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Novel payloads for immunotoxin-based treatment of neuroblastoma

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Abstract

Neuroblastoma is the most common form of extracranial solid tumour in childhood and the second biggest cause of cancer-related deaths in infants. Despite intensive, multi-modal treatment of advanced stage disease, prognosis is poor and relapse is common. This means that alternative forms of treatment are urgently needed.

Targeted toxins are cytotoxic enzymes fused to a targeting ligand or antibody that bind to a receptor or antigen highly expressed on the cancer cell surface. Upon binding, the toxin is internalised and its cytotoxic activity leads to cell death. All currently used toxins function via the inhibition of protein synthesis, making them highly potent in both healthy and transformed cells. Low-level expression of target receptor, or non-specific uptake into healthy cells has caused dose-limiting side effects in all targeted toxins tested to date which has severely restricted their use in cancer treatment.

In this thesis, novel protein delivery techniques were used to investigate the use of two enzymes, Burkholderia lethal factor 1 (BLF1) and botulinum neurotoxin type C (BoNT/C) protease, as possible alternative payloads for targeted toxin therapy in the treatment of neuroblastoma. BLF1 inhibits translation initiation by inactivation of eIF4A, and BoNT/C protease disrupts important membrane recycling events by cleavage of SNARE proteins. Both of these cytotoxic mechanisms show a degree of selectivity towards neuroblastoma cells. Future targeted toxins based on these enzymes may therefore have higher specificity towards neuroblastoma cells than conventional enzymes, leading to an increased therapeutic window and decreased side effects.

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List of abbreviations

β-TrCP1	β-transducin repeat-containing protein 1
13-cis-RA	13-cis-retinoic acid
4E-BP	eIF4E binding protein
A site	aminoacyl site
ADC	Antibody drug conjugate
ADCC	Antibody dependent cell-mediated cytotoxicity
ALK	Anaplastic lymphoma kinase
ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukaemia
ASO	Anti-sense oligonucleotide
AT-RA	All-trans retinoic acid
AUF-1	Hypoxia-inducible factor 1
AURKA	Aurora A kinase
BDNF	Brain derived neurotrophic factor
BET	Bromodomain and extra terminal
BIG	Botulism immunoglobulin
BLF1	Burkholderia lethal factor 1
BoNT	Botulinum neurotoxin
BSA	Bovine serum albumin
CCK-8	Cell counting kit-8
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CDK	Cyclin dependent kinase
CGRP	calcitonin gene-related peptide
Chk1	Checkpoint kinase 1
DMDA-PatA	des-methyl, des-amino pateamine A
DMEM	Dulbecco's modified Eagle medium
DT	Diphtheria toxin
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
eRF	eukaryotic release factor
E site	Exit site
EGFR	Epithelial growth factor receptor
ER	Endoplasmic reticulum
ERD2	Endoplasmic reticulum protein retention receptor 2
FBS	Foetal bovine serum
GEF	Guanine nucleotide exchange factor
GLI1	Glioma-associated protein 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GRSF-1	G-rich RNA sequence binding factor
GSK3	Glycogen synthase kinase
HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
ICC	Immunocytochemistry
IFP	Interstitial fluid pressure
IGF1	Insulin growth factor 1
IL-2	Interleukin 2
INSS	International neuroblastoma staging system
INRG	International Neuroblastoma Risk Group
IRES	Internal ribosome entry site

ITAF	IRES trans-activating factor
LcTd	Light chain and translocation domain
LF3000	Lipofectamine 3000
mChe	mCherry
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
Max	Myc-associated factor X
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2
MEM	Minimum essential media
MHC	Major histocompatibility complex
Mnk	MAPK-interacting kinases
MSLN	Mesothelin
Munc18-1	Mammalian uncoordinated-18-1
NAD+	Nicotinamide adenine dinucleotide
NEAA	Non-essential amino acids
NF-κB	Nuclear-factor-κB
NSF	N-ethylmaleimide-sensitive factor
P site	peptidyl site
P/S	Penicillin/streptomycin
PABP	Poly(A) binding protein
PatA	Pateamine A
PBS	Phosphate buffered saline
PDCD4	Programmed cell death 4
PE	Pseudomonas exotoxin A
PE38	Truncated version of pseudomonas exotoxin A
PEGPH20	Pegylated hyaluronidase
Pgp	Permeability-glycoprotein
PI3K	Phosphoinositide 3-kinase
PI	Propidium iodide
PIC	Pre-initiation complex
PSF	Polypyrimidine tract binding protein-associated splicing factor
PSG	Polysialogangliosides
rRNA	ribosomal RNA
RAR	Retinoic acid receptor
Ras	Rat sarcoma
Rbd	Receptor binding domain
RCA	Ricinus agglutinin
RI	RNase inhibitor protein
RIM	Rab3 interacting molecule
RIP	Ribosome inactivating protein
RIT	Recombinant immunotoxin
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SM	Sec1/Munc18-like
SNAP	Soluble NSF attachment protein
SNAP25	Synaptosomal-associated protein of 25kDa
SNARE	Soluble *N*-ethylmaleimide sensitive factor attachment protein receptor
SV2	Synaptic vesicle protein 2
tRNA	transfer RNA

tRNAi	Initiator transfer RNA
T58	Threonine 58
TAA	Tumour associated antigen
TC	Ternary complex
Td	Translocation domain
TERT	Telomere reverse transcriptase
TIF1A	RNA polymerase I transcription factor
TrkB	Tropomysin receptor kinase B
UGT1A	UDP glucuronosyltransferase
VAMP	Vesicle associated membrane protein
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VLS	Vascular leak syndrome
WB	Western blot
YB-1	Y-box binding proteins

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All other work and experiments were carried out by the author.

Chapter 1: Introduction

1.1 Neuroblastoma

Neuroblastoma is the most common form of extra-cranial solid tumour in childhood and accounts for 6-10% of all childhood cancers and 15% of childhood cancer mortalities in the United States (Maris, Hogarty et al. 2007). It is a predominantly paediatric disease, with approximately 30% of cases occurring in the first year of life and around half of newly diagnosed patients between 1-4 years of age (Gurney, Severson et al. 1995). Neuroblastoma arises when sympaticoadrenal precursor cells found in the developing neural crest fail to differentiate into mature neurons and initiate uncontrolled proliferation (Jiang, Stanke et al. 2011). Although there have been many advances in treatment strategies for neuroblastoma, it remains a complex and unpredictable illness, with poor overall outcome in advanced-stage disease.

1.1.1 Disease staging and classification

A defining feature of neuroblastoma is its extreme level of heterogeneity and broad spectrum of clinical behaviour. The clinical course of neuroblastoma can vary considerably from spontaneous regression into a differentiated, benign state to aggressive, metastatic disease (Brodeur 1995). The heterogeneity of this disease makes classification and prediction of neuroblastoma behaviour a challenge.

The International Neuroblastoma Staging System (INSS) was established in 1988 and divides the disease into different stages dependent upon the level of surgical resection of the tumour, invasiveness into lymph nodes and other parts of the body, and the location of the primary tumour in relation to the midline (Brodeur, Seeger et al. 1988). Stage 1 is a tumour that is localised to the area of origin, most commonly in the adrenal gland, although tumours can originate anywhere along the developing sympathetic axis. Stage 2A is a tumour that has been incompletely removed by surgery, but has not spread to lymph nodes. Stage 2B is a single tumour that has spread to regional lymph nodes without crossing the midline. Stage 3 is an unresectable tumour with spreading to lymph nodes across the midline. Stage 4 is metastatic disease, where metastases can be found in the lymph nodes, skin, liver, bones and bone marrow. Finally, Stage 4S is a special stage designated for infants under the age of 12 months with a tumour that has not crossed the midline and has limited spreading to the skin, liver and bone marrow. This stage is considered non-high risk. The INSS is a useful system to give an idea of disease progression; however further factors such as age and biological features of the tumour need to be taken into account for accurate assessment of risk. For

example, infants under the age of 18 months generally have a good chance of survival, even with stage 4 cancer (London, Castleberry et al. 2005).

Risk stratification of neuroblastoma can vary considerably around the world, which is why the International Neuroblastoma Risk Group (INRG) classification system was established in 2009. For this system, event free survival was looked at in 8800 patients from the USA, Europe, Australia and Japan between 1990 and 2002. Stage, age, level of tumor differentiation, the status of the MYCN oncogene, chromosome 11q status, and DNA ploidy are all taken into account for stratification into very low, low, intermediate and high risk (Cohn, Pearson et al. 2009). Accurate risk assessment is vital, as treatment varies dramatically from observation and surgery in low risk patients to intensive multimodal regimens combining surgery, chemotherapy and radiotherapy in high risk patients (Hara 2012). Giving the right treatment is important for achieving a balance between efficacy and debilitating side effects which often accompany chemo and radiotherapy.

1.1.2 Molecular pathology

Several genomic abnormalities are common in neuroblastoma and can be used as prognostic markers. For instance, loss of heterozygosity on chromosome 1 occurs in 70% of tumours and correlates strongly with poor outcome (Gilbert, Feder et al. 1984). Deletions on chromosome 11q and 14q and gains on chromosome 17q have also been found to be clinically significant (Gilbert, Feder et al. 1984, Srivatsan, Ying et al. 1993). Despite the knowledge of these chromosomal aberrations, the corresponding genes that cause the observed phenotypes are still not fully understood (Nicolai, Pieraccioli et al. 2015).

One of the first discovered and perhaps most important known genetic changes in neuroblastoma is amplification of the proto-oncogene MYCN. MYCN amplification is found in around 25% of tumours and is a strong marker of advanced stage and rapid disease progression (Brodeur, Seeger et al. 1984). MYCN is a member of the Myc gene family and encodes a helix-loop-helix leucine zipper transcription factor that forms a heterodimer with myc-associated factor X (Max) and binds to E-Box motifs located in the promoter regions of many different target genes, causing an upregulation of transcription (Grandori and Eisenman 1997). MYCN expression is highly restricted and limited to the developing embryonic nervous system (Luscher and Larsson 1999). Amplification of MYCN is found in many late stage neuroblastomas and is known to drive cell proliferation, angiogenesis and metastasis, as well as contributing to chemo-resistance (Buechner and Einvik 2012). The level of MYCN amplification appears to be bi-modal with copy numbers ranging from 3- to 10-fold in some patients and as high as 100- to 300-fold in others (Brodeur, Seeger et al. 1984). Due to its clear

contribution to pathogenesis, a lot of work has been carried out in elucidating downstream targets of MYCN and their role in tumour progression. For instance, MYCN is known to promote cell survival and proliferation by directly upregulating expression of the p53 inhibitor mouse double minute 2 (MDM2) (Slack, Chen et al. 2005). p53 is a tumour suppressor that is considered a master regulator of various cellular processes and is necessary for arresting the cell cycle or inducing apoptosis after DNA damage (Fridman and Lowe 2003). It is also thought that MYCN can increase expression of cell cycle regulators such as Cyclin dependent kinase 4 (CDK4) to drive cell cycle progression (Bell, Lunec et al. 2007). MYCN contributes to a number of other processes such as cell invasion via upregulation of matrix metalloproteases and focal adhesion kinase (Beierle 2011), and cell survival by constitutive activation of pro-survival signalling cascades such as tropomyosin receptor kinase B (TrkB) (Brodeur, Minturn et al. 2009). MYCN has also been shown to cause a large increase in the levels of protein synthesis machinery that is necessary for increased cell growth and division (Boon, Caron et al. 2001). The wide and diverse number of biological processes that MYCN influences makes it a key marker of advanced stage neuroblastoma and poor prognosis.

