl\(_1\) required 408.0990 (\(\Delta -1.8\) ppm).

2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-2-(4-chlorophenyl)acetic acid (9)

Prepared following general method 7 using NaHCO\(_3\) (1.80 g, 21.5 mmol), H\(_2\)O (120 mL), MeCN (120 mL), 4-chloro-DL-phenylglycine (1.00 g, 5.39 mmol) and \(N\)-(9-fluorenyl)methoxycarbonyloxy)succinimide (2.00 g, 5.93 mmol). The mixture was stirred at rt for 48 hours. After the work-up, the crude product was purified by flash chromatography on silica gel (0 to 20% MeOH in CH\(_2\)Cl\(_2\)) to yield 9 as a white solid (2.20 g, 5.39 mmol, quantitative).

\(R_f = 0.47\) (20% MeOH/CH\(_2\)Cl\(_2\)); \(M_p = 213.0-216.7^\circ\)C; \(\delta_H\) (400 MHz, DMSO-d\(_6\)): 7.89 (2H, d, \(J = 7.4\) Hz, H13), 7.72-7.70 (2H, m, H10), 7.41-7.29 (8H, m, H1, H2, H11, H12), 4.89 (1H, d, \(J = 6.8\) Hz, H8), 4.28-4.22 (3H, m, H7, H15); \(\delta_C\) (101 MHz, DMSO-d\(_6\)): 171.7 (C5), 155.8 (C6), 144.4 (C9), 144.2 (C14), 141.2 (C4), 129.5 (C1 or C2), 128.3 (C1 or C2), 128.0 (C13), 127.6 (C11 or C12), 125.7 (C3), 125.7 (C11 or C12), 120.5 (C10), 66.0 (C7), 59.2 (C15), 47.2 (C8); \(\nu_{\text{max}}\): 3303 (OH, br), 1677 (C=O, m); HRMS (ESI+): \(m/z\) found [M+Na]\(^+\) 430.0817, C\(_{23}\)H\(_{18}\)O\(_4\)N\(_3\)Cl\(_2\)Na required 430.0817 (\(\Delta 0.2\) ppm).

Fmoc-Azido ornithine (12)

HCl in (4 M in 1,4-dioxane, 14 mL) was added to a solution of Fmoc-Orn(Boc)-OH (2.00g, 4.39 mmol) in 1,4-dioxane (14 mL). After stirring at rt for 16 h, the solvent was removed under a stream of N\(_2\) and the resulting residue was suspended in Et\(_2\)O (70 mL). The solid was filtered and washed with Et\(_2\)O (70 mL) to give the Fmoc-Orn-OH HCl as a white solid (1.70 g, 99%). Fmoc-Orn-OH HCl (2.96 g, 7.54 mmol) was dissolved in a biphasic mixture of H\(_2\)O (44 mL), MeOH (87 mL) and CH\(_2\)Cl\(_2\) (72 mL). CuSO\(_4\)-5H\(_2\)O (14.5 mg, 0.0580 mmol) and imidazole-1-
sulfonyl azide hydrogen sulfate 6 (5.80 g, 22.7 mmol) were added. The mixture was adjusted to pH 9 with K₂CO₃ aqueous solution. After stirring vigorously for 18 hours, the organic solvents were removed under reduced pressure. The remaining aqueous phase was washed with Et₂O (2 x 50 mL), acidified to pH 2 with concentrated HCl and extracted with Et₂O (3 x 50 mL). The organic extracts were dried (MgSO₄) and concentrated under reduced pressure. The oily residue was dissolved in EtOAc and the solvent removed under a stream of N₂ to give 12 as a white solid (2.25 g, 5.91 mmol, 78%).

δₜ (400 MHz, DMSO-d₆): 7.79 (2H, d, J = 7.6 Hz, H10), 7.62-7.60 (2H, m, H13), 7.43 (2H, app t, J = 7.5 Hz, H11), 7.33 (2H, app tt, J = 7.4, 1.1 Hz, H12), 5.39 (1H, d, J = 8.1 Hz, CONH), 4.60-4.45 (3H, m, H1, H7), 4.23 (1H, t, J = 6.7 Hz, H8), 3.35-3.16 (2H, m, H5), 2.07-1.46 (4H, m, H3, H4); δₙ (126 MHz, DMSO-d₆): 175.6 (C2), 156.1 (C6), 143.7 (C14), 141.3 (C9), 127.8 (C11), 127.1 (C12), 125.0 (C13), 120.1 (C10), 67.2 (C7), 53.2 (C8), 50.8 (C1), 47.2 (C5), 29.6 (C4), 29.5 (C3); [α]_D^20 -2.02 (c = 1.0, MeOH).

Characterisation data in accordance with literature.²⁵¹
6.1.1.3. Constraints

1,2-Diethynylbenzene (C4)

1,2-dibromobenzene (200 mg, 0.850 mmol), Pd(PPh₃)₂Cl₂ (23.8 mg, 0.0340 mmol), CuI (6.50 mg, 0.0340 mmol), PPh₃ (18.0 mg, 0.0680 mmol) were dissolved in DIPEA (283 μL) and anhydrous toluene (1.41 mL). The mixture was stirred at rt for 5 minutes and TMS-acetylene was added. The mixture was refluxed under N₂ for 16 hours. The solution was cooled down to 0 °C and KOH (6N in MeOH, 1.30 mL, 3.40 mmol) was added and the mixture was stirred at rt for 6 hours. The reaction was quenched with saturated NH₄Cl and the volatiles removed under a stream of N₂. The aqueous phase was acidified to pH 5 and extracted with CH₂Cl₂ (3 x 5 mL). The combined organic layers were dried (MgSO₄), filtered, and the solvent evaporated under reduced pressure. The crude mixture was purified by flash chromatography on silica gel (100 % PE 30-40) to yield C4 as a 42% w/v solution in PE 30-40 (107 mg, 0.850 mmol, quantitative).

R_f = 0.4 (100% hexane); δ_H (400 MHz, CDCl₃): 7.55-7.53 (2H, m, H2), 7.34-7.32 (2H, m, H1), 3.35 (2H, s, H₅); δ_C (101 MHz, CDCl₃): 132.6 (C2), 128.5 (C1), 125.1 (C3), 81.8 (C4), 81.1 (C5); \nu_{max}: 3287 (C≡C), 756 (C=C), 731 (C=C); HRMS (ASAP): m/z found [M]^+ 126.0465, C₁₀H₆ required 126.0470 (Δ -4.0 ppm).

Methyl 3,5-bis((trimethylsilyl)ethynyl)benzoate (C5-1)

Trimethylsilylacetylene (6.00 mL, 42.2 mmol) was added to a stirring mixture of methyl 3,5-dibromobenzoate (900 mg, 3.10 mmol), Pd₂(dba)₃ (54.0 mg, 0.0590 mmol), CuI (11.4 mg, 0.00600 mmol) and PPh₃ (77.4 mg, 0.290 mmol) in dry triethylamine (15.0 mL). The reaction mixture was refluxed for 16 hours under N₂. The solvent was removed under reduced pressure, the residue was diluted with EtOAc and washed with H₂O. The organic phase was dried (MgSO₄) and the crude residue was purified by flash chromatography on silica gel (0-5% EtOAc in PE 40-60) to give C5-1 as a yellow oil (693 mg, 2.11 mmol, 68%).
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**3,5-Diethylbenzoic acid (C5)**

Prepared following general method 8 using aqueous 6 M KOH (3.00 mL, 17.9 mmol), C5-1 (590 mg, 1.79 mmol), MeOH (4.00 mL). After the work-up, C5 was obtained as an orange solid (300 mg, 1.76 mmol, 98%).

\[ R_f = 0.40 \text{ (20\% MeOH/CH}_2\text{Cl}_2); \delta_H (400 MHz, CD}_2\text{OD): 8.05 \text{ (2H, app d, } J = 1.32 \text{ Hz, H1), 7.73 \text{ (1H, app s, H2), 3.66 \text{ (2H, s, H5); } \delta_C (101 MHz, CD}_2\text{OD): 166.4 \text{ (C7), 138.5 \text{ (C2), 132.6 \text{ (C1), 131.6 \text{ (C3), 123.3 \text{ (C6), 81.0 \text{ (C4), 79.2 \text{ (C5); } } \nu_{max}: 3282 \text{ (C≡C, s), 1682 \text{ (C=O, s); } HRMS \text{ (ESI+): } m/z \text{ found [M+H]}^+ 171.0441, C_{11}H_{21}Si_2 \text{ required 171.0441 (Δ 1.9 ppm).} \]

Characterisation data in accordance with literature.\(^{219}\)

**1,5-bis(Trimethylsilyl)pent-1,4-diyne-3-ol (C6-1)**

Prepared following general method 9 using \textit{nBuLi} (1.6 M in hexane, 3.15 mL, 0.100 mmol), trimethylsilylacetylene (0.700 mL, 0.100 mmol), THF (15.0 mL), 3-(trimethylsilyl)-propirolaldehyde (0.500 mL, 0.610 mmol). After the work up, the crude residue was purified by preparative TLC (5\% EtOAc in toluene) to give C6-1 as an orange oil (136 mg, 0.610 mmol, quantitative).

\[ R_f = 0.3 \text{ (100\% hexane); } \delta_H (400 MHz, CDCl}_3): 5.10 \text{ (1H, s, H1), 3.49 \text{ (1H, br s, OH), 0.20 \text{ (18H, s, H4); } \delta_C (101 MHz, CDCl}_3): 101.8 \text{ (C2), 89.4 \text{ (C3), 52.8 \text{ (C1), -0.4 \text{ (C4); } } \nu_{max}: 3295 \text{ (O-H, br, w), 2961 \text{ (C≡C-H, m); } HRMS \text{ (ESI+): } m/z \text{ found [M+H]}^+ 225.1121, C_{11}H_{21}Si_2 \text{ required 225.1131 (Δ -4.4 ppm).} \]

Characterisation data in accordance with literature.\(^{220}\)
3-(4-Bromophenyl)-1,5-bis(trimethylsilyl)penta-1,4-diyn-3-ol (C6-6)

Prepared following general method 9 using trimethylsilylacetylene (0.950 mL, 7.00 mmol) in THF (5.50 mL), nBuLi (1.6 M in hexane, 4.30 mL, 7.00 mmol), ethyl 4-bromo benzoate (300 μL, 2.30 mmol) in THF (2.00 mL). After the work-up, the crude residue was purified by column chromatography on silica gel (100% hexane) to yield C6-6 as a white solid (800 mg, 2.13 mmol, 93%).

R_f = 0.2 (100% hexane); Mp = 87.0-88.5°C; δH (400 MHz, CDCl₃): 7.67 (2H, d, J = 8.6 Hz, H2), 7.53 (2H, d, J = 8.6 Hz, H3), 2.97 (1H, br s, OH), 0.23 (18H, s, H8); δC (101 MHz, CDCl₃): 140.7 (C1), 131.5 (C2), 127.8 (C3), 122.8 (C4), 104.1 (C6), 90.5 (C7), 65.0 (C5), -0.4 (C8); ν_max: 2960 (C≡C, m), 838 (C=C, s), 760 (C=C, s); HRMS (ESI+): m/z found [M+Na]^+ 401.0367, C₁₇H₂₃ON⁷⁹BrNa required 401.0363 (Δ 0.9 ppm).

