DISRUPTION OF CLATHRIN MEDIATED-ENDOCYTOSIS THROUGH SMALL MOLECULE INHIBITION OF DYNAMIN AND CLATHRIN

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A thesis submitted in fulfilment of the requirements for the award of the degree Doctor of Philosophy from the University of Newcastle

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DECLARATION

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ABSTRACT

Since the first evidence of clathrin-mediated endocytosis was reported almost 50 years ago, extensive research has been devoted to understanding the mechanisms of this process. Whilst molecular tools have contributed significantly to elucidating the mechanism of CME and the protein interaction network that underlies it, these tools suffer from pitfalls that limit their utility. Chemical inhibitors of endocytosis have been proposed as an attractive alternative to the current methods for disrupting protein function, but despite the extensive structural and biochemical knowledge about CME that is available, the development of chemical tools to interfere with this process is still in its infancy. The development of novel inhibitors to use in conjunction with existing inhibitors will assist in the molecular and functional dissection of the endocytic pathway, resulting in an increased understanding of many physiological phenomena and disease processes that rely on this pathway. Such understanding may also contribute to the rational discovery and development of novel, targeted therapeutic agents.

This thesis focused on the development of novel series of compounds that specifically targeted the endocytic proteins dynamin and clathrin. Parent compounds for further development were sourced from both virtual screening and high-throughput screening strategies. Using an approach combining focused library development and molecular modelling-guided drug design, preliminary structure-activity profiles were generated, and a number of noteworthy analogues were identified. The cellular effects of selected dynamin and clathrin inhibitors were also investigated, specifically their effects on clathrin-mediated endocytosis and cell proliferation.

The 1,4-benzoquinone derivative 2,5-bis(2-carboxyanilino)-1,4-benzoquinone (31-1) was selected as a parent compound for the development of novel dynamin inhibitors based on the benzoquinone scaffold. Compound 31-1 was found to possess a good level of dynamin inhibitory activity (IC$_{50}$: 22 ± 5 μM), and was predicted to bind to the GTP-binding site of dynamin, and possess a GTP-competitive mechanism of action. Examination of the binding conformation of 31-1 revealed that the inhibitory activity of this compound is due to a favourable hydrogen bonding, electrostatic and hydrophobic interactions with the binding site. The synthesis of four discrete analogue libraries revealed a number of structure-activity relationships, including a preference for polar substituents that are capable of increased electrostatic and hydrogen bonding interactions with the binding site. The most potent and noteworthy analogue in this series was 31-7, which exhibited a dynamin I inhibitory activity 4-
fold greater than that of the parent compound. Further biological evaluation of 31-7 revealed that this compound could inhibit RME in cells, presumably via inhibition of dynamin.

Through the synthesis of discrete analogue libraries based on 4-amino-3-sulfo-N-(2-hydroxyethyl)-1,8-naphthalimide (D1), a family of naphthalimides with dynamin inhibitory activity were developed. Biological evaluation of these analogue libraries allowed for the development of a preliminary structure-activity relationship profile for dynamin inhibitors based on the naphthalimide scaffold. Of the 61 analogues synthesised and subjected to biological evaluation, 21 were found to exhibit dynamin inhibitory activities comparable to, or better than, the parent compound. The most potent analogue, L1, exhibited a dynamin inhibitory activity 15-fold greater than the parent compound. The effects of selected inhibitors on transferrin uptake were evaluated, revealing that dynamin inhibitors based on the naphthalimide scaffold can inhibit endocytosis, provided the inhibitor is sufficiently lipophilic to enter the cell.

Following a HTS and scaffold simplification approach, C12 was identified as an inhibitor of the interaction between clathrin-TD and amphipysin B/C (IC50: 18 μM). Co-crystallisation of C12 in complex with the clathrin-TD revealed that the binding site of C12 largely overlaps that used by clathrin-box-motif-containing accessory proteins. Synthesis of two discrete analogue libraries based on C12 revealed that the presence of a 3-sulfonate moiety is pivotal to activity, whilst hydrophobic substituents on the benzyl imide moiety improved activity due to increased hydrophobic interactions with the binding site. Examination of the cellular effects of selected inhibitors revealed an inability to inhibit RME, and this was attributed to poor membrane permeability. However, intracellular application of C12 by microinjection resulted in an inhibition of SVE, indicating that inhibition of clathrin-TD can result in inhibition of clathrin-mediated endocytosis.
ABBREVIATIONS

Ala  Alanine
AP180 Assembly Protein 180
AP2  Adaptor Protein 2
Arg  Arginine
Asn  Asparagine
Asp  Aspartic Acid
BAR  Bin-Amphiphysin-Rvs
CBM  Clathrin-Box Motif
CCV  Clathrin-Coated Vesicle
CHC  Clathrin Heavy Chain
CHC-TD  Clathrin Heavy Chain – Terminal Domain
CME  Clathrin-Mediated Endocytosis
Cys  Cysteine
DEPTQ Distortionless Enhancement by Polarisation Transfer Quaternary
EGF  Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
EM  Electron Microscopy
Eps15 Epidermal Growth Factor Receptor Substrate 15
FCHo F-BAR Domain-containing Fer/Cip4 Homology Domain-only Protein
GAP  GTPase Activating Protein
GDP  Guanosine Diphosphate
GED  GTPase Effector Domain
Gln  Glutamine
Glu  Glutamic Acid
Gly  Glycine
GPCR G-Protein Coupled Receptor
GTP  Guanosine-5’-triphosphate
GTPase Guanosine Triphosphatase
hDynI Human Dynamin I
His  Histidine
HMBC Heteronuclear Multiple Bond Correlation
HSC70 Heat Shock Cognate 70
HSQC  Heteronuclear Single Quantum Coherence
HTS   High-Throughput Screening
Ile   Isoleucine
kDa   Kilodalton
LDL   Low-Density Lipoprotein
LDLR  Low-Density Lipoprotein Receptor
Leu   Leucine
Lys   Lysine
Met   Methionine
NMR   Nuclear Magnetic Resonance
NTD   N-Terminal Domain
PDB   Protein Data Bank
PH    Pleckstrin Homology
Phe   Phenylalanine
Pi    Inorganic Phosphate
PIP2  Phosphatidinyl-4,5-bisphosphate
PPI   Protein-Protein Interaction
PRD   Proline-Rich Domain
Pro   Proline
RME   Receptor-Mediated Endocytosis
RTIL  Room-Temperature Ionic Liquid
SAR   Structure-Activity Relationships
Ser   Serine
SH3   Src Homology 3
SV    Synaptic Vesicle
SV2A  Synaptic Vesicle Glycoprotein 2A
SVE   Synaptic Vesicle Endocytosis
Tf    Transferrin
Tf-A594 Alexa Fluor-594 Conjugated Transferrin
TfR   Transferrin Receptor
Thr   Threonine
Trp   Tryptophan
Tyr   Tyrosine
Val   Valine
VLS   Virtual Library Screening