As well as MYCN, other markers have been identified that play an important role in tumour development. For instance, mutations in Anaplastic Lymphoma Kinase (ALK) are observed in around 8% of patients, and germline mutations in ALK show particular importance in familial cases of neuroblastoma (Bresler, Weiser et al. 2014). ALK is a cell surface receptor tyrosine kinase that is usually expressed in the embryonic and neonatal brain (Iwahara, Fujimoto et al. 1997). Mutations can cause single substitutions of amino acids in the tyrosine kinase domain that lead to constitutive activation of the receptor, independent of ligand binding (Chen, Takita et al. 2008). Amplification of the ALK gene has also been found to be a contributing factor in some cases. Activation of ALK in neuroblastoma leads to dysregulated mitogen-activated protein kinase (MAPK) signalling and an increase in cell growth and survival (Barone, Anderson et al. 2013).

Another marker that has recently been identified in high risk neuroblastoma is genetic rearrangement and activation of telomerase reverse transcriptase (TERT) (Valentijn, Koster et al. 2015). TERT is the catalytic subunit of the telomerase enzyme, which is involved in the lengthening of telomeres (Kirkpatrick and Mokbel 2001). Telomeres are long segments of TTAGGG nucleotide repeats that act as a cap on the end of chromosomes to prevent deterioration. Telomere length has been found to be important in cell aging, and it has been shown that over activation of telomerase is a contributor to cell immortality and division (Sundin and Hentosh 2012). Rearrangement of the TERT gene is found in approximately 30% of high risk patients and is mutually exclusive to MYCN amplification. This suggests that TERT is an important contributor to disease progression and a key

marker for a subgroup of high risk patients (Valentijn, Koster et al. 2015). Although TERT rearrangements are absent in MYCN amplified neuroblastoma, TERT activation is nevertheless higher in MYCN amplified high risk patients compared to the low risk cohort. This is because MYCN is also a known transcriptional activator of TERT, which shows the importance of telomere length in this disease.

Loss of function mutations or deletion of the RNA helicase ATRX has also been shown to be an important marker for poor prognosis, especially in older age groups (Cheung, Zhang et al. 2015). Interestingly, loss of function of ATRX is only found in high risk patients that lack MYCN amplification and are TERT normal (Valentijn, Koster et al. 2015). ATRX is also associated with telomere length, as studies have shown that it represses alternative lengthening of telomeres (ALT) (Napier, Huschtscha et al. 2015). ALT, as the name suggests, is a mechanism of telomere lengthening that occurs independently of telomerase, and is thought to be carried out via homologous recombination-mediated DNA replication. This further highlights the importance of telomere length in neuroblastoma.

Despite the fact that genetic mutations in neuroblastomas are relatively low compared to other solid cancers, identification of key mutations in high risk disease shows the critical roles they play in disease progression. Identification of these mutations may open up avenues for possible targeted therapies.

1.1.3 Current Treatments

Current treatment for neuroblastoma varies considerably depending upon the risk stratification. Patients with low risk neuroblastomas will often be placed under observation until spontaneous regression of the tumour occurs, or completely cured with surgery (Davidoff 2012). Patients with intermediate risk will be subjected to surgical intervention as well as moderate levels of chemotherapy (Haase, Perez et al. 1999). Survival rates for low and intermediate risk neuroblastomas are very good, with over 90% of low risk patients and 70-90% of intermediate risk patients being completely cured. Research into treatment of these groups is now focusing on reducing therapies whilst maintaining efficacy (Baker, Schmidt et al. 2010). Although, great progress has been made in the treatment of low and intermediate risk disease, treatment of high risk disease has remained a stumbling block and patients are faced with only a 40-50% chance of long-term, event free survival (Castel, Segura et al. 2013).

Due to the fact that high risk neuroblastoma is a systemic disease, the use of localised therapies like surgical resection and focal radiotherapy are limited, which means that chemotherapy is the main

modality used for the treatment of neuroblastoma (Hara 2012). However, surgery and radiation therapy are used to help de-bulk the primary tumour (Wagner and Danks 2009). Treatment consists of induction, consolidation and maintenance phases (Maris 2010). The induction and consolidation phases aim to de-bulk and reduce the tumour burden as much as possible; and the maintenance phase is employed to prevent relapse following remission. Conventional chemotherapeutic agents such as vincristine, a microtubule inhibitor, and cyclophosphamide, an alkylating agent, show efficacy in the early phase of treatment, but relapse occurs in almost all patients after 3-4 months (Hara 2012). This suggests that neuroblastoma acquires chemo resistance faster and at a higher rate than other paediatric cancers. Other chemotherapeutic agents that have shown efficacy include the platinum containing compound cisplatin, the topoisomerase II inhibitor doxorubicin, and more recently the topoisomerase I inhibitor topotecan (Shafford, Rogers et al. 1984, Saylors, Stine et al. 2001). All of these compounds function by interfering with DNA replication. A lot of trials have been carried out to find the most effective combination and dosage of different chemotherapeutic agents. Results have shown that to get the best response rate, high dosages with short intervals are necessary. This does however lead to increased side effects, necessitating the use of stabilising treatments such as haematopoietic stem cell transplant to replenish white blood cells (Berthold, Boos et al. 2005). Although high dose, myeloablative therapy has helped to increase response rates and event free survival, the overall survival rate remains poor due to acquisition of drug resistance and the inability to clear minimal residual disease before relapse (Morgenstern, Baruchel et al. 2013, Yalçin, Kremer et al. 2016). As mentioned previously, resistance to conventional chemotherapeutic drugs in neuroblastoma is high which necessitates the use of alternative forms of treatment.

Retinoids are derivatives of vitamin A that have been shown to induce differentiation in neuroblastoma cells. They are increasingly used in the maintenance phase of therapy to help prevent relapse during minimal residual disease (Reynolds, Matthay et al. 2003). Retinoid compounds that have been tested in neuroblastoma include all-trans retinoic acid (AT-RA) and 13-cis-retinoic acid (13-cis-RA). 13-cis-RA is predominantly used as it shows greater stability and more sustained drug levels in trials (Reynolds and Lemons 2001). High dose, pulse treatment with 13-cis-RA after chemotherapy treatment has been shown to significantly increase event free survival (Matthay, Villablanca et al. 1999). *In vitro* studies demonstrate that RA is able to rapidly induce differentiation, leading to arrest of cell growth and down-regulation of MYCN, in different cell lines (Thiele, Reynolds et al. 1985). The mechanisms by which RA induces differentiation is still not clear, although it is partly due to activation of RA receptors (RARs) or retinoid X receptors (RXRs). RARs and RXRs are transcription factors that contain discrete RA and DNA binding domains (le Maire and Bourguet 2014). Overexpression of these receptors either naturally or artificially *in vitro* leads to an

increase in sensitivity to RA (Cheung, Hocker et al. 1998). Although the use of retinoic acid has shown benefit, resistance to this form of treatment and relapse is still common, meaning that other forms of treatment are necessary. There is promise in other, synthetic retinoids such as 4-HPR, which has been shown to cause cytotoxicity and apoptosis instead of differentiation, even in retinoic acid resistant tumours (Reynolds, Matthay et al. 2003). The exact mechanism by which 4-HPR functions is also not fully understood, although it has been shown to cause a release of the pro-apoptotic molecule ceramide, as well as generating reactive-oxygen species (Maurer, Metelitsa et al. 1999). Clinical trials of 4HPR have shown promising results, although low bioavailability remains an issue (Villablanca, London et al. 2011).

Therapeutic monoclonal antibodies are becoming an increasingly attractive form of treatment in the cancer field. The ganglioside GD2 is a tumour associated antigen (TAA) that is highly expressed on a range of different cancers and has relatively restricted expression in healthy tissue (Yang and Sondel 2010). Anti-GD2 antibody has now been incorporated into the maintenance phase of the treatment regimen for high risk neuroblastoma and has helped to improve the rate of event free survival. Anti-GD2 antibodies bind to cancer cells leading to activation of the immune system and induction of cell death (Croce, Corrias et al. 2015). This can happen via antibody dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) (Stevenson 2014). During ADCC, the antibody binds to the TAA and then recruits neutrophils, macrophages and natural killer cells via its Fc region to initiate tumour cell lysis. CDC relies on recruitment of the complement complex after binding to the TAA, which leads to the formation of pores in the tumour cell membrane and lysis. Dose limiting side effects include fever, chills and anaphylactoid reactions which are most likely caused by cytokine and complement activation (Yang and Sondel 2010). A number of anti-GD2 antibodies have been developed for clinical use including the murine 3F8 and 14.18 antibodies (Cheung, Lazarus et al. 1987, Mujoo, Cheresh et al. 1987). An issue encountered with the use of these antibodies is the development of human anti-mouse antibodies which neutralise the anti-GD2 after only a few treatments, rendering them ineffective (Kushner, Kramer et al. 2001). To overcome this issue, the human-murine chimeric anti-GD2 antibody ch14.18 was generated which is less immunogenic (Barker, Mueller et al. 1991). A completely humanised anti-GD2 antibody Hu14.18K322A is also in development to further reduce immunogenicity (Yang and Sondel 2010). To increase the efficacy of GD2 antibodies, investigations have been carried out into co-administration with ADCC augmenting cytokines to help boost the immune response (Parsons, Bernhardt et al. 2013). This is particularly important for patients in the maintenance phase that have completed myeloablative therapy and may have compromised immune function. Interleukin 2 (IL-2) is a strong pro-inflammatory cytokine which affects innate immunity via activation of natural killer cells as well

as adaptive immunity by stimulation of antigen specific T-cells (Sondel and Hank 1997). A phase I clinical trial using 14.18 and IL-2 showed a limited response, with only one child out of 33 showing reduced tumour burden (Frost, Hank et al. 1997). Granulocyte macrophage colony stimulating factor (GM-CSF) is a cytokine that stimulates differentiation of haematopoietic stem cells into granulocytes and monocytes (Francisco-Cruz, Aguilar-Santelises et al. 2014). A phase III trial that incorporated ch14.18, IL-2 and GM-CSF in combination with 13-cis-RA after myeloablative therapy showed a large increase in 2 year event free survival from 46% in patients with 13-cis-RA alone to 66% in patients with 13-cis-RA, GD2 and cytokines (Yu, Gilman et al. 2010). The ch14.18 antibody (also known as Dinutuximab or Unituxin) has recently received FDA approval as part of combination therapy for the front line treatment of high risk neuroblastoma (Dhillon 2015).

In summary, long-term survival of patients with advanced stage neuroblastoma remains low when compared to other paediatric cancers. Conventional chemotherapeutic drugs remain the treatment of choice despite high incidence of resistance and relapse after initial treatment. The use of retinoic acid and anti-GD2 antibodies has helped to reduce this; however it is clear that new treatments are needed at the induction, consolidation and maintenance phases of therapy to improve overall survival.

1.1.4 Future Treatments

Great strides have been made into gaining a better understanding of the molecular pathology underlying advanced stage neuroblastoma. This has led to the identification of a number of novel targets that can be exploited for neuroblastoma treatment. Despite the fact that there have been relatively few advances in treatment of neuroblastoma in recent years, a number of pre-clinical studies and early clinical trials show some promise for future therapies.

One of the most obvious targets for the treatment of high-risk neuroblastoma is MYCN; as amplification is found in approximately half of patients with advanced stage disease and it is one of the primary contributors to poor prognosis (Barone, Anderson et al. 2013). Its expression is also strictly limited to the developing neural crest, making it specific for neuroblastoma cells. However, MYCN, like Myc, has long been considered ‘undruggable’ as it is a nuclear transcription factor with no active site amenable to binding by conventional small molecule drugs (Huang and Weiss 2013). Several strategies have been developed to overcome this issue, leading to the development of a range of different classes of drugs (Fig. 1.1).