3-(4-Bromophenyl)penta-1,4-diyn-3-ol (C6-7)

Prepared following general method 8 using aqueous 6 M KOH (0.500 mL, 2.40 mmol), C6-6 (100 mg, 0.260 mmol) and MeOH (0.500 mL). After the work-up, the crude material was purified by flash chromatography on silica gel (0 to 10% EtOAc in hexane) to yield C6-7 as a yellow oil (33.0 mg, 0.140 mmol, 54%).

R_f = 0.11 (10% EtOAc/Hexane); δH (400 MHz, CDCl₃): 7.69 (2H, d, J = 8.6 Hz, H2), 7.55 (2H, d, J = 8.6 Hz, H3), 3.04 (1H, br s, OH), 2.82 (2H, s, H7); δC (101 MHz, CDCl₃): 139.8 (C1), 131.7 (C2), 127.6 (C3), 123.2 (C4), 83.0 (C6), 74.0 (C7), 64.2 (C5); ν_max: 3283 (C≡C, s), 944 (C=C, s), 826 (C=C, s); HRMS (ESI+): m/z found [M-H]^- 232.9602, C₁₁H₆O⁷⁹Br required 232.9602 (Δ 0.0 ppm).
4-(3-Hydroxy-1,5-bis(trimethylsilyl)penta-1,4-diyn-3-yl)benzoic acid (C6-9)

Prepared following general method 9 using trimethylsilylacetylene (5.00 mL, 34.7 mmol) in THF (50.0 mL), nBuLi (1.6 M in hexane, 25 mL, 36.0 mmol) and methyl-4-carboxybenzoate (2.50 g, 13.8 mmol) in THF (10.0 mL). After the work-up, the crude residue was purified by flash chromatography on silica gel (100% CH$_2$Cl$_2$) to yield C6-9 as an off white solid (2.20 g, 6.38 mmol, 46%).

$R_f = 0.02$ (0.5% MeOH/CH$_2$Cl$_2$); $Mp = 199.8$-200.6 °C $\delta_H$ (400 MHz, CD$_3$OD): 8.05 (2H, d, $J = 8.4$ Hz, H3), 7.84 (2H, d, $J = 8.4$ Hz, H4), 0.22 (18H, s, H9); $\delta_C$ (101 MHz, CD$_3$OD): 168.0 (C1), 147.4 (C2), 130.4 (C5), 129.3 (C3), 125.6 (C4), 105.1 (C7), 88.6 (C8), 64.3 (C6), -1.7 (C9); $\nu_{\text{max}}$: 3340 (O-H, br, w), 1683 (C=O, s); HRMS (ESI+): $m/z$ found [M+H]$^+$ 345.1336, C$_{18}$H$_{25}$O$_3$Si$_2$ required 345.1342 ($\Delta$ 1.7 ppm).

4-(3-Hydroxypenta-1,4-diyn-3-yl)benzoic acid (C6)

Prepared following general method 8 using aqueous 6 M KOH (5.00 mL, 29.0 mmol), C6-9 (1.00 g, 2.84 mmol) and MeOH (6.00 mL). After the work-up, C6 was yielded as a yellow solid (350 mg, 1.75 mmol, 62%).

$R_f = 0.4$ (20% CH$_2$Cl$_2$/MeOH); $Mp = 154.4$-155.6 °C; $\delta_H$ (400 MHz, CD$_3$OD): 8.07 (2H, d, $J = 8.4$ Hz, H3), 7.88 (2H, d, $J = 8.4$ Hz, H4), 3.22 (2H, s, H8); $\delta_C$ (101 MHz, CD$_3$OD): 167.9 (C1), 147.2 (C2), 130.5 (C5), 129.3 (C3), 125.6 (C4), 83.6 (C7), 73.2 (C8), 63.5 (C6); $\nu_{\text{max}}$: 3275 (C≡C, m), 1658 (C=O, s); HRMS (ESI+): $m/z$ found [M+Na]$^+$ 223.0369, C$_{12}$H$_{15}$O$_3$Na required 223.0366 ($\Delta$ 1.7 ppm).
A solution of N-bromosuccinimide (1.17 g, 6.60 mmol) in DMF (66.0 mL) was added dropwise to an ice-cold suspension of carbazole (500 mg, 3.00 mmol) in toluene (2.50 mL). The reaction mixture was warmed to rt, stirred for 30 minutes then poured onto H₂O (100 mL). The precipitate formed was then washed with H₂O (20 mL) and cold MeOH (20 mL). The crude product was triturated in cold MeOH/hexanes (1:1) to give the desired product as an off-white solid (673 mg, 2.10 mmol, 69%).

**Rf** = 0.30 (30% CH₂Cl₂/PE 40-60); **Mp** = 250-256 °C (lit. 210-211 °C); **δH** (400 MHz, DMSO-d₆): 11.57 (1H, s, H7), 8.44 (2H, app. d, J = 1.44 Hz, H3), 7.55-7.47 (4H, m, H5, H6); **δC** (101 MHz, DMSO): 139.3 (C1), 129.2 (C3), 123.8 (C5), 123.8 (C4), 113.7 (C6), 111.4 (C2); **νmax**: 3420 (N-H, s), 800 (C-Br, s); **HRMS** (ESI+): m/z found [M+H]+ 323.9012, C₁₂H₁₈N₇Br₂ required 323.9024 (Δ - 3.7 ppm).

Characterisation data in accordance with literature²⁵²,²⁵³

3,6-Diethynyl-9H-carbazole (C₈)

A suspension of C₈₁ (5.00 g, 15.5 mmol), Pd(PPh₃)₂Cl₂ (500 mg, 0.775 mmol), CuI (100 mg, 0.465 mmol), ethynyltrimethylsilane (10.0 mL, 69.8 mmol), and PPh₃ (100 mg, 0.388 mmol) in NEt₃ (160 mL) was refluxed for 48 hours. The reaction mixture was filtered through Celite, washed with H₂O (50 mL), brine (50 mL) and dried (MgSO₄). The mixture was concentrated under reduced pressure and the residue resuspended in 500 mL of MeOH. K₂CO₃ (14.1 g, 101 mmol) was added and the suspension stirred at rt for 18 hours. The reaction mixture was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel (30% CH₂Cl₂/PE 40-60) to yield the desired product as a dark orange solid (2.69 g, 12.5 mmol, 81%).
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R_f = 0.28 (20% EtOAc/hexanes); Mp = 210 °C decomp. (lit. Mp = 212-213 °C); δ_H (400 MHz, DMSO-d_6): 11.71 (1H, s, H1), 8.37 (2H, s, H6), 7.50 (4H, app. s, H3, H4), 4.04 (2H, s, H9); δ_C (101 MHz, DMSO-d_6): 140.5 (C2), 130.2 (C4), 125.2 (C6), 122.4 (C7), 112.6 (C5), 112.0 (C3), 85.3 (C9), 78.9 (C8); ν_max: 3404 (NH s), 3269 (C≡C-H, m), 2103 (C≡C, s); HRMS (ESI): m/z found [M+H]^+ 216.0818, C_{16}H_{10}N required 216.0813 (Δ 2.3 ppm).

Data in accordance with the literature.¹²⁴

6-(3,6-Diethynyl-9H-carbazol-9-yl)hexanoic acid (C9)

[Chemical structure image]

Prepared following a literature procedure.²⁵⁴ To a solution of 3,6-diethynyl-9H-carbazole (1.00 g, 2.78 mmol) in DMF (5.60 mL) was added powdered KOH (1.23 g, 22.0 mmol). The reaction mixture was stirred at rt for 20 minutes. After this time 6-bromohexanoic acid (1.08 g, 5.56 mmol) was added and the reaction was irradiated at 37 kHz ultrasonic waves at 35 °C for 2 hours. The reaction was acidified to pH 5 with conc HCl, portioned with Et₂O and H₂O and the organic phase washed with brine. The organic extract was dried (MgSO₄) and the solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel (0-50% EtOAc/hexane) to yield C9 as a gummy yellow oil (880 mg, 1.86 mmol, 67%).

R_f = 0.20 (20% EtOAc/hexane); δ_H (400 MHz, CDCl₃): 8.22 (2H, d, J = 1.0 Hz, H13), 7.61 (2H, dd, J = 8.4, 1.00 Hz, H9), 7.32 (2H, J = 8.4 Hz, H8), 4.27 (2H, t, J = 9.4 Hz, H6), 3.11 (2H, s, H12), 2.34 (2H, t, J = 7.4 Hz, H2), 1.88 (2H, app quint., J = 7.4 Hz, H5), 1.68 (2H, app quint., J = 6.8, H3), 1.46-1.39 (2H, m, H4); δ_C (101 MHz, CDCl₃): 179.6 (C1), 140.6 (C7), 130.2 (C13), 124.8 (C9), 122.3 (C14), 112.8 (C10), 108.9 (C8), 84.7 (C11), 75.6 (C12), 43.0 (C6), 33.7 (C2), 28.6 (C5), 26.6 (C4), 24.3 (C3); ν_max: 3260 (C≡C, m), 1698 (C=O, s); HRMS (ESI+): m/z found [M+H]^+ 330.1491, C_{22}H_{20}O₂N required 330.1494 (Δ -0.9 ppm).
6.1.2. Peptides

General method 10: Manual peptide synthesis

Manual peptide synthesis was performed on Merck LL MHBA Rink amide resin (0.33 mmol/g, 1 equiv). Couplings were carried out by adding HATU (4 equiv) to a solution of the Fmoc-protected amino acid (4 equiv) in DMF (~0.4 M). After 10 seconds, DIPEA (8 equiv) was added to the mixture. This pre-activated mixture was then added to the resin in DMF and shaken for 3 minutes. The coupling time was extended in the case of N-terminal capping with C5 and C9 (1 hour), for coupling of Arg and unnatural amino acids (30 minutes). The side chain protecting groups used were: tBu for Asp, Glu, Ser, Thr, Tyr; Boc for Lys, Trp; Pbf for Arg; Trt for Asn, Gln, His. Fmoc-Lys(ddve)-OH was used for conjugation of FITC with Lys. Fmoc-Gly(Dmb)-OH was used for the synthesis of peptide P7.

Fmoc deprotection was carried out with 20% piperidine in DMF (3 x 3 minutes).

N-terminal capping with FITC and Ac₂O (2 equiv) was achieved using DIPEA (4 equiv) in CH₂Cl₂ overnight (FITC) or for 1 hour (Ac₂O).

On-resin attachment of FITC via Lys was achieved by orthogonal deprotection of the Lys(ddve) with 5% NH₂NH₂ in DMF (2 x 10 minutes) followed by conjugation with FITC (2 equiv) in the presence of DIPEA (4 equiv) overnight.

Completion of amide couplings and Fmoc deprotection was determined by a chloranil test, in which acetaldehyde (200 μL) and a saturated solution of chloranil in toluene (50 μL) were added to a small amount of resin swelled in CH₂Cl₂. After 10 seconds shaking at rt, no change in colour indicated complete coupling, whilst green colouration of the resin indicated presence of a free amine. Any incomplete couplings was submitted to a second round of coupling.

Side chain deprotection and cleavage from the resin was achieved with TFA containing 2.5% TIPS and 2.5% H₂O for 3 hours at rt or 1 hour at 42 °C. In case of methionine and cysteine-containing peptides, cleavage was achieved with TFA containing 5% EDT, 5% H₂O and 2.5% TIPS. After cleavage, the mixture was filtered through a sintered funnel, the beads washed with MeOH and the filtrate was concentrated under a stream of N₂. The crude residue was triturated with cold Et₂O before purification by preparative HPLC.

General method 11: Automated Fmoc solid-phase peptide synthesis

Automated peptide synthesis was carried out on a CEM Liberty Automated Microwave Peptide Synthesiser using Merck LL MHBA Rink Amide resin (0.33 mmol/g, 1 equiv). All peptide couplings were performed with Fmoc-protected amino acids (5 equiv), Oxyma pure (10 equiv) and DIC (5 equiv) in DMF. Arg was coupled using double couplings for 15 min each without
microwave irradiation. All other amino acids were coupled with 25 W power at 75 °C over 15 min.

Fmoc deprotection was achieved with a solution of 20% piperidine in DMF, using 45 W power at 75 °C over 3 min. N-terminal capping, cleavage and HPLC purification of peptides were carried out as previously described for manual SPPS (general method 10).

**General method 12: Peptide stapling via Copper-catalysed azido-alkyne click (CuAAC).**

A solution of diazido peptide (1 equiv) and dialkynyl linker (1 equiv) in 1:1 BuOH/H2O (0.8 mL/mg peptide) was degassed with N2 for 15 min, followed by the addition of CuSO4 · 5H2O (1 equiv), THPTA (1 equiv), and sodium ascorbate (3 equiv). Peptides bearing sulfur atoms required CuSO4 · 5H2O (2 equiv), THPTA (2 equiv), and sodium ascorbate (6 equiv). All the reactions were stirred under N2 and monitored by LCMS. When no starting material could be detected by LCMS, the reaction mixture was diluted with H2O and lyophilised prior to purification. The absence of azido peak on IR (~ 2100 cm⁻¹) was checked for all the purified, dried peptide.

**General method 13: Peptide macrocyclisation via disulphide bridge formation.**

To a solution of the linear peptide in aqueous AcOH (50%) was added 2 mM I2 in MeOH dropwise. After 5 minutes the reaction was quenched by adding ascorbic acid (1 M). The crude mixture was directly purified on preparative HPLC.

**General method 14: Peptide macrocyclisation via ring-closing metathesis (RCM).**

RCM of the peptides was carried out on resin. After N-terminus capping, the resin was deswollen in MeOH, then washed with CH2Cl2 and resuspended in the minimum amount of CH2Cl2. Grubb’s II catalyst (20 mol%) was dissolved in CH2Cl2 and added to the resin under bubbling N2. The reaction was agitated for 2 hours. The resin was then washed with CH2Cl2 and the process repeated. The peptide was then cleaved from the resin and purified as described above (general method 10).
6.1.2.1. Conversion of CuAAC macrocyclisation reactions

The conversions of the peptides that underwent macrocyclisation via CuAAC 2C PS refer to LCMS conversion of the crude material (Table 18). All the reactions were quenched when no starting material could be detected.

Table 18 - LCMS conversion of peptides that underwent CuAAC cyclisation. CK2 peptides are shown in the left column, BH3 peptides in the right column.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO-C1</td>
<td>82</td>
</tr>
<tr>
<td>P1-C2</td>
<td>65</td>
</tr>
<tr>
<td>P1-C3</td>
<td>62</td>
</tr>
<tr>
<td>P1-C4</td>
<td>79</td>
</tr>
<tr>
<td>P1-C5</td>
<td>92</td>
</tr>
<tr>
<td>P1-C6-7</td>
<td>52</td>
</tr>
<tr>
<td>P1-C6</td>
<td>85</td>
</tr>
<tr>
<td>FITC-P1-C5</td>
<td>56</td>
</tr>
<tr>
<td>P1-F1C5</td>
<td>79</td>
</tr>
<tr>
<td>P1-F2C5</td>
<td>54</td>
</tr>
<tr>
<td>P1-F3C5</td>
<td>87</td>
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<td>P2'-C5</td>
<td>67</td>
</tr>
<tr>
<td>P2”'-C5</td>
<td>NA</td>
</tr>
<tr>
<td>P3-C5</td>
<td>53</td>
</tr>
<tr>
<td>P4-C5</td>
<td>60</td>
</tr>
<tr>
<td>P5'-C5</td>
<td>53</td>
</tr>
<tr>
<td>P5”'-C5</td>
<td>47</td>
</tr>
<tr>
<td>P6-C5</td>
<td>88</td>
</tr>
<tr>
<td>P7-C5</td>
<td>83</td>
</tr>
<tr>
<td>P7-F1C5</td>
<td>60</td>
</tr>
<tr>
<td>P7-F2C5</td>
<td>72</td>
</tr>
<tr>
<td>P8-F1C5</td>
<td>72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9-C5</td>
<td>58</td>
</tr>
<tr>
<td>P9-C7</td>
<td>81</td>
</tr>
<tr>
<td>P9-F4C5</td>
<td>59</td>
</tr>
<tr>
<td>P9-F5C5</td>
<td>87</td>
</tr>
<tr>
<td>P11-F4C5</td>
<td>NA</td>
</tr>
<tr>
<td>P14-C8</td>
<td>NA</td>
</tr>
<tr>
<td>P15-C8</td>
<td>90</td>
</tr>
<tr>
<td>P16-C8</td>
<td>60</td>
</tr>
<tr>
<td>P17-C5</td>
<td>92</td>
</tr>
<tr>
<td>P18-C5</td>
<td>68</td>
</tr>
<tr>
<td>P19-C5</td>
<td>97</td>
</tr>
<tr>
<td>P17-F1C5</td>
<td>92</td>
</tr>
<tr>
<td>P18-F1C5</td>
<td>77</td>
</tr>
<tr>
<td>P19-F1C5</td>
<td>72</td>
</tr>
<tr>
<td>FITC-Ahx-P9-F4C4</td>
<td>74</td>
</tr>
<tr>
<td>FITC-Ahx-P9-F5C5</td>
<td>90</td>
</tr>
<tr>
<td>FITC-Ahx-P11-F4C4</td>
<td>79</td>
</tr>
</tbody>
</table>

NA = not available due to complicated chromatogram.
6.1.2.2. LCMS and purity of CK2 peptides