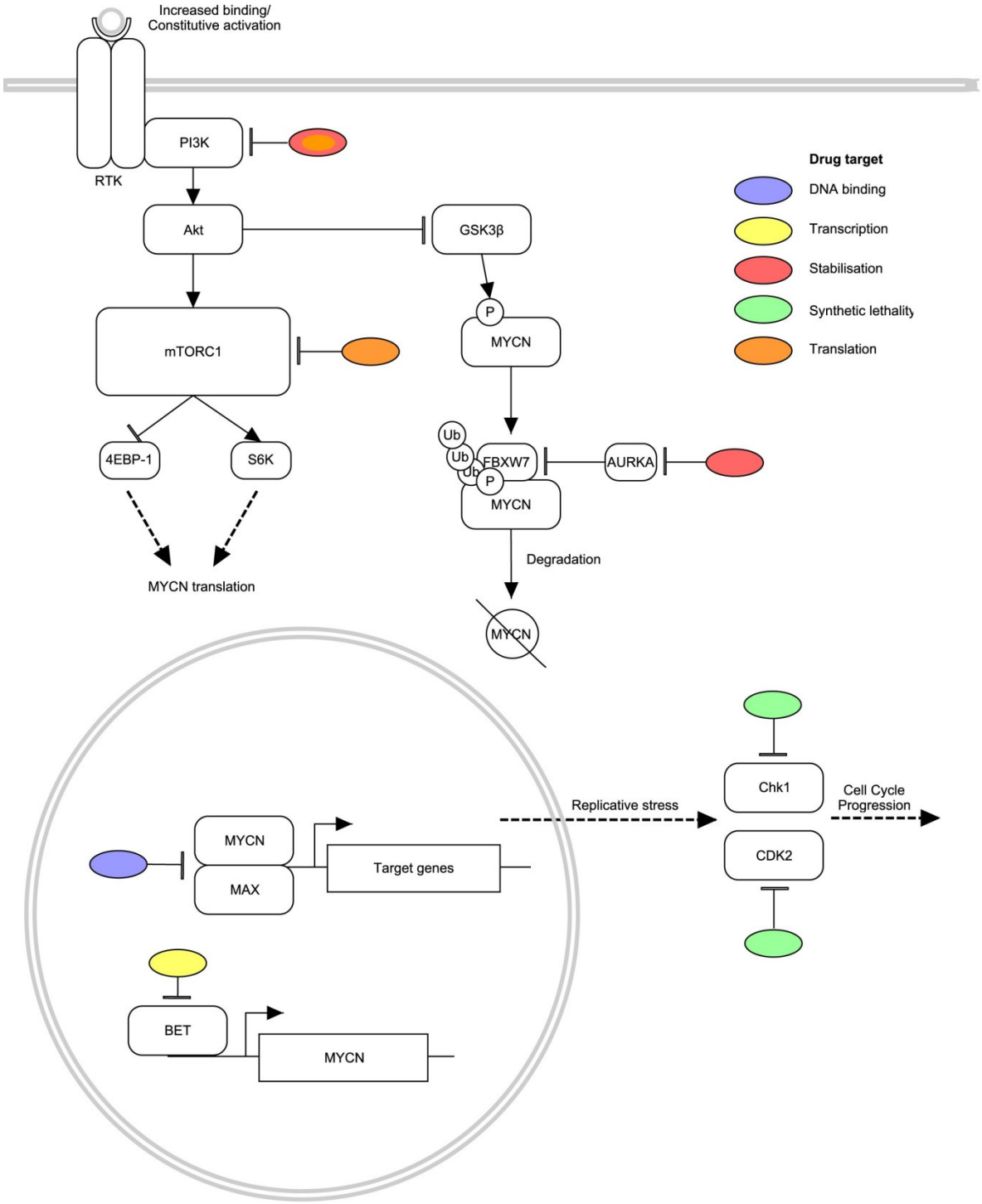

Figure 1.1: Schematic showing the different classes of drugs being developed to inhibit MYCN activity. Inhibition of the interaction between MYCN and MAX (blue) prevents DNA binding and the ability of MYCN to initialise target gene transcription. BET inhibitors (yellow) stop MYCN expression at a transcriptional level. Inhibition of AURKA (red) activates FBXW7 leading to ubiquitination of MYCN and subsequent degradation. Inhibition of Chk1 or CDK2 (green) leads to cell death in a synthetically lethal manner. mTORC1 increases MYCN translation by inhibiting eIF4E binding protein (4EBP-1) and activating S6 kinase, resulting in the increased activity of a number of translation initiation factors. Inhibition of mTORC1 (orange) therefore down regulates MYCN translation. Inhibition of PI3K (red and orange) leads to decreased MYCN translation, as it is an upstream regulator of mTORC1. PI3K inhibition also decreases MYCN stability as it allows for the phosphorylation of MYCN by GSK3β that is necessary to initiate degradation.

One possible approach is the inhibition of binding of MYCN to DNA, thus preventing target gene transcription. The small molecule 10058-F4 inhibits the interaction between MYCN and its cofactor Max, which is necessary for binding to DNA and regulation of gene expression (Huang, Cheng et al. 2006). *In vitro* testing showed that 10058-F4 caused a concentration dependent decrease in MYCN and induced apoptosis at high concentrations and differentiation at low concentrations in neuroblastoma cell lines (Zirath, Frenzel et al. 2013). The same study also found that 10058-F4 led to prolonged survival in TH-MYCN mice, a transgenic model for advanced stage neuroblastoma. However, this response was modest, and no decrease in overall tumour size was observed in xenografts.

Another approach for targeting MYCN is at a transcriptional level. The Bromodomain and extra terminal (BET) adaptor proteins BRD2, BRD3 and BRD4 have been found to localise to the promoter region of Myc, as well as other oncogenes, and activate transcription (Fu, Tian et al. 2015). The small molecule JQ1 binds to the bromodomain of these proteins, inhibiting binding to DNA. A screen of different cancer cell lines using JQ1 highlighted MYCN amplification in neuroblastoma as a contributor to sensitivity (Puissant, Frumm et al. 2013). In the same study, *in vivo* testing with the TH-MYCN transgenic mice and xenografts showed that JQ1 causes a significant increase in survival. There are currently no BET inhibitors in clinical trials for neuroblastoma, although I-BET762 is an inhibitor currently in phase I clinical trials for other forms of cancer (Zhao, Yang et al. 2013).

Synthetic lethality is the principle by which over-expression or mutation of a particular gene (such as MYCN) can lead to a dependence on another mutated or over-expressed gene. This acquired dependence can be exploited to cause cell death in cancer treatment. A number of synthetically lethal partners of MYCN have been identified, including the cyclin dependent kinases (CDKs) 1 and 2 as well as checkpoint kinase 1 (Chk1) (Molenaar, Ebus et al. 2009, Cole, Huggins et al. 2011). Over-activation of MYCN causes a large increase in the rate of DNA replication and cell division. This replicative stress is known to cause DNA damage and gross chromosomal rearrangement that would lead to apoptosis in a healthy cell. Chk1 is a serine/threonine kinase that coordinates the DNA damage response and progression through the cell cycle (McNeely, Beckmann et al. 2014). Activation of Chk1 activates a series of check points leading to cell cycle arrest, DNA repair or apoptosis. This led many to believe that Chk1 acts as a tumour suppressor. However, increasing evidence demonstrates that depletion of Chk1 does not enhance tumorigenicity, and no loss of function mutations have been found in any tested cancers. Indeed, over-activation of Chk1 is often observed in cancer cells, suggesting that it may be oncogenic and therefore a promising therapeutic target (Zhang and Hunter 2014). MYCN causes an over-activation of Chk1 which leads to aberrant

checkpoint activation and stabilisation of replicative forks during cell division. Inhibition of Chk1 is thought to abrogate the cells ability to cope with the replicative stress induced by MYCN (Cole, Huggins et al. 2011). The Chk1 inhibitor CCT244747 has shown efficacy on its own and in combination with other cytotoxic drugs in TH-MYCN mice (Walton, Eve et al. 2012). CDK2 is a serine/threonine kinase that is also involved in cell cycle regulation and is thought to play a role in progression through the G1/S phases of the cell cycle, via its interactions with cyclins E and A (Woo and Poon 2003). Initial *in vitro* studies using the small molecule CDK2 inhibitor roscovitine showed that CDK2 inhibition led to apoptosis in MYCN amplified cell lines and had little effect on non-MYCN amplified cells (Molenaar, Ebus et al. 2009). Use of the MYCN inducible SHEP-TET21N cell line confirmed that toxicity mediated by CDK2 inhibition is MYCN dependent. Pre-clinical *in vivo* studies using the CDK2 inhibitor AT7519 led to an 86% reduction in tumour size in TH-MYCN mice after just 7 days of treatment (Dolman, Poon et al. 2015).

MYCN is required for embryonic neural development and terminal differentiation of these neurons requires degradation of MYCN. This means that MYCN is tightly controlled in a cell cycle specific manner (Barone, Anderson et al. 2013). During normal development, MYCN is upregulated by growth factor signalling at the start of the cell cycle, but is then quickly ubiquitinated by the E3 ubiquitin ligase FBXW7 and subsequently degraded by the proteasome (Otto, Horn et al. 2009). The interaction of FBXW7 is dependent upon phosphorylation at threonine 58 (T58) and a 'priming' phosphorylation at serine 62 of MYCN (Sjostrom, Finn et al. 2005). The T58 status of MYCN is critical for activity and is directly regulated by glycogen synthase kinase 3-β (GSK3β) which is controlled by growth factor signalling and the PI3K (phosphoinositide 3-kinase)/Akt pathway (Barone, Anderson et al. 2013). In neuroblastoma, oncogenic stabilisation of MYCN prevents its degradation, increasing activity. This stabilisation can occur through over-activation of Akt by increased growth factor binding or constitutive receptor activation, leading to inhibition of GSK3β and preventing FBXW7 mediated degradation of MYCN (Sjostrom, Finn et al. 2005). Akt is a viable target for targeting MYCN-amplified neuroblastoma; however a lack of specific inhibitors has prevented progress in this area (Sartelet, Oligny et al. 2008). Inhibition of PI3K upstream of Akt is another possible target for de-stabilising MYCN and use of the small molecule inhibitor NVP-BEZ235 led to decreased angiogenesis and increased survival of neuroblastoma mice models in a MYCN dependent manner (Chanthery, Gustafson et al. 2012). Another mechanism employed to stabilise MYCN in neuroblastoma is up regulation of Aurora A kinase (AURKA), which inhibits the activity of FBXW7 and prevents MYCN ubiquitination (Otto, Horn et al. 2009). The AURKA inhibitor MLN8273 causes a conformational change of AURKA, independently of enzymatic activity, that prevents binding to MYCN (Brockmann, Poon et al. 2013). Early phase trials have shown limited anti-tumour activity,

although it is thought that this is due to a lack of efficacy of MLN8273, necessitating research into other AURKA inhibitors (Barone, Anderson et al. 2013).

Recent studies have demonstrated that targeting translation initiation may be a viable approach for down-regulating Myc as well as other short-lived oncogenic proteins (Bhat, Robichaud et al. 2015). The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that can form two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 is a key regulator of translation initiation which activates various factors that make up the eIF4F initiation complex (described in more detail later) (Thoreen 2013). It is thought that inhibition of mTOR shows efficacy as it leads to a decrease in Myc levels as well as attenuating the enhanced levels of protein synthesis necessary for cell growth in Myc-driven cancers (Pourdehnad, Truitt et al. 2013). Analysis of neuroblastoma tissue samples shows that mTOR is significantly over expressed and that inhibition of mTOR causes a decrease in MYCN levels and has anti-proliferative effects both *in vitro* and *in vivo* (Johnsen, Segerstrom et al. 2008). Inhibition of translation of MYCN could also be a possible mechanism of action for the PI3K inhibitors, as PI3K is an upstream regulator of mTOR (Siddiqui and Sonenberg 2015). Direct targeting of translation initiation factors downstream of mTOR using small molecule inhibitors may also be a viable approach as this has been shown to induce cell cycle arrest and apoptosis of various different Myc-driven cancers, including leukaemia and non-small cell lung carcinoma (Moerke, Aktas et al. 2007, Tsumuraya, Ishikawa et al. 2011). This shows that translation initiation could be a promising target for the treatment of MYCN amplified neuroblastoma, although whether the observed down-regulation of Myc translates to MYCN is yet to be elucidated.

Another therapeutic target that shows promise for treatment of neuroblastoma is ALK. As mentioned earlier, ALK is a receptor tyrosine kinase that is over expressed or constitutively active in a subset of high-risk neuroblastomas. Normal ALK is only expressed in the developing neural tissue, making it a highly specific target for neuroblastoma (Iwahara, Fujimoto et al. 1997). The structure of ALK makes it much more amenable to small molecule inhibition and more straight-forward to target than MYCN. Crizotinib is a small molecule inhibitor of ALK that functions by competitively binding to the ATP-binding pocket of both ALK and the proto-oncogene c-Met (Matthay, George et al. 2012). It is currently an approved treatment for non-small cell lung carcinoma in the US (Iragavarapu, Mustafa et al. 2015). A shortfall of crizotinib is that its efficacy is greatly reduced by one of the most common activating point mutations found in neuroblastoma, F1174L, which leads to increased ATP binding affinity at the intracellular tyrosine kinase domain of ALK (Bresler, Wood et al. 2011). It is therefore only of use for cells with amplified wild-type ALK or that harbour specific ALK mutations. A phase I trial using crizotinib found one complete and two partial responses in eleven patients with

known activating ALK mutations (Mosse, Lim et al. 2013). However, the small test sample makes the overall effectiveness of this drug uncertain. Other inhibitors with alternative mechanisms are in development to overcome the lack of efficacy in neuroblastoma with activating point mutations. For instance, the novel inhibitor AZD3463 has been shown to effectively inhibit ALK and induce apoptosis in crizotinib resistant neuroblastoma cell lines (Wang, Wang et al. 2016).

GD2 antibodies have already been shown to increase survival rates in patients with advanced stage neuroblastoma. However, it is thought that attachment of GD2 to a second, biologically active molecule, could improve the efficacy of this approach even further (Ahmed and Cheung 2014). The high level of GD2 expressed on the surface of neuroblastoma cells makes it ideal for the targeted delivery of cytotoxic components into cancer cells. Immunotoxins are the fusion of a targeting antibody with a toxin, usually of plant or bacterial origin (Pastan, Hassan et al. 2007). Studies using the ribosome inactivating protein (RIP) gelonin showed that fusion to a GD2 antibody led to almost 1000-fold increased cytotoxicity in GD2-positive cells, than gelonin alone (Mujoo, Reisfeld et al. 1991). GD2 antibodies have also been fused to diphtheria toxin, ricin A chain and pseudomonas exotoxin A (Wargalla and Reisfeld 1989, Tur, Sasse et al. 2001, Thomas, Delatte et al. 2002). All of these conjugates showed cytotoxicity independent of ADCC or CDC. However, issues with these toxins include off target toxicity and immunogenicity, and there are no trials being carried out for neuroblastoma at present (Choudhary, Mathew et al. 2011). As well as toxins, highly cytotoxic drugs can also be fused to GD2 antibodies to make antibody-drug conjugates (ADCs). Current drugs that are being developed as ADCs include tubulin polymerisation inhibitors such as auristatin and DNA damaging agents such as clicheamicin (Ahmed and Cheung 2014). The high potency of these drugs means that they are too toxic to be used without targeting.

Neuroblastoma is an incredibly diverse disease, with a range of molecular aberrations that have led to the development of a number of therapeutic targets and drugs. Some of these new drugs have shown promise in pre-clinical testing and clinical trials and may lead to improved survival rates in patients with advanced-stage neuroblastoma. However, the ability of neuroblastoma to develop resistance may well extend to these new targets, meaning that it is unlikely one treatment will lead to a cure, but a combination of different treatments targeting different oncogenic mechanisms, or the same mechanism but in different ways. This means that there is still a need for alternative or synergistic strategies to combat this disease and help improve the lives of patients.

1.2 Targeted toxins

General inhibition of protein synthesis by plant and bacterial toxins such as ricin and diphtheria toxin is being investigated for therapy in cancer (Alewine, Hassan et al. 2015). The catalytic nature of these toxins conveys a high level of potency not possible with small molecule drugs, meaning that only a small number of molecules need to enter the cytosol for induction of cell death (Shan, Liu et al. 2013). However, the enzymatic action of these toxins causes a complete block of protein synthesis making them toxic to both healthy and transformed cells. This means that they must first be targeted to cancer cells, either by conjugation to an antibody that is specific for a TAA on the cell surface (an immunotoxin), or to a ligand complementary to a receptor highly expressed on the cell surface (Fig. 1.2) (Pastan, Hassan et al. 2007). Although a lot of work has been carried out on optimising the targeting to cells and attachment of targeting moieties to toxins, comparatively little has been carried out to identify novel payloads for targeted toxin therapy.

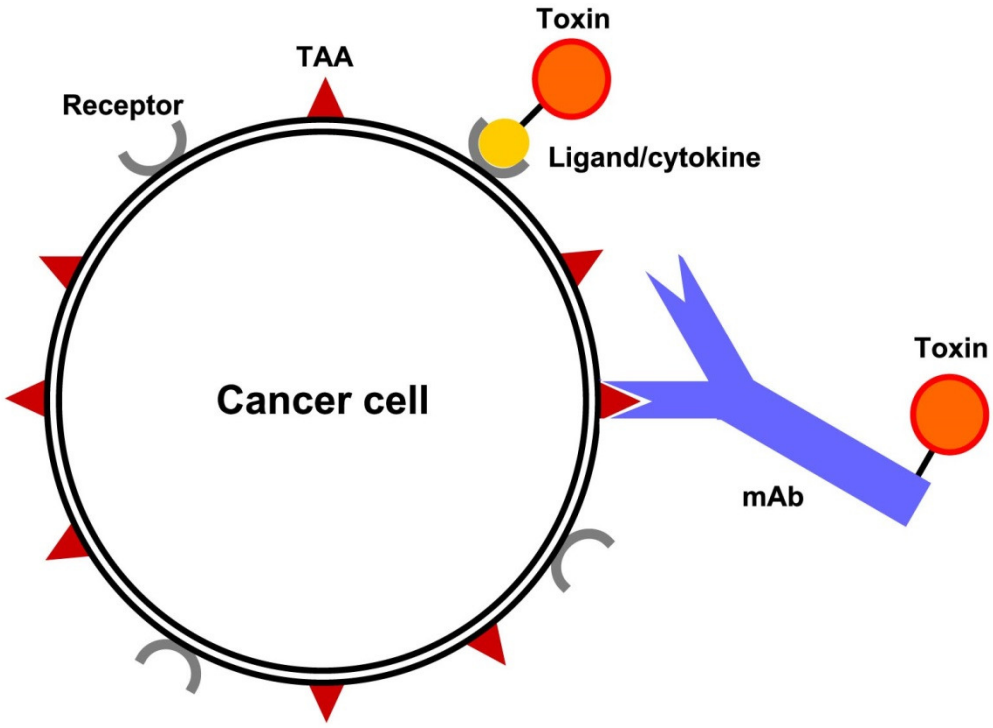

Figure 1.2: Schematic showing how toxins are targeted to cancer cells. Toxins can be targeted to tumour associated antigens (TAA) that are highly expressed on the cancer cell membrane via fusion to a monoclonal antibody (mAb). Alternatively, toxins can be fused to a ligand or cytokine that will bind receptors highly expressed on the cancer cell surface.

1.2.1 Protein synthesis

Protein synthesis, or translation, is the process by which messenger RNA (mRNA) gets translated into protein and is a key mechanism vital for the function and survival of almost every cell in the body. Generation of new proteins is the most energy consuming process in the cell and is necessary for cell growth and division, as well as responses to environmental cues (Buttgereit and Brand 1995). Regulation of protein production at a translational level allows for rapid responses to changing environments not possible through altered gene expression (Bhat, Robichaud et al. 2015). Translation is made up of three major stages; initiation, elongation and termination (Merrick 1992). Following the transcription of DNA into mRNA and export of the mRNA out of the nucleus, translation occurs on ribosomes either in the cytoplasm or across the membrane of the endoplasmic reticulum (ER). Translation involves three different types of RNA; mRNA, which carries the base sequence that is translated into protein, transfer RNA (tRNA) which recognises the mRNA sequence via its anticodon loop and brings the corresponding amino acid to the ribosome, and ribosomal RNA (rRNA) which, along with various proteins, makes up the structure of the ribosome (Merrick 1992). Ribosomes are highly conserved and found in all living cells (Fox 2010). They consist of two distinct large and small subunits (40S and 60S in eukaryotes and 30S and 50S in prokaryotes), which make up the site for protein synthesis (Doudna and Rath 2002). The ribosome contains three different binding sites for tRNA. These are the A site (aminoacyl) which is generally the first site that tRNA binds to, the P site (peptidyl) which holds the tRNA in place while the polypeptide chain is formed, and the E site (Exit) which is the last place the tRNA occupies before leaving the ribosome (Doudna and Rath 2002). During translation initiation, the 48S initiation complex is assembled, which includes the small, 40S ribosomal subunit, the mRNA and various initiation factors (Jackson, Hellen et al. 2010). The complex also contains the initiator tRNA (tRNAi) which carries a methionine amino acid and recognises the start codon (AUG) of the mRNA. The tRNAi binds to the mRNA at the P site of the small ribosome and this complex then associates with the large, 60S ribosomal subunit to make the 80S ribosomal complex (Lomakin and Steitz 2013). Translation initiation is the most highly regulated and rate limiting step of translation and will be described in more detail later. After initiation, elongation occurs (Fig. 1.3) which is where the mRNA gets decoded and the corresponding polypeptide chain is formed (Proud 1994). During elongation the next complementary tRNA, guided by GTP-bound eukaryotic elongation factor (eEF) eEF1, binds to the mRNA at the A site next to the initiator tRNA and the amino acids covalently bond together (Merrick 1992). This peptide bond formation is catalysed by the peptidyl transferase activity of the large ribosome (Doudna and Rath 2002). The ribosome then translocates 3 base pairs (or 1 codon) along the mRNA, assisted by eEF2 in a GTP-dependent manner so that the tRNAi moves to the E site and the subsequent tRNA moves to

the P site. The next tRNA in the sequence can then bind to the A site and the tRNAi is released. This process is repeated until the full polypeptide is generated and the stop codon is reached (Merrick 1992). The dependence of eEF1 and eEF2 on GTP and a dependence on ATP for tRNA generation make elongation a highly energy consuming process (Browne and Proud 2002). Termination occurs after elongation once the stop codon (usually AUG, UGA or UAA) is reached. These codons are recognised by GTP-bound eukaryotic release factor (eRF), which initiates hydrolysis of the polypeptide chain followed by hydrolysis of GTP to GDP (Merrick 1992). This process releases the completed polypeptide as well as the eRF, terminating protein synthesis and recycling the ribosomal complex constituents.