CK2 peptides sequence, mass observed on LCMS, purity and retention time (Rt) by analytical HPLC are shown in Table 19.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass</th>
<th>m/z found</th>
<th>m/z calcul.</th>
<th>Species</th>
<th>Purity</th>
<th>Rt* (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc</td>
<td>GCRLYGFKHHC(G-S)</td>
<td>1448.7</td>
<td>1449.5</td>
<td>1449.7</td>
<td>M+H</td>
<td>87%</td>
<td>7.26</td>
</tr>
<tr>
<td>Pc linear</td>
<td>GCRLYGFKHGC</td>
<td>1450.7</td>
<td>1451.49</td>
<td>1451.7</td>
<td>M+H</td>
<td>99%</td>
<td>9.62</td>
</tr>
<tr>
<td>Short Pc linear</td>
<td>CRLYGFKHGC</td>
<td>1336.6</td>
<td>1337.3</td>
<td>1337.7</td>
<td>M+H</td>
<td>90%</td>
<td>9.92</td>
</tr>
<tr>
<td>Short Pc</td>
<td>CRLYGKFIHG (S-S)</td>
<td>1334.6</td>
<td>1335.4</td>
<td>1335.6</td>
<td>M+H</td>
<td>84%</td>
<td>14.32</td>
</tr>
<tr>
<td>P0</td>
<td>XRLYGFKHIX</td>
<td>1297.7</td>
<td>1298.9</td>
<td>1298.7</td>
<td>M+H</td>
<td>&gt;99%</td>
<td>10.16</td>
</tr>
<tr>
<td>P0-C1</td>
<td>GX2RLYGFKIHXc1</td>
<td>1376.7</td>
<td>1377.5</td>
<td>1377.7</td>
<td>M+H</td>
<td>97%</td>
<td>6.96</td>
</tr>
<tr>
<td>P1</td>
<td>GXRLYGFKHXGG</td>
<td>1468.7</td>
<td>1470</td>
<td>1469.8</td>
<td>M+H</td>
<td>91%</td>
<td>7.29</td>
</tr>
<tr>
<td>P1-C2</td>
<td>GX2RLYGFKIHXc2GG</td>
<td>1594.8</td>
<td>1595.61</td>
<td>1595.8</td>
<td>M+H</td>
<td>91%</td>
<td>7.78</td>
</tr>
<tr>
<td>P1-C3</td>
<td>GX2RLYGFKIHXc3GG</td>
<td>1532.8</td>
<td>1533.6</td>
<td>1533.8</td>
<td>M+H</td>
<td>97%</td>
<td>6.98</td>
</tr>
<tr>
<td>P1-C4</td>
<td>GX2RLYGFKIHXc4GG</td>
<td>1423.7</td>
<td>1424.5</td>
<td>1424.7</td>
<td>M+H</td>
<td>97%</td>
<td>7.85</td>
</tr>
<tr>
<td>P1-C5</td>
<td>GX2RLYGFKIHXc5GG</td>
<td>1638.9</td>
<td>1639.4</td>
<td>1639.9</td>
<td>M+H</td>
<td>89%</td>
<td>7.46</td>
</tr>
<tr>
<td>P1-C6-7</td>
<td>GX05-RLYGFKIHXc6-7GG</td>
<td>1702.7</td>
<td>568.6</td>
<td>568.3</td>
<td>M+3H</td>
<td>&gt;99%</td>
<td>7.74</td>
</tr>
<tr>
<td>P1-C6</td>
<td>GCXLRLYGFKIHXc6GG</td>
<td>1671.0</td>
<td>836.2</td>
<td>836.2</td>
<td>M+2H</td>
<td>92%</td>
<td>7.14</td>
</tr>
<tr>
<td>FITC-P1</td>
<td>FITC-Ahx-GXRLYGFKHXGG</td>
<td>1929.7</td>
<td>644.6</td>
<td>644.3</td>
<td>M+3H</td>
<td>62%</td>
<td>8.96</td>
</tr>
<tr>
<td>FITC-P1-C5</td>
<td>FITC-Ahx-GXR2LYGFKHXc5GG</td>
<td>1709.7</td>
<td>1710.1</td>
<td>1710.7</td>
<td>M+H</td>
<td>94%</td>
<td>7.89</td>
</tr>
<tr>
<td>P1-F1C5</td>
<td>GX3CRLYGFKIHXF1C5GG</td>
<td>2343.4</td>
<td>1168.1</td>
<td>1168.1</td>
<td>M+2H</td>
<td>&gt;99%</td>
<td>6.96</td>
</tr>
<tr>
<td>P1-F2C5</td>
<td>GX3CRLYGFKIHXF2C5GG</td>
<td>2851.4</td>
<td>1427.3</td>
<td>1426.7</td>
<td>M+2H</td>
<td>98%</td>
<td>7.69</td>
</tr>
<tr>
<td>P1-F3C5</td>
<td>GX3CRLYGFKIHXF3C5GG</td>
<td>1191.1</td>
<td>1192.7</td>
<td>1192.1</td>
<td>M+H</td>
<td>89%</td>
<td>8.44</td>
</tr>
<tr>
<td>P2</td>
<td>GXRLYG(L-3-C1-F)KHXGG</td>
<td>1502.7</td>
<td>752.3</td>
<td>752.4</td>
<td>M+2H</td>
<td>74%</td>
<td>7.57</td>
</tr>
<tr>
<td>P2’</td>
<td>GXRLYG(3-C1-F)KHXGG</td>
<td>1502.7</td>
<td>752.3</td>
<td>752.4</td>
<td>M+2H</td>
<td>74%</td>
<td>7.57</td>
</tr>
<tr>
<td>P2”</td>
<td>GXRLYG(3-C1-F)KHXGG</td>
<td>1502.7</td>
<td>752.3</td>
<td>752.4</td>
<td>M+2H</td>
<td>74%</td>
<td>7.57</td>
</tr>
<tr>
<td>P2”-C5</td>
<td>GX3CRLYG(L-3-C1 F)KHXCG</td>
<td>1674.7</td>
<td>1675</td>
<td>1675.7</td>
<td>M+H</td>
<td>90%</td>
<td>7.46</td>
</tr>
<tr>
<td>P2”-C5</td>
<td>GX3CRLYG(L-3-C1 F)KHXCG</td>
<td>1674.7</td>
<td>1675.5</td>
<td>1675.7</td>
<td>M+H</td>
<td>85%</td>
<td>7.24</td>
</tr>
<tr>
<td>P3</td>
<td>GXRLYG(4-C1-F)KHXHGG</td>
<td>1502.7</td>
<td>752.3</td>
<td>752.4</td>
<td>M+2H</td>
<td>87%</td>
<td>7.71</td>
</tr>
<tr>
<td>P3-C5</td>
<td>GX3CRLYG(4-C1-F)KHXC5GG</td>
<td>1677.8</td>
<td>1676.9</td>
<td>1676.8</td>
<td>M-H</td>
<td>92%</td>
<td>7.33</td>
</tr>
<tr>
<td>P4</td>
<td>GXRLYG(3-C1)pheglyKHXGG</td>
<td>1490.7</td>
<td>1491.1</td>
<td>1491.7</td>
<td>M+H</td>
<td>&gt;99%</td>
<td>7.56</td>
</tr>
<tr>
<td>P4-C5</td>
<td>GX3CRLYG(3-C1)pheglyKHXCG</td>
<td>1659.8</td>
<td>1659.6</td>
<td>1658.4</td>
<td>M-H</td>
<td>82%</td>
<td>8.26</td>
</tr>
<tr>
<td>P5</td>
<td>GXRLYG(4-C1)pheglyKHXGG</td>
<td>1490.7</td>
<td>1491.2</td>
<td>1491.7</td>
<td>M+H</td>
<td>&gt;99%</td>
<td>7.61</td>
</tr>
<tr>
<td>P5-C5</td>
<td>GX3CRLYG(4-C1)pheglyKHXCG</td>
<td>1659.8</td>
<td>1661.3</td>
<td>1660.8</td>
<td>M+H</td>
<td>92%</td>
<td>7.25</td>
</tr>
<tr>
<td>P5”-C5</td>
<td>GX3CRLYG(4-C1)pheglyKHXCG</td>
<td>1659.8</td>
<td>1661.3</td>
<td>1660.8</td>
<td>M+H</td>
<td>97%</td>
<td>7.38</td>
</tr>
<tr>
<td>P6</td>
<td>GXRLYGFKHXGG</td>
<td>1403.7</td>
<td>1402.1</td>
<td>1402.7</td>
<td>M-H</td>
<td>89%</td>
<td>7.15</td>
</tr>
<tr>
<td>P6-C5</td>
<td>GX3CRLPGFKHXC5GG</td>
<td>1573.6</td>
<td>1574.7</td>
<td>1573.7</td>
<td>M+H</td>
<td>98%</td>
<td>6.95</td>
</tr>
<tr>
<td>P7</td>
<td>GXRLYGFKWHXGG</td>
<td>1543.7</td>
<td>1544</td>
<td>1544.7</td>
<td>M+H</td>
<td>95%</td>
<td>7.48</td>
</tr>
<tr>
<td>P7-C5</td>
<td>GX3CRLYGFKWHXCG</td>
<td>1713.6</td>
<td>1714.7</td>
<td>1714.6</td>
<td>M+H</td>
<td>&gt;99%</td>
<td>7.29</td>
</tr>
<tr>
<td>P7-F1C5</td>
<td>GX3CRLYGFKWHXF1C5GG</td>
<td>2407.3</td>
<td>1205</td>
<td>1204.5</td>
<td>M+2H</td>
<td>&gt;99%</td>
<td>7.22</td>
</tr>
<tr>
<td>P7-F2C5</td>
<td>GX3CRLYGFKWHXF2C5GG</td>
<td>2929.5</td>
<td>1462.2</td>
<td>1463.7</td>
<td>M+2H</td>
<td>95%</td>
<td>7.62</td>
</tr>
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</table>
6.1.2.3. LCMS and purity of CPPs

CPPs sequence, mass observed on LCMS, purity and retention time (Rt) by analytical HPLC are shown in Table 20.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass</th>
<th>m/z found</th>
<th>m/z calc.</th>
<th>Species</th>
<th>Purity</th>
<th>Rt* (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1C5</td>
<td>C5-(Abx)_3+(D)R[1]_1</td>
<td>863.5</td>
<td>864.9</td>
<td>864.5</td>
<td>M+H</td>
<td>99%</td>
<td>7.47</td>
</tr>
<tr>
<td>F2C5</td>
<td>C5-(Abx)_3(D)R[1]_1-FITC</td>
<td>1381.7</td>
<td>1382.4</td>
<td>1382.7</td>
<td>M+H</td>
<td>83%</td>
<td>9.18</td>
</tr>
<tr>
<td>F3C5</td>
<td>C5-(Abx)_3+(D)R[1]_1</td>
<td>911.3</td>
<td>912.4</td>
<td>911.3</td>
<td>M+H</td>
<td>97%</td>
<td>7.18</td>
</tr>
<tr>
<td>F4C4</td>
<td>C5-RRR</td>
<td>637.4</td>
<td>638.7</td>
<td>638.4</td>
<td>M+H</td>
<td>93%</td>
<td>6.6</td>
</tr>
<tr>
<td>F5C5</td>
<td>C5-(Abx)_3-PKKKRKV</td>
<td>1259.8</td>
<td>1261.2</td>
<td>1260.9</td>
<td>M+H</td>
<td>99%</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*rt (retention time) on a 5 to 95 % B over 15 minutes run on analytical HPLC.

6.1.2.4. LCMS and purity of BH3 peptides

BH3 peptides sequence, mass observed on LCMS, purity and retention time (Rt) by analytical HPLC are shown in Table 21.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass</th>
<th>m/z found</th>
<th>m/z calc.</th>
<th>Species</th>
<th>Purity</th>
<th>Rt* (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>IWIAQELRXscGDXscFNAYYARR</td>
<td>2677.4</td>
<td>1339.7</td>
<td>1337.6</td>
<td>M+2H</td>
<td>94%</td>
<td>13.7</td>
</tr>
<tr>
<td>P9-C5</td>
<td>IWIAQELRXscGDXscFNAYYARR</td>
<td>2846.4</td>
<td>1424.9</td>
<td>1424.2</td>
<td>M+2H</td>
<td>93%</td>
<td>9.92</td>
</tr>
<tr>
<td>P9-F4C5</td>
<td>IWIAQELRXscGDXscFNAYYARR</td>
<td>3315.2</td>
<td>1658.1</td>
<td>1658.1</td>
<td>M+H</td>
<td>96%</td>
<td>14.32</td>
</tr>
<tr>
<td>P9-F5C5</td>
<td>IWIAQELRXscGDXscFNAYYARR</td>
<td>3940.0</td>
<td>1314.1</td>
<td>1314.3</td>
<td>M+3H</td>
<td>&gt;99%</td>
<td>8.49</td>
</tr>
<tr>
<td>P9-C7</td>
<td>IWIAQELRXscGDXscFNAYYARR</td>
<td>2975.2</td>
<td>1487.6</td>
<td>1487.6</td>
<td>M+2H</td>
<td>99%</td>
<td>10.09</td>
</tr>
<tr>
<td>FITC-P9</td>
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<td>1046.6</td>
<td>1046.2</td>
<td>M+3H</td>
<td>&gt;99%</td>
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<td>3773.8</td>
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<td>1887.9</td>
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<td>1465.3</td>
<td>1466.4</td>
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<td>&gt;99%</td>
<td>9.82</td>
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<td>1629.2</td>
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<td>97%</td>
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<td>10.11</td>
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<tr>
<td>SAHBa</td>
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<td>2646.4</td>
<td>1324.24</td>
<td>1324.2</td>
<td>M+2H</td>
<td>91%</td>
<td>11.13</td>
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<tr>
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<td>3106.5</td>
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<td>1554.2</td>
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<tr>
<td>P12</td>
<td>RYGRELRRMSDEVYDSF</td>
<td>2204.6</td>
<td>2205.7</td>
<td>2205.7</td>
<td>M+H</td>
<td>87%</td>
<td>7.81</td>
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*rt (retention time) on a 5 to 95 % B over 15 minutes run on analytical HPLC.
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<th></th>
<th>Peptide</th>
<th>M+2H Retention Time</th>
<th>M+H Retention Time</th>
<th>M+2H Percent</th>
<th>M+H Percent</th>
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<tr>
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<td>IIRNIARHLAQVGDMSDRS</td>
<td>2192.2 1098 1098.1</td>
<td>M+2H 96% 8.66</td>
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<tr>
<td>P14</td>
<td>IAXELRRIGDEFNXYAA</td>
<td>2151.1 2152.4 2152.4</td>
<td>M+H 86% 9.21</td>
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<tr>
<td>P14-C8</td>
<td>IAX₈ELRRIGDEFNX₈Y₈AA</td>
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<td>M-2H 92% 9.95</td>
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<td>XYGRELRRMSDXFVDSF</td>
<td>2199.6 2200.5 2200.6</td>
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<tr>
<td>P15-C8</td>
<td>X₈YGRELRRMSDX₈FVDSF</td>
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<tr>
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<tr>
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<tr>
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<td>M+2H 85% 8.74</td>
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<tr>
<td>P18-F1C5</td>
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<td>M+2H &gt;99% 8.59</td>
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<td>P19</td>
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<td>P19-C5</td>
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<td>3097.0 1548.8 1548.5</td>
<td>M+2H 90% 9.09</td>
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</tbody>
</table>

*Unless otherwise specified, rt (retention time) refers to a 5 to 95 % B over 15 minutes run on analytical HPLC.
* rt on a 30 to 100% solvent B over 15 minutes.
CHAPTER 7:
Computational Chemistry
Experimental
With the exclusion of the docking studies, all the experiments described in this dissertation were performed by Dr Yaw Sing Tan, A*STAR, Singapore.

## 7.1. Docking studies for CK2 fragments

All the docking studies herein reported were performed using Glide of the suite Maestro, produced by Schrodinger. The protein was prepared starting from the PDB file of the X-ray structures generated by Dr Paul Brear (Department of Biochemistry, Hyvönen Group) and using the PrepWinz feature of Maestro. To keep the conformation of the protein as close as possible to the X-ray structures, no modifications such as ‘cap termini’, ‘filling loops’ or ‘side chain’, ‘original hydrogen deletion’ were performed. Only the orientation of the water molecules was sampled, and the ionisation state of the protein was calculated at pH 7 ± 2. Ligands were prepared with the LigPrep functionality, using OPLS_2005 as the force field. All other parameters were left as default. Conformational search of the ligands was performed after ligand preparation. The parent ligand of the crystal structure was used as the template for the grid generation and positional constraints were given to the benzylamine nitrogen of the ligand. All other parameters were left as default. Ligand input partial charges were used and, in addition to default parameters, the planarity of conjugated pi groups was enhanced. For the conformer generation, enhanced sampling was used, and the energy window for ring sampling was increased to 3.5 kcal mol\(^{-1}\).

## 7.2. Computational chemistry for CK2 peptides

### 7.2.1 Molecular dynamics

Chains A and D from the crystal structure of human CK2α in complex with a CK2β-derived cyclic peptide called Pc (1-GCRLYGFKHGCG-13) (PDB: 4IB5) were used as the initial structures for molecular dynamics (MD) simulations. The unresolved CK2α residue Met1 was added using PyMOL. CK2α was capped at its C-terminus by N-methyl while Pc was capped by acetyl and amide groups. Crystallographic water molecules were retained. PDB2PQR was then used to determine the protonation states of residues. Using the LEaP program in the AMBER 14 package, each complex was solvated with TIP3P water molecules in a periodic truncated octahedron box such that its walls were at least 10 Å away from the complex, followed by neutralisation of the system with either sodium or chloride ions.

Energy minimisations and MD simulations were carried out by the PMEMD module of AMBER 14, using the ff14SB force field for protein residues and the generalised AMBER force field (GAFF) for the stapled residues. Atomic charges for the stapled residues were derived using the R.E.D. Server, which fits restrained electrostatic potential (RESP) charges to a molecular electrostatic potential (MEP) computed by the Gaussian 09 program at the HF/6-31G* theory level.
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level. A total of three independent explicit-solvent MD simulations using different initial atomic velocities were carried out. The SHAKE algorithm\textsuperscript{266} was applied to constrain all bonds involving hydrogen atoms, allowing for a time step of 2 fs. A cutoff distance of 9 Å was implemented for nonbonded interactions. The particle mesh Ewald method\textsuperscript{267} was used to treat electrostatic interactions with a grid spacing of 1.0 Å and the order of B-spline interpolation set to 4. Energy minimisation was performed for 500 steps with the steepest descent algorithm, followed by another 500 steps with the conjugate gradient algorithm. The system was then heated gradually to 300 K over 50 ps at constant volume before equilibration at a constant pressure of 1 atm for another 50 ps. During the minimisation and equilibration, weak harmonic positional restraints with a force constant of 2.0 kcal mol\(^{-1}\) Å\(^{-2}\) were imposed on the non-hydrogen atoms of the solute. These restraints were removed in a subsequent equilibration run (2 ns) and the production run (100 ns), which were carried out at 300 K and 1 atm. The Langevin thermostat\textsuperscript{268} was used to maintain the temperature with a collision frequency of 2 ps\(^{-1}\). Pressure was maintained by a Berendsen barostat\textsuperscript{269} with a pressure relaxation time of 2 ps.

7.2.2 Ligand mapping simulations

Ten different distributions of chlorobenzenes around apo CK2α were created using Packmol.\textsuperscript{270} The LEaP module in the AMBER 14 package was then used to solvate each system with TIP3P water molecules in a periodic truncated octahedron box, such that its walls were at least 10 Å away from the protein, resulting in a final chlorobenzene concentration of \(\sim 0.15 \text{ M}\). Minimisation, equilibration and production (20 ns) MD simulations were carried out as described above for the CK2α complexes, for a cumulative sampling time of 200 ns. The GAFF6 force field was used to describe the chlorobenzenes during the simulations. Atomic charges for chlorobenzene were used as previously described.\textsuperscript{271}

The 10 individual runs were combined into a single trajectory for analysis. Chlorobenzene occupancy grids were generated using the cpptraj module of AMBER 14 to bin both carbon and chlorine atoms of chlorobenzenes into \(1 \times 1 \times 1\) Å grid cells. The cutoff isocontour value used for visualisation of chlorobenzene carbon and chlorine atom occupancies is five times the threshold bulk value, which is defined as the highest isovalue at which the respective atoms are detected in the bulk solvent. In order to compare the overlap of the chlorobenzene occupancy maps with known CK2α ligands, the respective ligand-bound CK2α structures were aligned using PyMOL\textsuperscript{272} to the average protein structure sampled during the LMMD simulations.

7.2.3 Binding free energy decomposition

The contribution of each Pc peptide residue to the binding free energy of the complex was computed by applying the free energy decomposition method\textsuperscript{273} on 200 equally-spaced structures extracted from the last 40 ns of the MD simulations of the CK2α–Pc complex. Binding
free energies were calculated in AMBER 14\textsuperscript{259} using the molecular mechanics/generalised Born surface area (MM/GBSA) method.\textsuperscript{274} The molecular mechanical energies and polar contribution to solvation free energy were computed by the sander module and pbsa program using the modified GB model described by Onufriev et al.\textsuperscript{275} respectively. The nonpolar contribution to solvation free energy was estimated from the solvent accessible surface area (SASA) using the ICOSA method.\textsuperscript{276}

7.2.4 Computational alanine scanning (CAS)

Computational alanine scanning was carried out on 200 equally-spaced structures extracted from the last 40 ns of the MD simulations of the CK2α–Pc complex. The difference in the binding free energy ($\Delta\Delta G_{\text{bind}}$) of the wild-type and alanine mutants was calculated using the MM/GBSA method with modules in AMBER 14.\textsuperscript{259} Molecular mechanical energies were calculated with the sander module. The polar contribution to the solvation free energy was calculated by the pbsa module\textsuperscript{277} using the modified GB model described by Onufriev et al.\textsuperscript{275} while the nonpolar contribution was estimated from the SASA using the linear combinations of pairwise overlaps method,\textsuperscript{278} with $\gamma$ set to 0.0072 kcal mol$^{-1}$ Å$^{-2}$ and $\beta$ to zero.\textsuperscript{276} The entropy term was not considered due to the high computational cost and the assumption that the entropy of the mutant does not differ considerably from that of the wild-type.\textsuperscript{279}

7.2.5 Binding free energy calculations

Binding free energies for the CK2α complexes were calculated using the molecular mechanics/generalised Born surface area (MM/GBSA) method implemented in AMBER 14.\textsuperscript{259} Two hundred equally-spaced snapshot structures were extracted from the last 40 ns of each of the trajectories, and their molecular mechanical energies calculated with the sander module. The polar contribution to the solvation free energy was calculated by the pbsa\textsuperscript{277} module using the modified generalised Born (GB) model described by Onufriev et al.\textsuperscript{275} while the nonpolar contribution was estimated from the solvent accessible surface area using the molsurf\textsuperscript{280} program with $\gamma = 0.0072$ kcal Å$^{-2}$ and $\beta$ set to zero. Entropies were estimated by normal mode analysis\textsuperscript{281} using the nmode program. Due to its computational expense, only 50 equally-spaced snapshots from the last 40 ns of the trajectories were used for entropy calculations.
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7.3. Computational chemistry for BH3 peptides

7.3.1 Preparation of structures

The structure of Bcl-xL (Δ45-84) in complex with Bim BH3 (residues 141-166) (PDB: 4QVF) was used as the initial structure for MDs. Bim BH3 was truncated to residues 146-166. The unresolved Bim residues 165 and 166 were then added using PyMOL to give peptide P9-C2. Bcl-xL was capped at its N- and C-termini by acetyl and N-methyl groups respectively while peptide P9-C2 was capped at its N- and C-termini by acetyl and amide groups respectively. Peptide P9-C2 was modified into the stapled peptide by replacing residues 154 and 158 with a two-component triazole staple formed by a double-click reaction between two azido-ornithine residues and 1,3-diethylbenzene. PDB2PQR was used to determine the protonation states of residues. Each Bcl-xL complex was solvated with TIP3P water molecules in a periodic truncated octahedron box, such that its walls were at least 9 Å away from the complex, and neutralised with sodium ions.

7.3.2 Molecular dynamics

Energy minimisations and MD simulations were performed with the sander and PMEMD modules of AMBER 14 respectively. Three independent MD simulations were carried out on each of the Bcl-xL complexes using the ff14SB and GAFF. Atomic charges for the stapled residues were derived using the R.E.D. Server, which fits RESP charges to a MEP computed by the Gaussian 09 program at the HF/6-31G* theory level. All bonds involving hydrogen atoms were constrained by the SHAKE algorithm, allowing for a time step of 2 fs. Nonbonded interactions were truncated at 9 Å, while the particle mesh Ewald method was used to account for long range electrostatic interactions under periodic boundary conditions. Weak harmonic positional restraints with a force constant of 2.0 kcal mol⁻¹ Å⁻² were placed on the protein and peptide non-hydrogen atoms during the minimisation and equilibration steps. Energy minimisation was carried out using the steepest descent algorithm for 500 steps, followed by the conjugate gradient algorithm for another 500 steps. The systems were then heated gradually to 300 K over 50 ps at constant volume before equilibration at a constant pressure of 1 atm for another 50 ps. Subsequent unrestrained equilibration (2 ns) and production (100 ns) runs were carried out at 300 K using a Langevin thermostat with a collision frequency of 2 ps⁻¹, and 1 atm using a Berendsen barostat with a pressure relaxation time of 2 ps.