Translational regulation is an incredibly important regulatory process in the cell, as it allows for faster responses to environmental cues than the upstream components of gene expression (Bhat, Robichaud et al. 2015). The importance of translation in gene control is highlighted by the disparity between steady state mRNA levels and proteomic changes. It is thought that around 60% of changes in protein abundance can't be explained by measuring mRNA levels and that these changes are instead controlled at the translation and degradation stages (Vogel and Marcotte 2012). Translation also has an important role in localised protein synthesis, for instance in maintaining neural plasticity and responses to extracellular signals in nerve terminals that are a long distance from the cell stroma (Jung, Yoon et al. 2012). Hijacking of the translation process is considered a hallmark of cancer and changes in protein synthesis contribute to proliferation, survival, angiogenesis and altered immune response (Bhat, Robichaud et al. 2015).

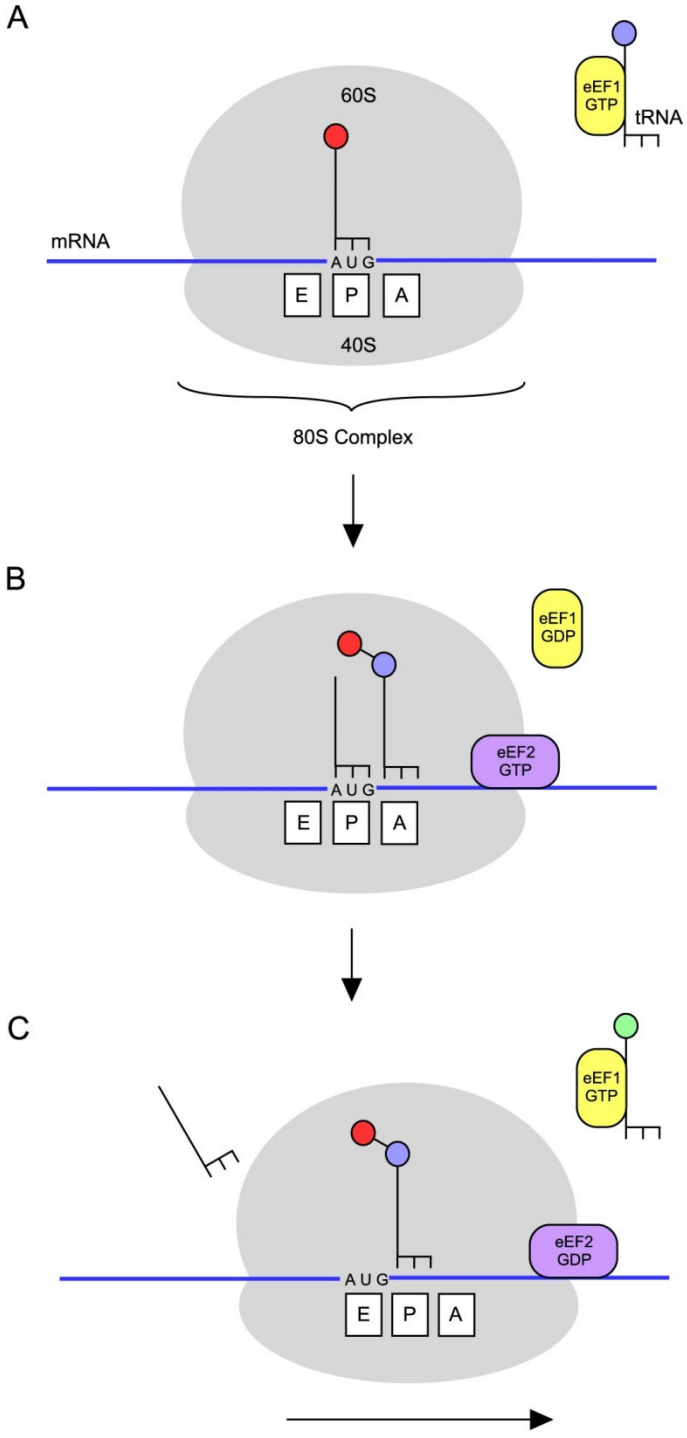

Figure 1.3: Schematic showing the process of elongation during protein synthesis. **A,** Following translation initiation, eEF1 recruits the next complementary tRNA to the 80S ribosomal complex. **B,** Hydrolysis of eEF1-bound GTP leads to the release of eEF1 and placement of the tRNA in the A-site of the ribosome, next to the tRNAi. The amino acids are then covalently bonded together. **C,** eEF2 translocates the ribosome 3 base pairs along the mRNA in a GTP-dependent manner moving the tRNAi to the E-site and the subsequent tRNA to the P-site. This leads to the release of the tRNAi from the ribosomal complex and allows binding of the next tRNA to the A-site.

1.2.2 Plant derived ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) are toxins of plant origin that act as N-glycosidases and function by depurination of the 28S rRNA within the 60S large ribosomal subunit (Schrot, Weng et al. 2015). This completely and irreversibly inactivates the ribosome, leading to a block of global protein synthesis. RIPs are found almost ubiquitously among plants and are thought to act as a form of immune defence, as upregulation of expression can be seen following viral infection and contamination with microorganisms (Wong, Mak et al. 1995). There are currently almost 250 RIPs that have been scientifically described and they can be divided into two main groups; type 1 and 2 (Gilabert-Oriol, Weng et al. 2014). Type 1 RIPs are monomeric proteins of approximately 30 kDa with enzymatic activity, and type 2 RIPs are heterodimeric proteins that contain an enzymatic domain of 30 kDa (A chain) as well as a second, lectin-like, domain of 35 kDa (B chain) which is able to bind to cells and facilitate internalisation (Stirpe and Battelli 2006). A type 3 RIP has also been proposed, which is a proenzyme that becomes active only after the removal of a short peptide fragment, although only a small number of type 3 RIPs have been identified so far (Gilabert-Oriol, Weng et al. 2014). The N-glycosidase activity of RIPs has been shown to act predominantly by removal of a single adenine residue (A^{4324} in rat rRNA) from a GAGA sequence at the universally conserved α-sarcin/ricin loop of the 28S rRNA (Endo and Tsurugi 1988). This inhibits the ability of the ribosome to bind eEF2, stopping translocation along the mRNA during elongation and causing a block of protein synthesis (Montanaro, Sperti et al. 1975). It was initially thought that cells underwent apoptosis after exposure to RIPs solely due to the ribotoxic stress response after inhibition of protein synthesis. However, more recent data suggests that RIPs may also exhibit action independent of protein synthesis. For instance, it has been shown that RIPs show adenine glycosidase activity in DNA, RNA and poly(A) (Barbieri, Valbonesi et al. 1997). Additionally, the type 2 RIP ricin has been shown to cause early nuclear DNA damage independently of protein synthesis inhibition, and the type 1 RIP saporin-6 was shown to induce apoptosis through mitochondrial cascade prior to the onset of protein synthesis inhibition (Brigotti, Alfieri et al. 2002, Sikriwal, Ghosh et al. 2008). It has therefore been proposed that RIPs may induce apoptosis by a number of different mechanisms, of which inhibition of protein synthesis plays an important, but not always essential, role (Das, Sharma et al. 2012).

Type 2 RIPs such as ricin are able to bind to sugars on the cell surface via their lectin-like B chain. Although this means that type 2 RIPs are generally more potent than type 1 RIPs, cell binding alone is not enough to confer potency, as there are type 2 RIPs that are considered non-toxic. For instance, Ricinus agglutinin (RCA) and ricin are both type 2 RIPs found in castor beans, but ricin shows around

68-fold higher potency than RCA in cells, likely due to a decreased ability of RCA to translocate into the cytoplasm (Stirpe and Battelli 2006). Nigrin b is another non-toxic type 2 RIP which was found to enter cells as efficiently as ricin, but was more rapidly degraded and excreted by cells (Battelli, Citores et al. 1997). These examples highlight the importance of intracellular trafficking following binding for mediating cytotoxicity. Studies using ricin show that, following binding, the toxin is taken up by both clathrin-dependent and independent endocytosis and that a small percentage localises with the trans-Golgi network, followed by retrograde transport to the ER (Sandvig and van Deurs 1996). Once in the lumen of the ER it is thought that the A chain is cleaved from the B chain by the protein disulphide isomerase and is then processed by the ER as a misfolded protein, meaning that it is exported to the cytosol for degradation (Roberts and Lord 2004, Spooner, Watson et al. 2004). Evasion of this degradation is essential for ricin toxicity.

Type 1 RIPs such as saporin and gelonin lack the cell binding B chain and are therefore much less cytotoxic than most type 2 RIPs. It is thought that uptake generally occurs through a passive manner, such as by fluid-phase pinocytosis (Polito, Bortolotti et al. 2013). However, they still have a highly active enzymatic action and artificial delivery into the cell or attachment to a targeting ligand leads to cytotoxicity with high potency (Stirpe and Battelli 2006). The mechanism of endocytosis of type 1 RIPs remains unclear, but studies with saporin appear to show an internalisation mechanism that is independent of the Golgi apparatus, suggesting that it follows a distinct pathway to ricin (Vago, Marsden et al. 2005). It has also been proposed that saporin can enter cells in a receptor-dependent manner, via binding to α_2-macroglobulin receptors (Cavallaro, Nykjaer et al. 1995). However, similar sensitivities to saporin have been observed between α_2-macroglobulin receptor expressing and non-expressing cell lines which would indicate that saporin internalisation does not occur via this receptor (Bagga, Hosur et al. 2003).

1.2.3 Bacteria derived translation inhibiting enzymes

As well as RIPs, a number of translation inhibiting enzymes derived from bacteria have been the subject of investigation for targeted toxin generation (Pastan, Hassan et al. 2007). The most commonly used toxins are diphtheria toxin (DT) and pseudomonas exotoxin A (PE), which both show very high levels of potency similar to RIPs. DT is secreted by the Gram-positive bacterium *Corynebacterium diphtheria* and is a key virulence factor of the diphtheria disease in humans (Collier 2001). It is made up of a single 58 kDa, 535 amino acid polypeptide chain that is functionally divided into two major domains; the amino terminal domain A (1-193 amino acids) which exhibits ADP-ribosylase function necessary for translation inhibition, and the carboxyl terminal domain B (194-535 amino acids) which promotes toxin binding to cells and internalisation into the cytosol

(Chandramohan, Sampson et al. 2012). The B domain is split into two further domains; the translocation domain (Td), and the receptor binding domain (Rbd) (Zhao and London 2005). The Rbd domain recognises and binds to heparin-binding epidermal growth factor-like precursor (HB-EGF) which acts as a receptor on the cell surface (Naglich, Metherall et al. 1992). Upon binding, the DT is internalised into endosomes via receptor-mediated endocytosis, where decreasing pH in the endosome induces a partial unfolding of the Td domain making it more hydrophobic and triggering penetration into the endosome membrane and pore formation (Collier 2001). An arginine rich site means that the A domain is concomitantly cleaved from the B domain by proteases within the endosome and enters the cytosol through the pore generated by the Td domain (Zhao and London 2005). Upon entry into the cytosol, the A domain catalyses the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD+) to a highly conserved diphthamide residue on eEF2 (Li, Vallera et al. 2013). This inactivates eEF2, inhibiting ribosome translocation during elongation, and abolishing protein synthesis. DT has been shown to be extremely potent, with only one molecule needing to enter the cytosol for the induction of cytotoxicity (Yamaizumi, Mekada et al. 1978).

Pseudomonas exotoxin A is a 638 amino acid single polypeptide chain secreted by the Gram-negative bacterium *Pseudomonas aeruginosa,* which has a similar structure and function as DT (Chandramohan, Sampson et al. 2012). Upon synthesis, a 25 kDa section of the toxin (Domain Ib) proprotein is cleaved off to leave a 66 kDa, 613 amino acid mature toxin, which contains three major domains; an N-terminal domain Ia which is involved in cell binding, domain II which is necessary for translocation, and the C-terminal catalytic domain III (Chandramohan, Sampson et al. 2012). Domain Ib targets the α2-macroglobulin receptor which is ubiquitously expressed in multiple tissues. Upon binding, PE is internalised through clathrin-coated pits into endosomes, where proteolytic cleavage releases a 37 kDa fragment containing the catalytic domain III and part of the translocation domain II (Pastan, Hassan et al. 2007). This fragment contains a REDL (Arg-Glu-Asp-Leu) sequence in its C-terminus, which is recognised by the ER protein retention receptor 2 (ERD2) (Kreitman and Pastan 1995). This receptor carries the fragment to the ER via retrograde transport through the Golgi and once in the lumen of the ER, the toxin can translocate out into the cytosol and carry out its catalytic activity (Hessler and Kreitman 1997). The catalytic domain III is similar to the enzymatic activity of DT in that it ADP-ribosylates and inactivates eEF2, causing a block of protein synthesis (Chandramohan, Sampson et al. 2012).

1.2.4 Development of targeted toxins

RIPs and other translation inhibiting enzymes show very high levels of cytotoxicity upon entry into the cytosol. However, this potency is achieved in both healthy and malignant cells, meaning that

these toxins must be efficiently targeted to cancer cells to convey specific anticancer activity (Alewine, Hassan et al. 2015). The main methods by which toxins are targeted to cancer cells are either by conjugation to an antibody, to make an immunotoxin, or to a targeting ligand such as a growth factor or cytokine (Gilabert-Oriol, Weng et al. 2014). Immunotoxins are becoming the predominant choice as they allow for greater selectivity and flexibility when choosing a target. Selecting an appropriate target is of high importance when generating a targeted toxin, as it has a large impact on specificity and potency. The chosen target must be highly expressed on the surface of the cancer cell, but have relatively restricted expression in healthy tissue, as this limits on-target off-tumour toxicity (Alewine, Hassan et al. 2015). For immunotoxins, this generally means targeting to TAAs, which are highly expressed on the cell surface as a result of transformation (Madhumathi and Verma 2012). As mentioned previously, the GD2 ganglioside is a TAA that is highly expressed on the surface of neuroblastoma cells, as well as a number of other cancers (Yang and Sondel 2010). Its restricted expression in healthy tissue makes it a suitable choice for targeted therapy. Other examples of receptors used for targeted therapy include cytokine receptors, growth factor receptors and clusters of differentiation (CDs) (Madhumathi and Verma 2012).

Design of targeted toxins is constantly changing and evolving with advances in recombinant technology and knowledge of toxin and antibody structure and function. First generation immunotoxins consisted of a full toxin chemically linked to a full antibody (Shan, Liu et al. 2013). Despite showing high cytotoxicity *in vitro*, side effects *in vivo* were common due to the presence of the cell binding domain on the toxin and non-specific killing of healthy cells (Pastan, Hassan et al. 2007). A better understanding of toxin structure and function led to the second generation of targeted toxins which lacked binding domains. These toxins were much better tolerated *in vivo*, leading to some early phase clinical trials (Becker and Benhar 2012). However, problems still persisted, including low stability due to the chemical conjugation, large size (>190 kDa) leading to poor tumour penetration, immunogenicity, and dose limiting side effects such as vascular leak syndrome and hepatotoxicity (Shan, Liu et al. 2013). Advances in recombinant technology have led to the current generation of immunotoxins, which are also known as recombinant immunotoxins (RITs). These RITs are genetically designed to negate problems from previous targeted toxin design. Changes include smaller overall size, by the use of antibody fragments leading to greater tumour penetration, removal of immunogenic epitopes on the toxin and antibody humanisation leading to decreased immunogenicity, and greater stability by the use of peptide linkers between the targeting and catalytic moieties (Pranchevicius and Vieira 2013).

Since the generation of the first targeted toxins in the early 1980s, there have been more than 450 targeted toxins described for RIPs alone (Gilabert-Oriol, Weng et al. 2014). The most commonly used toxins used in targeted toxin development are the RIPs ricin, saporin and gelonin, and the bacterial toxins DT and PE. There are currently a number of targeted toxins in clinical trials for a range of different cancers. SS1P is an immunotoxin which consists of a 38 kDa truncated version of PE (PE38) fused to an antibody against the glycoprotein mesothelin (MSLN) (Hassan, Lerner et al. 2002). MSLN is normally only expressed on mesothelial cells which line the pleura, pericardium and peritoneum. However, it is robustly expressed on a number of solid tumours including mesothelioma, pancreatic adenocarcinoma, lung adenocarcinoma and triple-negative-type breast cancer, making it a highly attractive TAA (Hassan, Bera et al. 2004). A phase I trial looking at the use of SSP1 in combination with pemetrexed and cisplatin for frontline therapy of mesothelioma found a 77% partial response in patients given the maximum tolerated dose (Hassan, Sharon et al. 2014). This compares favourably to a phase III trial which showed a 41% response rate in mesothelioma patients treated with cisplatin and pemetrexed without SSP1 (Vogelzang, Rusthoven et al. 2003). SSP1 is the only targeted toxin currently in clinical trials for the systemic treatment of a solid tumour. Some success has been seen with TP-38, a targeted toxin composed of TGF-α, a ligand for epithelial growth factor receptor (EGFR), fused to P38. TGF-α is not considered specific enough to deliver systemically, but intracerebral microinfusion into the close vicinity of malignant brain tumours in a phase I trial led to two partial responses and one complete response in 15 patients (Sampson, Akabani et al. 2003). The limited responses are thought to be due to inefficient delivery of the toxin to the tumour as well as heterogeneity of receptor expression on tumour cells (Alewine, Hassan et al. 2015). This latter issue could be addressed by the use of bi-specific targeting modalities, or combination toxin delivery. The limited success of targeted toxins in trials for solid tumours is partly due to the fact that they have only been tested in advanced stage disease, where large tumours are present. Targeted toxins may be more effective for use following chemotherapy to kill residual cells and prevent relapse. This would help to overcome issues with poor tumour penetration.

Most success has been seen with the treatment of haematological malignancies as there is no issue of tumour penetration, patients are often immunocompromised and so less likely to elicit an immune response, and blood cells are able to regenerate, so targeted entry into healthy blood cells will not have as severe side effects as in other tissues. Moxetumomab pasudotox consists of a high affinity anti-CD22 Fv (targeting region of the antibody) fused to PE38 (Bang, Nagata et al. 2005). CD22 is a B-cell differentiation marker that is highly expressed on mature B-cells and in many B-cell malignancies (Sullivan-Chang, O'Donnell et al. 2013). A phase I trial of 28 patients with hairy cell leukaemia showed highly promising results with an 86% response rate and 46% of patients achieving

complete remission (Kreitman, Tallman et al. 2012). Patients are currently being recruited for a phase III multicentre trial for the treatment of hairy cell leukaemia (NCT01829711). DT2219 consists of a modified form of DT which is fused to two Fv fragments that target both CD22 and CD19, another B-cell differentiation antigen (Vallera, Todhunter et al. 2005). This bispecific targeting technique allows binding to a larger number of cells and increased potency. A phase I clinical trial in 25 patients with refractory B-cell malignancies showed that DT2219 is well tolerated, although only two patients showed a response with one entering complete remission (Bachanova, Frankel et al. 2015). Despite some promising clinical trial data, issues such as immunogenicity and off-target effects have limited further progression in this field and there is only one targeted toxin, denileukin diftitox, currently approved for treatment. Denileukin diftitox (also known as Ontak) is a targeted toxin made up of the enzymatic and translocation domains of DT recombinantly fused to IL-2 (Alewine, Hassan et al. 2015). The IL-2 domain binds to IL-2 receptors which are highly expressed on activated T-cells as well as in a number of haematological malignancies (FitzGerald, Wayne et al. 2011). A phase III trial showed a 30% overall response rate and 10% complete response in 71 patients with cutaneous T-cell lymphoma (Olsen, Duvic et al. 2001). However, despite FDA approval, use of denileukin diftitox is infrequent due to a large number of possible side effects such as vascular leak syndrome, hypoalbuminemia, visual changes, rash and hepatobiliary disorders (McCann, Akilov et al. 2012).

1.2.5 Limitations and future directions for targeted toxin development

Advances in antibody therapy and recombinant technology have sparked a resurgence in interest for targeted toxins as a possible therapeutic approach, and a number of promising results have been obtained for the treatment of haematological malignancies. However, a number of issues still need to be addressed to make these therapies more effective in solid tumours, including increasing bioavailability and tumour penetration, reducing immunogenicity and reducing off-target toxicity.

Delivery of most macromolecules into solid tumours presents a challenge due to the lack of functional lymphatics, high interstitial fluid pressure (IFP) and irregular vascularisation found in the tumour microenvironment (Kreitman 2006). A recent study utilised flow cytometry to analyse the number of RG7787 (a modified, less immunogenic version of SSP1) molecules that reached various different xenograft tumours (Mason-Osann, Hollevoet et al. 2015). Results showed that at 7.5mg/kg, 20% of cells accumulated around 20 300 molecules per cell, which is sufficient to kill cells *in vitro,* and led to tumour regression. However, at 2.5mg/kg the top 20% of cells only internalized enough molecules to induce growth arrest, which caused tumour stabilisation but not regression. The maximum tolerated dose in a phase I trial using SSP1 was 45μg/kg and this study therefore suggests

that current targeted toxins need unfeasibly high amounts to be delivered to show efficacy (Hassan, Bullock et al. 2007). The size of the targeted toxin is a key determinant of efficacy as it influences tumour penetration. Attempts to make immunotoxins using engineered antibody fragments led to the generation of small RITs of approximately 60 kDa (Shan, Liu et al. 2013). Although these showed increased tumour penetration, they were rapidly cleared from the blood leading to decreased therapeutic efficacy. Another issue with smaller RITs is that they are cleared by renal filtration and can accumulate in the kidney leading to renal toxicity (Vallera, Panoskaltsis-Mortari et al. 1997). It is therefore thought that RITs should be over 65 kDa in size as this is the cutoff limit for macromolecules cleared by renal filtration (Pennell and Erickson 2002). Thus, a balance needs to be found between decreasing size to increase tumour penetration, without affecting plasma half-life. Another possible technique to increase tumour penetration is the co-administration of agents that help to break down components of the extra-cellular matrix and increase tumour perfusion. For instance, pegylated hyaluronidase (PEGPH20) can deplete intra-tumoural hyaluronic acid (HA) (Stromnes, DelGiorno et al. 2014). Large molecular weight HA is secreted into the interstitium of some solid tumours and accumulates there, triggering an increase in IFP. Break down of HA using PEGPH20 has been shown to normalise IFP and re-expand the microvasculature in pancreatic ductal carcinoma (Provenzano, Cuevas et al. 2012). Another approach for increasing efficacy in solid tumours would be to improve cytosolic delivery of the toxin so that fewer molecules would need to reach the cell to induce cytotoxicity. This is particularly important for type 1 RIPs as they lack a translocation domain, and it is thought that only a small percentage of internalised toxin is able to evade lysosomal degradation and enter the cytosol. Indeed, a study using targeted gelonin found a near universal requirement of 5 x 10^6 molecules needed to be internalised for cell killing, despite different routes of binding and internalisation (Pirie, Hackel et al. 2011). Considering the high potency of RIPs upon entry into the cytosol, this strongly suggests that escape from the endolysosomal compartment is the rate-limiting step that determines efficacy. Various methods are in development to improve endosome escape, including the co-administration of endosome disrupting agents. For instance, one study found that co-administration of targeted gelonin with listeriolysin targeted to the same antigen lead to a large increase in efficacy and was well tolerated *in vivo* (Pirie, Liu et al. 2013). Lysteriolysin is a cytolysin protein produced by the bacterium *Listeria monocytogenes* which can lyse endosomes in a pH dependent manner. Co-administration of plant-based saponins, glycosides that can form pores in membranes, has also been found to increase potency whilst maintaining target specificity of targeted toxins (Fuchs, Bachran et al. 2009). An alternative method is the use of photochemical internalisation which utilises an endocytic vesicle-localising photosensitizer that generates reactive oxygen species upon exposure to light triggering