7.3.3 Binding free energy calculations

Binding free energies for the Bcl-xL complexes were calculated using the molecular mechanics/generalised Born surface area (MM/GBSA) method implemented in AMBER 14.
Two hundred equally-spaced snapshot structures were extracted from the last 40 ns of each of the trajectories, and their molecular mechanical energies calculated with the sander module. The polar contribution to the solvation free energy was calculated by the pbsa program\textsuperscript{277} using the modified GB model described by Onufriev \textit{et al.}\textsuperscript{275} while the nonpolar contribution was estimated from the solvent accessible surface area using the molsurf program\textsuperscript{280} with $\gamma = 0.0072$ kcal Å$^{-2}$ and $\beta$ set to zero. Entropies were estimated by normal mode analysis\textsuperscript{281} using the nmode program. Due to its computational expense, only 50 equally-spaced snapshots from the last 40 ns of the trajectories were used for entropy calculations.
CHAPTER 8:

Biophysics Experimental
Chapter 8: Biophysics Experimental

8.1. Biophysics experimental for CK2 fragments and peptides

8.1.1. Protein expression and purification

8.1.1.1. CK2α

Three constructs of CK2α were used in this study. For kinase activity assays and competition experiments, CK2α_WT was used (residues 2-329). For ITC CK2α_KA construct was used whilst CK2α_FP10 was used for crystallisation purposes. CK2α_KA (residues 2-329) contained four mutations designed to aid crystallisation by reducing the overall charge of the protein; R21S, K74A, K75A and K76A. CK2α_FP10 contained one mutation (R21S) and an N-terminal extension GSMDIEFDDDADDDGSGSGSGS aimed at mimicking a substrate peptide for CK2α. CK2α_FP10 was cloned into pHAT4 vector and CK2α_KA was cloned into pHAT2 vector to give constructs with cleavable His6-tags. Recombinant plasmids containing one of the three constructs (CK2α_WT/ CK2α_KA/ CK2α_FP10) were introduced into *Escherichia coli* BL21(DE3) for protein production. Single colonies of the cells were grown in 6 x 1L of 2 x TY with 100 μg/mL ampicillin at 37°C. Isopropylthio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression when the optical density at 600 nm reached 0.6. The cells were incubated overnight at 25°C then harvested by centrifugation at 4,000 g for 20 minutes. The same extraction and purification procedure were used for all three constructs, with the exception that CK2α_KA used 350 mM NaCl in the buffer, whereas, CK2α_WT and CK2α_FP10 required 500 mM NaCl. The cell pellets were suspended in 20 mM Tris, 350/500 mM NaCl, pH 8.0) and lysed using a high-pressure homogeniser. Protease inhibitor cocktail tablets (one tablet per 50 mL extract; Roche Diagnostics) and DNase I were then added. The crude cell extract was then centrifuged at 10,000 g for 45 minutes, the supernatant was filtered with a 0.22 μm filter. The soluble supernatant was applied on a Ni Sepharose Fast Flow6 column at pH 8.0, washed and eluted in 20 mM Tris pH 8.0, 350/500 mM NaCl, 200 mM imidazole. After overnight dialysis into 20 mM Tris, pH 8.0, 350/500 mM NaCl the N-terminal His6-Tag was cleaved overnight by TEV protease and passed through a second metal affinity column to remove uncleaved protein and the protease. The cleaved protein was further purified on a Sepharose Q HP anion-exchange column and the main peak fraction from this column was further purified by gel filtration on a Superdex 75 16/60 HiPrep column equilibrated with Tris 20 mM, pH 8.0, 350/500 mM NaCl. Pure protein was concentrated to 15 mg/mL and flash frozen in liquid N₂.

8.1.1.2. CK2β

pGEX-CK2β construct (1-198) obtained from Victor Bolanos-Garcia (Prof. Tom Blundell’s lab) was introduced into *Escherichia coli* BL21 (DE3) for protein production. Single colonies of the cells were grown in 6x1 L of 2 x TY with 100 μg/mL ampicillin at 37 °C. Isopropyl thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression when
the optical density at 600 nm reached 0.6. The cells were incubated overnight at 25 °C then harvested by centrifugation at 4,000 g for 20 minutes. The cell pellets were suspended in 20 mM Tris, 500 mM NaCl, pH 8.5) and lysed using a high-pressure homogeniser. Protease inhibitor cocktail tablets (one tablet per 50 mL extract; Roche Diagnostics) and DNase I were then added. The crude cell extract was then centrifuged at 10,000 g for 45 minutes, the supernatant was filtered with a 0.22 μm filter. The soluble supernatant was applied on a Glutathione Sepharose column and washed with 5 column volumes of loading buffer (20 mM Tris, 500 mM NaCl, pH 8.5) followed by washing with 10 column volumes of cleavage buffer (20 mM Tris 500 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 8.5). 100μL of the precision protease was loaded onto the column and incubated for 5 hours at 4°C and eluted in the cleavage buffer. The cleaved protein was further purified on a Sepharose Q HP anion-exchange column (gradient 0-500 mM NaCl) and the main peak fraction from this column was further purified by gel filtration on a Superdex 75 16/60 HiPrep column equilibrated with Tris 20 mM, pH 8.5, 500 mM NaCl. Pure protein was concentrated to 15 mg/mL and flash frozen in liquid N2.

8.1.2. Fluorescent Polarisation

Approximate % inhibition of the holoenzyme assembly was determined using a PHERAstar FS plate reader (BMG labtech). The fluorescein probe was measured using 485 nm excitation and 530 nm emission. The fluorescein probe was covalently linked to the N-terminal of the linear CK2β-based peptide RLYGFKIHPMAYQLQ (CK2β_pep). Inhibition was measured using 15 μM and 0 μM of the test compounds at a constant concentration of 3% DMSO. The experiments were performed in a 384 well plate with final concentrations of 450 nM CK2α_KA, 7.4 nM CK2β_pep, 350 mM NaCl, 20 mM MES pH 6.5. The plates were read after a 30 min incubation period. The experiment was ran in triplicates.

8.1.3. Isothermal Calorimetry Titration

Direct binding assay

All ITC experiments were performed at 25 °C using a MicroCal ITC-200 (GE Healthcare). CK2α_KA (20 mg/mL, 20 mM tris pH 8.0, 500 mM NaCl) was diluted in Tris buffer (200 mM, NaCl 300 mM, 10% DMSO) and concentrated to 20-50 μM. Compounds in 100× stock solutions were diluted into the same buffer. In a typical experiment CK2α (25 μM) was loaded into the sample cell and 19 injections (2 μL each) with a 2 second duration were performed at 150 second intervals. The syringe was loaded with 200-250 μM peptides or with 0.4-2 mM fragments and rotated at 750 rpm. Control titrations were performed, and the data fitting was performed with a single site binding model using Origin software.
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Competition assay

Experiments were performed at 25 °C using a MicroCal ITC-200 (GE Healthcare). CK2α (0.2 mg/mL, 20 mM Tris pH 8.0, 500 mM NaCl) was diluted in Tris buffer (200 mM, NaCl 300 mM, 10% DMSO) and concentrated to 4-6 μM. CK2β (9.3 mg/mL, 20 mM Tris pH 8.5, 500 mM NaCl) was diluted in Tris buffer (200 mM, NaCl, 300 mM, 10% DMSO) and concentrated to 40 μM. In a typical experiment, CK2α was loaded into the sample cell and 19 injections (2 μL each) with a 2 second duration were performed at 150 second intervals. The syringe was loaded with CK2β and rotated at 750 rpm. The same experiment was repeated in the presence of the peptides: compounds in 10 mM DMSO stock solutions were diluted to 1 mM and added to the solutions of CK2α and CK2β (prepared as described above) to a final concentration of 100 μM. Control titrations were performed, and the data fitting was performed with a single site binding model using Origin software.

8.1.4. Kinase assay

The kinase assays were performed using the ADP-Glo™ kinase assay kit (Promega). 50 nM CK2α_WT was incubated in the kinase reaction buffer (40 mM Tris pH 7.5, 200 mM NaCl, 20 mM MgCl₂, 0.1 mg/mL BSA, 25 μM ATP, 50 μM substrate peptide (RRRADDSDDD, Enzo Life Sciences Inc. or eIF2β peptide, MSGDEMIFDPTMSKKKKKKK), 5% (v/v) DMSO) in the presence of different concentrations of the inhibitor at 25 °C for 120 min. In the kinase assay using eIF2β substrate 50 nM CK2β_WT was added to the reaction mixture. 5 μL aliquots of the kinase reaction were quenched with 5 μL of ADP-glo™ solution. After 45 min 10 μL of the kinase detection reagent was added and maintained at 25 °C for 20 minutes. The luminescence was recorded using a PHERAstar FS plate reader (BMG LABTECH) with an integration time of 1 s. Percentage inhibition was calculated relative to a DMSO control and a baseline measurement without substrate. All measurements were performed in duplicates. The IC₅₀ curves were fitted using GraphPad Prism software.

8.1.5. X-ray crystallography

X-ray crystallography was performed by Dr Paul Brear, Department of Biochemistry, University of Cambridge.

For fragment crystallisation the following conditions were used: CK2α_KA at 5 mg/mL in 20 mM Tris, pH 8.0, 350 mM NaCl, 1 mM DTT, and 25 mM ATP was crystallised with 112.5 mM MES pH 6.5, 35% glycerol ethoxylate and 180 mM ammonium acetate in a 1:1 ratio with a total volume of 2 μL by the hanging drop vapour-diffusion method. The fragments were soaked as singletons at 2-100 mM into these crystals for 15–20 h in 107 mM MES pH 6.5, 35% glycerol ethoxylate and 1.04 M ammonium acetate after which the crystals were cryo-cooled in liquid nitrogen for data collection. CK2α_FP10 at 10 mg/mL in 20 mM Tris, pH 8.0, 500 mM NaCl, 4 mM DTT, 13 mM
ATP, 2 mM phytic acid was crystallised with 107 mM MES, pH 6.5, 29% glycerol ethoxylate, 1.04 M ammonium acetate in a 1:1 ratio with a total volume of 2 μL by the hanging drop vapour-diffusion method. The fragments were soaked into the crystals of CK2α_FP10 for 15–20 h at 100 mM in 107 mM MES pH 6.5, 29% glycerol ethoxylate and 1.04 M ammonium acetate.

Co-crystals of CK2α and P1-C5 were generated by screening CK2α_FP10 at 10 mg/mL in 20 mM Tris, pH 8.0, 500 mM NaCl and 500 μM peptide with the JCSG+ screen (molecular dimensions). Drops were set up at 0.2 μL protein solution 0.2 μL screen using the sitting drop vapour-diffusion method. Crystals were observed in a number of conditions in the JCSG+ screen. The condition that yielded the crystals from which the final data set was collected grew in 0.1 M Hepes pH 7.5, 10% (w/v) PEG 8K, 8% v/v Ethylene glycol. The crystals were cryo-cooled in liquid nitrogen in the same solution for data collection.

Crystals of P2'-C5 and P7-C5 were generated using matrix seeding from the co-crystals grown of P1-C5. Seeds of CK2α were generated using Micro seed beads (Molecular Dimensions). Co-crystals of CK2α and P2'-C5 or P7-C5 were generated by screening CK2α_FP10 at 10 mg/mL in 20 mM Tris, pH 8.0, 500 mM NaCl and 500 μM peptide with the JCSG+ screen (Molecular Dimensions) and the seed stocks obtained from P1-C5. Drops were set up at 0.2 μL protein solution + 0.2 μL screen +0.01 μL seed stock using the sitting drop vapour-diffusion method. Crystals were observed in a number of conditions in the JCSG+ screen. The condition that yielded the crystals from which the final data set was collected grew in 0.16 M calcium acetate, 0.08 M sodium cacodylate, 14.4% PEG 8K, 20% glycerol.