vesicle rupture (Berg, Folini et al. 2007). Preferential retention of the sensitizer in tumour cells and focused light application using a laser adds a degree of selectivity to this technique which, combined with specific targeting by the toxin, can reduce side effects and increase the therapeutic window (Weyergang, Selbo et al. 2011). Photochemical internalisation has been used successfully *in vivo* to enhance the efficacy of a saporin based immunotoxin that targets the cancer stem cell marker CD133 (Bostad, Olsen et al. 2015)

Another limitation of current targeted toxins is immunogenicity due to the foreign nature of the toxins and antibodies used. Again, this is less of an issue in haematological malignancies because patients are often severely immunocompromised (Pastan, Hassan et al. 2007). Most patients with solid tumours however, have functioning immune systems and will develop neutralising antibodies to the targeted toxins which limits the number of treatment cycles and renders the treatment ineffective (Kreitman 2006). Development of humanised antibodies has largely stopped immune reactions to the targeting moiety of immunotoxins, but problems persist with the toxins themselves. Methods employed to reduce immunogenicity of the toxins include chemical modification, removal of immunogenic epitopes, and the use of human derived cytotoxic enzymes. Chemical modification with polyethene glycol (PEGylation) is a common technique used to increase the plasma half-life of therapeutic proteins (Molineux 2002). Moreover, site-specific PEGylation of an IL-2 targeting P38 based immunotoxin was found to dramatically reduce immunogenicity in mice, and was thought to act by reducing protein degradation in antigen presenting cells as well as shielding some epitopes following degradation (Tsutsumi, Onda et al. 2000). Removal of immunogenic epitopes is being increasingly utilised in the design of new RITs. For instance, the immunogenicity of P38 has been almost completely abolished by the identification and silencing of human B-cell epitopes using deletions and point mutations (Liu, Onda et al. 2012). This should allow for a higher number of treatment cycles and increase efficacy in solid tumours. Another approach to prevent immune reactions is to use toxic enzymes of human origin. The pancreatic RNase A superfamily of proteins, which catalytically degrade rRNA and tRNA leading to cell death, show particular promise (Pennell and Erickson 2002). Initial work with these enzymes showed that they were around 100-1000 fold less potent than plant and bacterial toxins, and that this was likely due to sensitivity to the RNase inhibitor protein (RI) which is present in the cytosol of most mammalian cells. RI resistant RNase proteins have now been developed which show higher toxicity and are being investigated for future use as targeted toxins (Erickson, Jund et al. 2006).

Non-specific, dose-limiting toxicity remains a problem with all current targeted toxins. This toxicity can occur from on-target, off-tumour toxicity due to expression of the target antigen on healthy

cells, or from off-target toxicity caused by non-specific internalisation of the toxin into cells. In order to reduce targeted toxicity, it is necessary to find a target found exclusively on cancer cells (Pennell and Erickson 2002). However, antigens found exclusively on cancer cells are rare which severely limits targeting choices. Indeed unexpected, low level expression of the target antigen in certain tissues has limited the use of a number of targeted toxins (Alewine, Hassan et al. 2015). For instance, an immunotoxin consisting of PE38 fused to an antibody targeting the Lewis Y antigen that is highly expressed on many endothelial cancers showed good *in vivo* activity and was used in phase I clinical trials (Pastan, Hassan et al. 2006). However, severe and dose-limiting gastritis was caused by the killing of Lewis Y expressing healthy cells in the stomach (Kuan, Pai et al. 1995). This side effect was prevented by blocking acid secretion using the proton pump inhibitor Omeprazole, but increasing toxin concentrations then led to dose limiting renal toxicity which was likely caused by low levels of Lewis Y expression on some tubular cells of the kidney (Pai, Wittes et al. 1996). This highlights the importance of antigen selection and the problems that can arise from on-target, off-tumour toxicity. Off-target toxicity due to non-specific uptake of toxin leads to dose limiting side effects such as vascular leak syndrome (VLS), hepatotoxicity, renal toxicity and cardiac dysfunction. VLS is the most common side effect observed and is the result of uptake of the toxin into endothelial cells which results in cell death and leakage of fluid from the circulatory system into the interstitial space, leading to edema, weight gain, hypoalbuminemia and orthostatic hypotension (Alewine, Hassan et al. 2015). All toxins used to date have induced VLS to some extent, but it is particularly severe in ricin based therapies, even resulting in fatalities in some trials (Fidias, Grossbard et al. 2002, Pennell and Erickson 2002). One study identified short structural motifs (x)D(y) (where x is L, I, G or V and y is V, L or S) which may bind to endothelial cells resulting in internalisation of the toxin and cell death (Baluna, Rizo et al. 1999). Modification of these motifs in ricin led to the generation of a toxin with reduced ability to cause vascular leak syndrome in mice (Smallshaw, Ghetie et al. 2003). It has also been suggested that other toxins such as PE and DT can cause VLS by initiating release of inflammatory cytokines that activate endothelial cells to produce nitric oxide (Baluna and Vitetta 1997). This triggers oxidative damage leading to compromised barrier function and permeability. Pre-treatment of patients with steroids to reduce inflammatory responses has been shown to help combat VLS (Pastan, Hassan et al. 2006). The majority of these dose-limiting side effects are likely due to the uptake of toxin into healthy cells in either a receptor mediated or non-specific manner, leading to cell death. Development of novel catalytic domains with a cytotoxic mechanism more specific for cancer cells could help to overcome these issues and create a greater therapeutic window.

1.2.6 Delivery of novel payloads into cells

Since the first immunotoxin was described in the early 1980s hundreds of different targeted toxins have been generated that target a range of different antigens and receptors (Pastan, Hassan et al. 2006). Despite the vast array of targeted toxins described, the toxic payloads used have remained restricted to a small number of translation inhibiting enzymes. One explanation for this is that the selection of an appropriate target and generation of a targeted fusion protein is a lengthy and costly process, particularly for immunotoxins where antibody fragments can't be recombinantly expressed in bacterial cultures (Yin, Li et al. 2007). This process makes it difficult to justify the use of novel enzymes that have an unproven cytotoxic effect and unknown potency. Pre-validation of cytotoxic enzymes by direct delivery into cells may help to establish potency and selectivity prior to targeting and justify targeted fusion protein development. A number of intracellular protein delivery techniques are currently available which have different strengths and limitations.

Microinjection involves the use of a micro-capillary needle to directly inject protein and other macromolecules into cells (Zhang and Yu 2008). An advantage of this technique is that the protein is delivered directly into the cytoplasm of the cell. This, combined with a high level of control over the amount of protein administered allows for highly accurate measurement of protein activity and a theoretical 100 percent delivery efficiency of viable cells. However, this system is complex and only allows delivery to individual cells, meaning that it is low-throughput and difficult for testing a wide range of conditions.

An alternative approach for protein delivery is to create pores in the membranes of cells via electroporation (Fox, Esveld et al. 2006). This technique involves the use of electrodes placed in the cell media to generate an optimised electrical pulse. This pulse disrupts the phospholipid bilayer, forming temporary pores in the cell membrane and allowing the movement of large macromolecules through these pores into the cell. As with microinjection, an advantage of this technique is that proteins are delivered directly into the cytosol of the cell. Electroporation can be used to deliver proteins into large numbers of cells which is not possible with microinjection. However, the need to remove the cells from growing conditions and resuspend them in electroporation buffer makes testing of multiple concentrations or treatments time consuming (Potter 2001). Furthermore, uneven distribution of the electrical pulse can cause high levels of cellular stress and toxicity.

Cell penetrating peptides (CPPs) are short polypeptides of between 5-30 amino acids in length that are able to cross the cell membrane (Milletti 2012). CPPs can have varying properties that can facilitate uptake including being cationic leading to electrostatic interaction with negatively charged

phospholipids, or amphipathic to encourage interaction with the cell membrane. Some studies have also shown that CPPs can cause uptake via activation of endocytosis. A number of studies have utilised CPP-fusion proteins to deliver protein into the cell. For instance, the cationic TAT peptide has been successfully used to deliver PE and RNase A across the cell membrane *in vitro* (Fawell, Seery et al. 1994). However, this technique often requires high micro-molar concentrations of proteins to observe effects, which suggests low efficiency. Another shortcoming of this technique is that it requires generation of fusion proteins which can be time consuming and costly.

One possible approach is to transfect DNA encoding the protein into cells using standard DNA transfection reagents. The cell will then express high levels of the protein and effects on cell growth and viability can be assessed. Expression of protein by DNA transfection is a very common technique used in biological research and transfection reagents are relatively cheap and widely available. The most commonly used transfection reagents are cationic lipid formulations that form a complex with negatively charged DNA and overcome the electrostatic repulsion between the cell membrane and DNA molecules (Koynova and Tenchov 2014). This is thought to promote internalisation via endocytosis where the lipids merge with the membrane and allow escape of molecules into the cytoplasm (Rao 2010). This method generally shows little toxicity and good delivery efficiency, but differences in delivery can be seen between different cell lines, and slow or non-dividing cells are considered particularly difficult to transfect. Another disadvantage of DNA transfection is that it is difficult to control the level of expression and accurately measure effective protein concentrations. This would be necessary for determining the relative potency of novel enzymes. Other disadvantages include the need to deliver the DNA into the nucleus and poor temporal control of protein levels. Direct delivery of protein into the cell would overcome these problems and formulations designed for protein are available but are more costly than DNA transfection reagents. It has previously been shown in the Davletov lab that the DNA transfection reagent lipofectamine LTX can be used to deliver the enzymatic regions of botulinum neurotoxins directly into cells (Arsenault, Cuijpers et al. 2013). Whether this is true for proteins of different size, charge and structure is yet to be elucidated. The successful use of DNA transfection reagents to efficiently deliver protein into cells would give a cheap, simple and non-toxic alternative to currently used protein delivery techniques.

1.3 Inhibition of translation initiation by Burkholderia Lethal Factor 1

Burkholderia Lethal Factor 1 (BLF1) is a recently characterised toxin from the bacterium *Burkholderia pseudomallei*. As with RIPs, DT and PE, BLF1 has been shown to cause cytotoxicity by inhibition of protein synthesis. However, BLF1 targets the translation initiation phase of translation via inactivation of the eukaryotic initiation factor (eIF) eIF4A. Translation initiation is the most complex and rate limiting step of translation, and the majority of regulatory control is carried out during this step (Watkins and Norbury 2002). As a result, a number of different cancers upregulate this process to cope with the demands of increased cell growth, which makes translation initiation a promising target for cancer interventions.