The crystals were cryo-cooled in liquid nitrogen in the same solution for data collection. The crystals were cryo-cooled in liquid nitrogen in the same solution for data collection.

X-ray diffraction data was collected at the Diamond synchrotron radiation source, then processed using the pipedream package by Global Phasing Ltd; structures were solved by using programs from the CCP4 package. Models were iteratively refined and rebuilt by using AutoBuster and Coot programs. Ligand coordinates and restraints were generated from their SMILES strings using the Grade software package.
Chapter 8: Biophysics Experimental

8.2. Biophysics experimental for BH3 peptides

8.2.1. Protein expression and purification of Bcl-xL

Bcl-xL protein was expressed and purified by Jha Rupam at AstraZeneca. The His6-TEV-Bcl-xL construct was expressed in *Escherichia coli* (BL21 Gold DE3) by induction with 0.2 mM IPTG for 16 h at 18 °C and purified by affinity purification followed by gel filtration. The pellets from 3 L culture were thawed and resuspended in lysis buffer (50 mM Hepes, pH 8.0, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM TCEP) supplemented with protease inhibitors (Complete-EDTA Free, Roche Applied Science), Benzonase, and 2.5 mg/mL lysozyme. The lysate was clarified by centrifugation (15000 rpm, 60 min, 4 °C) and incubated with 8 mL of Talon Superflow resin (Clontech) for 1 hour whilst rotating. The lysate was then passed through an Econocolumn (BioRad) and washed with 200 mL of lysis buffer. The protein was eluted by elution buffer (50 mM Hepes pH 8, 500 mM NaCl, 250 mM imidazole, 1 mM TCEP, 5% glycerol), and dialysed overnight against 5 L of dialysis buffer (50 mM Hepes pH 8, 300 mM NaCl, 1 mM TCEP). The dialysed protein was concentrated to 4 mL volume using centrifugal 10K MWCO concentrator (Millipore). The concentrated protein was loaded on to HiLoad 16/60 Superdex 75 PG (GE Healthcare) pre-equilibrated in the storage buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP). The fractions containing Bcl-xL protein from this size exclusion chromatography step were analysed on SDS-PAGE, pooled, snap frozen, and stored at -80 °C.

8.2.2. Surface plasmon resonance (SPR)

Biacore™ T200 (GE Healthcare) was used to conduct all experiments reported herein. Sensor surface preparation and all interaction analyses experiments were performed at 25 °C utilising a data collection rate of 10 Hz. Reagents were purchased from GE Healthcare. Running buffer containing 10 mM Hepes, pH 7.4, 150 mM sodium chloride, 1 mM DTT, 1% DMSO and 0.05% polysorbate 20 were utilised throughout surface preparation and all interaction analysis experiments. Histidine tagged Bcl-xL protein was diluted to 5 μg/mL and captured onto Sensor chip NTA via the following sequence of injections to achieve a target Bcl-xL surface density of 700 R.U. (response units). The carboxymethyl dextran surface of Sensor chip NTA was activated by injection of an aqueous solution containing 50 mM N-hydroxysuccinimide (NHS) and 200 mM N-ethyl-N’-(dimethylaminopropyl) carbodiimide (EDC). 500 μM nickel sulfate (NiSO₄) was subsequently injected for one minute and utilised to capture Bcl-xL from a 5 μg/mL solution injected for one minute. A further one-minute injection of 1 M ethanolamine, pH 8.5 was performed to deactivate any unreacted carboxymethyl esters. The flow-rate for all injections during surface derivatisation was 10 μL/min. A blank flow-cell was similarly treated with the exception of injection of Bcl-xL and served as a reference surface in the direct binding interaction analyses. Interaction analyses were performed by first equilibrating each sample within a 7-
point 2-fold peptide dilution series in the range 33.3 nM to 1.03 nM for 30 minutes during instrument start-up procedures. nM peptide concentrations were used to accommodate potential solubility issues for the panel screening assay. Running buffer samples (0 nM peptide) were injected following each cycle and later used to facilitate double-referencing procedures during data analysis. Each peptide sample was injected over each peptide surface 90 seconds at a flow-rate of 80 μL/min to monitor peptide association, and peptide dissociation was subsequently monitored for 300 seconds. Data analyses were carried out using Biacore™ T200 evaluation software v2.0 to validate assay quality, perform double-referencing (blank buffer sample and reference surface data subtraction) and evaluate steady state affinity plots. Briefly, equilibrium R.U. levels (R_{eq}) were collected 5 seconds before the end of the sample injection. R.U. levels at this time-point were plotted versus sample concentration and the resultant binding isotherm fitted using the following steady state equation:

\[ R_{eq} = \text{Conc} \times R_{\text{max}} / (\text{Conc} + K_d) \]

R_{eq} refers to the equilibrium binding response (R.U.), R_{max} is the total surface binding capacity (R.U.) and K_d is the apparent equilibrium dissociation constant, a parameter subsequently utilised to provide a relative ranking for the peptides studied in the current panel.

8.2.3. Circular dichroism experiments

CD spectra of selected peptides were recorded on an Applied Photo-physics Chirascan circular dichroism spectro-polarimeter (P9-C5 and P10) or on an AVIV 410 circular dichroism spectro polarimeter using a 1 mm path length quartz cuvette. CD measurements were performed at 298 K over a range of 185-260 nm using a response time of 0.5 s, 1 nm pitch and 0.5 nm bandwidth. The recorded spectra represent a smoothed average of three scans, zero-corrected at 260 nm and normalised against the solvent used (Mean Residue Ellipticity \( \theta_{\text{MRE}} \) is quoted in deg·cm\(^2\)·dmol\(^{-1}\)·residue\(^{-1} \) and defined as the ratio between the Molar Ellipticity and the number of residues in the peptide). Peptides were dissolved in a 1:1 mixture of MQ water and MeCN to a final concentration of 50-100 μM.

Percentage helicity was calculated based on mean residue ellipticity at 222 nm compared to the theoretical maximum helicity:

\[
\text{maximum MRE}_{222} = 40000 \times [1 - (2.5/n)]
\]

where n is the number of amide bonds, as previously reported.\(^{290} \)
CHAPTER 9:

Biology Experimental
CHAPTER 9: Biology Experimental

9.1. Biology experimental for CK2 peptides

9.1.1. Serum stability test

500 μL of PBS buffer supplemented with 20% (v/v) of human serum was allocated into an Eppendorf tube and temperature kept at 37 °C for 15 minutes before commencing the experiment. 5 μL of the peptide from 10 mM stock solution in DMSO was added. Caffeine was added as an internal standard (10 μL of a 15 mg/mL solution in MQ water). At specific intervals, 50 μL of the reaction mixture was taken and quenched with 100 μL of a 1:1 mixture of 96% Ethanol:DMSO. The suspension was spun at 13400 g for 10 minutes. 100 μL of the supernatant was analysed using C-18 HPLC with an eluting gradient 5-95% MeCN (0.05% TFA) in water (0.05% TFA) over 15 minutes (90 μL injection volume). Percentage of intact starting peptide was monitored over 24 h (calculated as the ratio of the area of the peak corresponding to the intact peptide to the area of the peak of caffeine). The experiment was performed in duplicates.

9.1.2. Tissue culture

All cell lines used were supplied by AstraZeneca cell bank as mycoplasma free. U2OS bone osteosarcoma and HCT116 colon carcinoma cells were maintained in RPMI-1640 (1x, Sigma Aldrich, R0883) supplemented with 2 mM L-glutamine (Gibco, 25030-149) and fetal bovine serum (FBS, Gibco Life Technologies, 10270-106) at a final concentration of 10%. All cells were grown at 37°C / 5% CO₂ in a humidified environment and all the assays were performed using these culturing conditions.

9.1.3. Proliferation assay

HCT116 cells were seeded at 2000 cells per well into two flat-bottomed tissue 384-well plates (PerkinElmer CellCarrier Ultra™) in a volume of 40 μL of growth medium. After 24 hours, the peptides dissolved in DMSO (10 mM) were added to the cells (to final concentrations of 100, 75, 50, 32, 25, 16, 10, 7.7, 5, 3.2, 0 μM) using HP Tecan dispenser and DMSO normalised to a final concentration of 1% (v/v). Cells were then incubated in the presence of the compounds for 4 days and the rate of growth monitored with IncuCyte Zoom™ (plate day 4). To one plate (day 0), 5 μL of Saponin (2.5% w/v in 5 mM EDTA in Tris buffer) and 10 μL of Sytox green™ reagent (2 μM Tris buffer containing 5 mM EDTA) were added after dosing. The plate was left in the dark at rt for 4 hours before imaging with Thermo Scientific™ CellInsight™ CX5 High Content Screening (HCS) Platform using a 10X lent. The same procedure was repeated for plate day 4 after 4 days of incubation.

Percentage of growth inhibition was calculated relative to DMSO controls, normalised against day 0 and GI₅₀ values were calculated using Graphpad Prism. The results are the average of at least 4 independent repeats.
9.1.4. Viability assay

HCT116 cells were seeded at 5000 cells per well into a flat-bottomed tissue 384-well plate (PerkinElmer CellCarrier Ultra™) in a volume of 40 μL of growth medium. After 24 hours, the compounds (10 mM stock in DMSO) were added to the cells (to final concentrations of 100, 75, 50, 32, 25, 16, 10, 7.7, 5, 3.2, 0 μM) using HP Tecan dispenser and DMSO normalised to a final concentration of 1% (v/v). After 4 hours, 20 μL of CytoTox-Glo™ Cytotoxicity Assay Reagent (Promega) were added to all well. After 15 minutes at room temperature, luminescence was read on a multimode plate reader (EnVision™, PerkinElmer). 40 µL of Lysis Reagent (prepared using 33 μL Digitonin in 5 mL of assay buffer) were added to all the wells, mixed, and incubated at room temperature for 15 minutes to cause lysis of all the cells. Luminescence of total dead cells was measured and used as normalisation. The results are the average of at least three independent repeats.

9.1.5. Co-localisation experiments

Confocal imaging of fixed U2OS cells was conducted using a Yokogawa Cell Voyager CV8000™ confocal microscope with a 40X water immersion objective. Images were acquired with excitation at 488, 561 and 405 nm and emissions detected with a 525/50, 600/37, 445/460 nm BP filter for the green, red and blue channel respectively. The following primary antibodies were used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier and catalogue number</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEA1</td>
<td>Abcam, ab2900</td>
<td>1:4000</td>
</tr>
<tr>
<td>Rab7</td>
<td>Abcam, ab50533</td>
<td>1:4000</td>
</tr>
<tr>
<td>LAMP1</td>
<td>Cell Signalling, 9091</td>
<td>1:4000</td>
</tr>
<tr>
<td>TGN46</td>
<td>Abcam, ab50595</td>
<td>1:750</td>
</tr>
<tr>
<td>ZFPL1</td>
<td>Sigma, HPA014909</td>
<td>1:500</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Invitrogen, PA5-34665</td>
<td>1:750</td>
</tr>
</tbody>
</table>

Secondary antibodies goat anti-rabbit IgG (H+L) AlexaFluor 568 and goat anti-mouse IgG (H+L) AlexaFluor 568 were used at a working concentration of 1:500. Hoechst nuclei stain was used to a working concentration of 1:5000. FITC-labelled peptides were used to 1.25 μM final concentration.

U2OS cells were seeded at 2500 cell per well into a flat-bottomed tissue 384-well plate (PerkinElmer CellCarrier Ultra™) in a volume of 40 μL of growth medium and cultured for 24 h before commencing the experiment. After 24 hours, FITC-labelled compounds dissolved in
DMSO (1 mM) were added to the cells using HP Tecan™ dispenser and DMSO normalised to a final concentration of 0.3% (v/v). Cells were fixed after 360, 180, 120, 60, 30, 15, and 5-minute incubation using 40 μL of 8% PFA (final PFA concentration 4%). Cells were fixed for 15 minutes, washed with PBS (3x) and blocked with 3% BSA + 0.1 % Triton X-1000 for 5 minutes. The blocking solution was then removed and 40 μL of the primary antibodies in BSA added. The plate was stored in the dark, at 4 °C overnight. The primary antibodies were washed with PBS (3x) and 40 μL of the secondary antibodies and Hoechst nuclei stain in BSA added. The plate was kept at 4 °C for 1 hour, washed with PBS (3x) and imaged as described above. Images were analysed using Fiji (ImageJ) software.
9.2. Biology experimental for BH3 peptides

Ethical approval was obtained from the Faculty Research Ethics Committee at Anglia Ruskin University, Cambridge, UK where the experiments were carried out. Unless otherwise stated, the experiments were performed on no fewer than three separate occasions using platelets from different donors. Data were analysed using AccuriC6 BD Software and GraphPad Prism.

9.2.1. Preparation of washed platelet suspension

Human blood was collected from healthy volunteers, who had not taken medication for two weeks, following informed consent in accordance with the Declaration of Helsinki. Blood was collected into 11 mM sodium citrate and washed platelets were prepared as follows: Platelet-rich plasma (PRP) was obtained by centrifugation (240 g, 15 min). Prostaglandin E1 (PGE$_1$, 2 µM) was added to prevent premature platelet activation, and the plasma was centrifuged at 640 g for 15 min. Platelets were suspended in calcium free Tyrode’s buffer (CFT buffer, containing: 140 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM Glucose, 0.42 mM NaH$_2$PO$_4$, 12 mM NaHCO$_3$, titrated to pH 7.4 with NaOH) to a final concentration of 2x10$^8$ platelet/mL and rested at 37 °C for 1 hour prior to experimentation.

9.2.2. Flow cytometry

Treated washed platelet suspensions (1x10$^7$/mL) were analysed by flow cytometry using a C6 Accuri Flow Cytometer (Beckton Dickinson, UK), in the presence or absence of Annexin-V-PE (BD Biosciences, UK), anti-PAC-1 (BD Biosciences, UK), anti-CD62P (BD Pharmingen, UK), or anti-CD63 (BD Pharmingen, UK). Peptides were used to a final concentration of 10 µM (from 1 mM DMSO stock solutions). At least 10,000 events were acquired per experiment. In all the experiments, the entire platelet population was primarily gated by FSC. Untreated platelets (no fluorescent markers, no compounds) were analysed by flow cytometer on the laser of interest and the measurement used as zero. The shift of the peak of treated platelets towards the right was normalised against untreated platelets (Figure 72).

![Figure 72](image)

**Figure 72** – Example of flow cytometry data normalisation. a) The fluorescence associated with untreated platelets is used to set the threshold for future measurements. b) After treatment, the platelet population that gives a shift towards the right of the threshold in the fluorescent signal is considered as positive platelets.
9.2.2.1. Peptide uptake experiments in a washed platelet suspension

250 μL of washed platelet suspension was incubated with 2.5 μL of the fluorescently labelled peptide. The suspension was kept in the dark at 37 °C and measurements taken at the designed time points (1, 2, 3 hours). For real time measurements, the peptides were added to the 250 μL of washed platelet suspension after events had been recorded for 1 minute and then event were monitored for a further 4 minutes.

9.2.2.2. Peptide uptake experiments in platelet reach plasma (PRP)

The PRP was rested for 30 minutes prior experimentation. 120 μL of PRP was added to 1.2 μL of the fluorescently labelled peptide (1 mM DMSO stock) and incubated in the dark at 37 °C for 1 h prior analysis by flow cytometry.

9.2.2.3. PS exposure and activation markers

PS exposure was assessed after incubation of 50 μL of washed platelet suspensions at 37 °C in 445 μL of Tyrode’s solution (pH 7.4) with the peptide (5 μL of 1 mM stock solution in DMSO). At the designed time point 25 μL of the suspension was added to 25 μL of Annexin V (10% Annexin V in Tyrode’s solution) under the protection from direct light and analysed by flow cytometry.

Platelet activation markers (PAC-1, CD62P and CD63 binding) were assessed after incubation of 3 μL of washed platelet suspensions at 37 °C in 105 μL of CFT buffer (pH 7.4) with 1.2 μL of the desired antibody and 1.1 μL peptide (1 mM DMSO stock). The suspensions were analysed by flow cytometry.

9.2.3. Light Transmission Aggregometry

Platelet aggregation was monitored using an AggRam™ aggregometer (Helena Biosciences, UK). 250 μL of washed platelet suspensions (1x10⁸/mL) were stimulated with 10 μM peptide under stirring conditions at 37 °C for 15 minutes and the percentage aggregation was acquired from the aggregation traces using proprietary software (Helena Biosciences, UK).

9.2.4. Confocal microscopy of live platelets

Peptides were used to a final concentration of 10 μM. Confocal imaging of live washed platelets suspensions (2x10⁸/mL) were conducted using a Ziess 510 confocal microscope with a Plan- APOCHROMAT 63x/1.4NA oil immersion objective, and a confocal aperture of 60 μm. Images of TAMRA-labelled model peptide stained platelets were acquired after 1-hour incubation using with excitation at 565 nm and emission was detected with a 560-615 nm BP filter. Images of
FITC-peptide stained platelets were acquired after 15-minute incubation\textsuperscript{38} using excitation at 488 nm and emission was detected with a 505-550 nm BP filter.

9.2.5. Serum stability test

One mL of RPMI-1640 media supplemented with 25\% (v/v) of human serum was allocated into an Eppendorf tube and temperature kept at 37 °C for 15 minutes before commencing the experiment. 40 μL of 10 mM peptide in DMSO were added to make a final peptide concentration of 100 μg/mL. At specific intervals, 100 μL of the reaction mixture was taken and quenched with 200 μL of a 1:1 mixture of 96\% Ethanol:DMSO. The suspension was cooled to 4 °C for 15 minutes and then spun at 13400 g for 4 minutes.\textsuperscript{283} Caffeine (0.5 μL of 7 mg/mL solution in MQ water) was then added to 100 μL of the supernatant and used as a reference. The supernatant was analysed using C-18 HPLC with an eluting gradient 5-95\% MeCN (0.05\% TFA) in water (0.05\% TFA) over 15 minutes. Percentage of intact starting peptide was monitored over time (calculated as the ratio of the area of the peak corresponding to the intact peptide to the area of the peak of caffeine). The results are the average of three independent repeats.

\textsuperscript{38} Difference in incubation times are due to the fact that the model peptides do not activate platelets while the BH3 peptides do. Therefore, to avoid complication in the imaging due to platelet aggregation or activation images were acquired after 15 minutes incubation.
CHAPTER 10:
Bibliography


H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. Degrado and D.


14143.


2012, **55**, 3923–3933.


Appendices
A.1. $^1$H, $^{19}$F and $^{13}$C NMR spectra
A.2. HPLC traces of selected compounds

CK2 peptides

Pc linear

Pc cyclic

Pc cyclic short

P0-C1

P1-C2

P1-C3
BH3 peptides

**P9-C5**

**P9-C7**

**P9-F4C5**

**P9-F5C5**

**P10**

**FITC-P10**

**SAHBa**

**FITC-SAHBa**

**FITC-P9-F4C5**

**FITC-P9-F5C5**
A.3. Structures of additional fragments docked
A.4. Selected ITC binding curves

P1-C5
$K_d = 460 \text{ nM}$

Pc
$K_d = 1 \mu M$

P1-C6
$K_d = 58 \mu M$

P2'-C5
$K_d = 2 \mu M$
\[ K_d = 56 \text{ } \mu\text{M} \]

\[ K_d = 800 \text{ } \text{nM} \]
$K_d = 9 \text{nM}$

$K_d = 44 \text{μM}$
## Thermodynamic data

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<th>Peptide</th>
<th>$K_D$ μM</th>
<th>$\Delta H$ cal/mol</th>
<th>$\Delta S$ cal/mol/deg</th>
<th>Stoichiometry</th>
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<tr>
<td>Pc</td>
<td>1</td>
<td>$-1.483E4 \pm 487.7$</td>
<td>$-22.7$</td>
<td>$1.15 \pm 0.0256$</td>
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<tr>
<td>P1-C5</td>
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<td>58</td>
<td>$-1.452E4 \pm 2.06E4$</td>
<td>$-29.3$</td>
<td>$1.45 \pm 1.25$</td>
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<td>P2'-C5</td>
<td>2</td>
<td>$-2.035E4 \pm 818.0$</td>
<td>$-42.3$</td>
<td>$0.807 \pm 0.0239$</td>
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<td>$-2.244E4 \pm 5273$</td>
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<td>$0.989 \pm 0.00421$</td>
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<td>P7</td>
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<td>$-4.372E4 \pm 4711$</td>
<td>$-126$</td>
<td>$1.00 \pm 0$</td>
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<tr>
<td>P7-F1C5</td>
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<td>$-2.712E4 \pm 826.6$</td>
<td>$-61.4$</td>
<td>$0.864 \pm 0.0188$</td>
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**P7-F1C5**

$K_D = 170 \text{ nM}$

**P7-C5**

$K_D = 150 \text{ nM}$
### Unsuccessful crystallisation attempts of CK2 peptides and CK2α

<table>
<thead>
<tr>
<th>Crystallisation technique</th>
<th>Protein construct</th>
<th>Conditions</th>
<th>Results</th>
</tr>
</thead>
</table>
| Soaking                   | WT, K74A          | No ATP     | Crystals of the protein from Brear *et al.*
| Soaking                   | WT, K74A          | No ATP     | Crystals of the protein from Brear *et al.*
| Soaking                   | WT, K74A          | No ATP     | Crystals of the protein from Brear *et al.*
| Co-crystallisation        | WT, K74A          | Conditions from Raaf *et al.* | No crystals |
| Co-crystallisation        | WT, K74A          | JCSG+™ screen | No crystals |
| Co-crystallisation        | WT, K74A          | JCSG+™ screen | No crystals |
| Co-crystallisation        | K74A              | JCSG+™ screen | No crystals |
| Co-crystallisation        | FP_10, K74A, WT   | JCSG+™ screen | No crystals |
| Co-crystallisation        | FP_10, K74A, WT   | Proplex™ screen | No crystals |
A.6. Papers published on this work