1.3.1 Translation initiation

Translation initiation is the formation of elongation competent 80S ribosomes where the mRNA initiation codon is base paired with tRNAi (Jackson, Hellen et al. 2010). To give an idea of the complexity of translation initiation compared to the other stages of protein synthesis, it is a good idea to look at the numbers of core factors involved in the different processes. Elongation has two factors, eEF1 and eEF2, termination has one factor, eRF, and initiation involves at least twelve core eIFs (summarised in table 1.1) as well as a number of auxiliary factor.

Initiation Factor	Function
eIF1	Prevents premature binding of the 40S and 60S ribosomal subunits, enhances binding of the ternary complex to the 40S ribosomal subunit, facilitates 5' UTR scanning, prevents eIF2-GTP hydrolysis by eIF5 before reaching the start codon
eIF1A	Prevents premature binding of the 40S and 60S ribosomal subunits, enhances binding of the ternary complex to the 40S ribosomal subunit, facilitates 5' UTR scanning
eIF2	Recruits tRNAi to the 40S ribosomal subunit by formation of a ternary complex
eIF2B	Guanine nucleotide exchange factor which promotes ternary complex formation by conversion of eIF2-GDP to eIF2-GTP
eIF3	Prevents premature binding of 40S and 60S ribosomal subunit, enhances binding of the ternary complex to the 40S ribosomal subunit, promotes 48S complex formation
eIF4A	RNA-helicase that facilitates 5' UTR scanning by resolution of mRNA secondary structure
eIF4B	Enhances activity of eIF4A and aids 48S complex formation
eIF4E	Binds the 5' Cap of the mRNA, recruiting mRNA to the eIF4F complex
eIF4G	Scaffold protein that is necessary for eIF4F complex formation, mRNA circularisation and 48S complex formation via interaction with eIF3
eIF4F	Complex made up of eIF4A, eIF4E and eIF4G
eIF4H	Shares homology with eIF4B and enhances eIF4A activity
eIF5	Hydrolyses eIF2 bound GTP upon recognition of the start codon, leading to dissociation of eIFs from the 48S complex
eIF5B	Ribosome dependent GTPase that mediates ribosomal subunit joining

Table 1.1 List of core initiation factors and their role in translation initiation

Translation initiation can occur via two pathways, cap-dependent and cap-independent. The cap-dependent pathway (Fig. 1.4) is considered the canonical pathway by which the bulk of proteins are synthesised and involves binding of the initiation complex to the 5' cap structure (m^7GpppN, where N is any nucleotide) on the mRNA. During cap-dependent translation, two separate complexes are formed, eIF4F and the 43S ribosomal pre-initiation complex (PIC). eIF1, eIF1A and eIF3 bind the 40S small ribosomal subunit which prevents pre-mature association with the 60S ribosomal subunit (Jackson, Hellen et al. 2010). At the same time, the tRNAi forms a stable ternary complex (TC) with eIF2-GTP which can then bind to the 40S ribosomal subunit, forming the 43S PIC. The binding of the TC is enhanced by the activities of eIF1, eIF1A, eIF3 and eIF5 (Lomakin and Steitz 2013). The guanine nucleotide exchange factor (GEF) eIF2B also facilitates TC formation by conversion of eIF2-GDP to eIF2-GTP. Recruitment of mRNA to the 43S PIC is carried out by the eIF4F complex which is made up of the mRNA cap binding protein eIF4E, the ATP-dependent DEAD-box RNA helicase eIF4A and the scaffold protein eIF4G. Following binding of eIF4F to the mRNA 5'Cap via eIF4E, the Poly(A) tail loops round and binds to the eIF4F complex to form a closed-loop structure (Derry, Yanagiya et al. 2006). The poly(A) tail is a long series of adenine nucleotides found at the end of the 3'UTR which plays a role in stabilisation and export of mRNA from the nucleus as well as enhancement of translation.

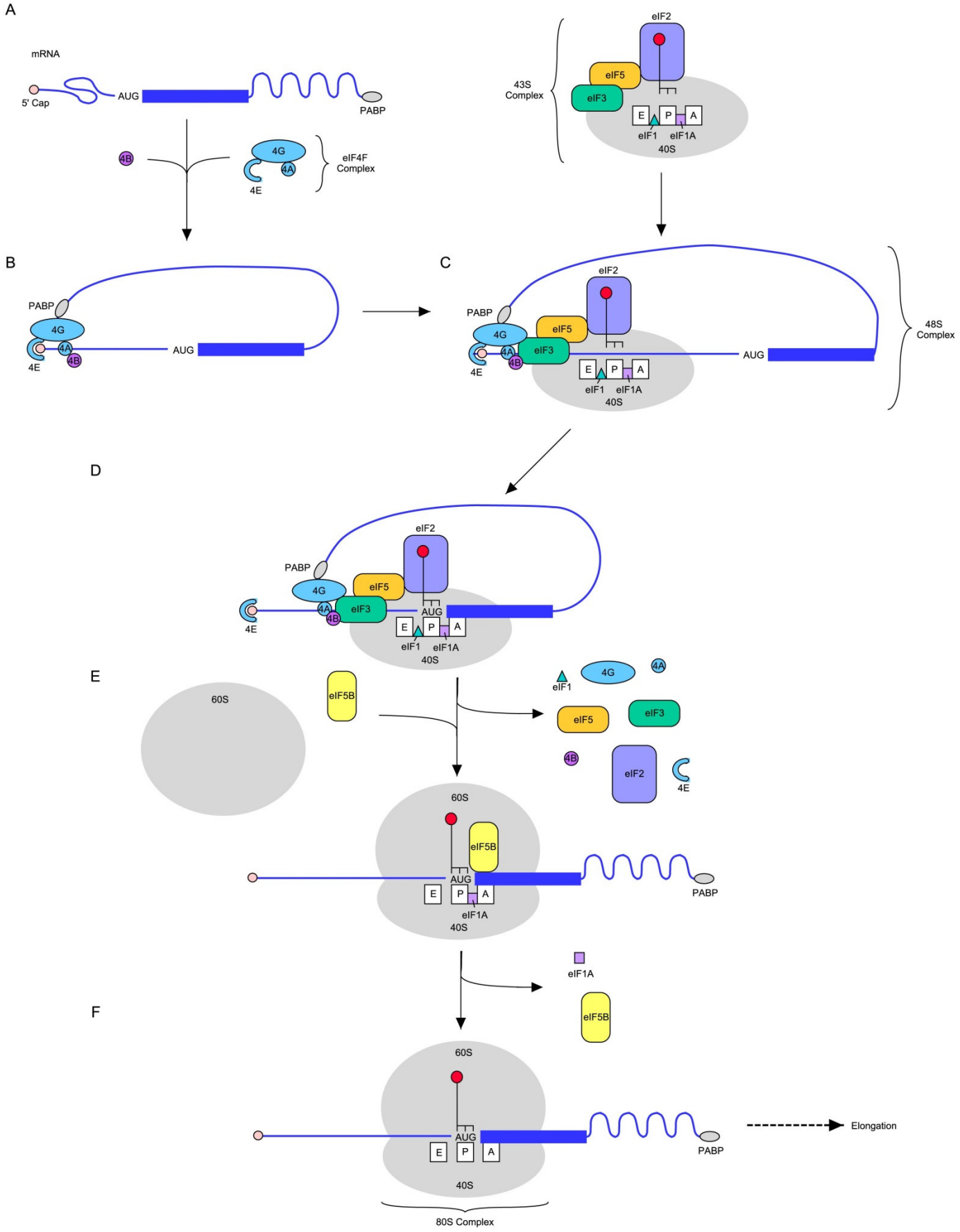
A
mRNA
5' Cap
AUG
PABP
4B
4G
4A
4E
eIF4F Complex
eIF2
43S Complex
eIF5
eIF3
E
P
A
eIF1
eIF1A
40S
B
PABP
4G
4A
4B
4E
AUG
C
eIF2
PABP
4G
eIF5
eIF3
4A
4B
4E
AUG
E
P
A
eIF1
eIF1A
40S
48S Complex
D
eIF2
PABP
4G
eIF5
4A
eIF3
4B
4E
AUG
E
P
A
eIF1
eIF1A
40S
E
60S
eIF5B
eIF1
4G
4A
eIF5
eIF3
4B
eIF2
4E
60S
eIF5B
AUG
E
P
A
eIF1A
40S
PABP
F
eIF1A
eIF5B
60S
AUG
E
P
A
40S
PABP
Elongation
80S Complex

Figure 1.4: Schematic showing the process of translation initiation. **A,** Two separate complexes are formed: the eIF4F complex which consists of eIF4E, eIF4A and eIF4G; and the 43S complex which consists of the 40S ribosomal subunit, eIF1, eIF1A, eIF2, eIF3, eIF5 and the tRNAi. **B,** The eIF4F complex binds to the 5' Cap of the mRNA via eIF4E and eIF4B is recruited to the complex. The poly-A tail loops round and joins to the eIF4F complex via PABP. **C,** The 43S complex associates with the mRNA-bound eIF4F complex via interactions between eIF3 and eIF4B to form the 48S complex. The association is strengthened by further interactions between eIF3 and eIF4G. **D,** eIF4A resolves secondary structures within the 5' UTR of the mRNA, allowing the 48S complex to scan along the mRNA until the start codon is reached. **E,** Upon reaching the start codon, all of the eIFs dissociate from the mRNA apart from eIF1A, and the 60S ribosomal subunit is recruited to the mRNA via eIF5B. **F,** eIF1A and eIF5B are released to leave the elongation competent 80S ribosome containing the tRNAi at the start codon.

Circularisation of the mRNA via the poly(A) tail is thought to enhance translation via a number of mechanisms including stimulation of recruitment of the 40S subunit to the mRNA, 60S ribosomal subunit joining, and binding of eIF4E to the 5' cap (Sachs and Davis 1989, Tarun and Sachs 1995, Kahvejian, Svitkin et al. 2005). Studies in yeast have shown that the poly(A) tail binds to eIF4F via poly(A) binding protein (PABP) which interacts directly with eIF4G, although it is also thought that PABP may interact with eIF4A in mammalian cells (Tarun, Wells et al. 1997, Craig, Haghighat et al. 1998). Following eIF4F binding the mRNA associates with the 43S PIC to form the 48S complex. This association is thought to be aided by eIF4B which binds to eIF3 in the 43S pre-initiation complex and also induces structural changes in the ribosome's mRNA entry channel, facilitating mRNA loading (Walker, Zhou et al. 2013). eIF3 also interacts with eIF4G to enhance 48S complex formation (Villa, Do et al. 2013). The 48S complex then scans along the 5'UTR until the start codon is reached. This scanning is dependent on eIF1 and eIF1A which promote an open, scanning-competent conformation (Passmore, Schmeing et al. 2007). The ATP-dependent RNA helicase eIF4A facilitates scanning by resolution of the often complex secondary structures found in the 5' UTR (Parsyan, Svitkin et al. 2011). eIF4B is thought to act with eIF4G to enhance eIF4A activity by changing its structure to a closed conformation and increasing affinity for ATP over ADP (Harms, Andreou et al. 2014). eIF4H is a protein that shares homology with eIF4B that is also thought to facilitate eIF4A activity (Richter-Cook, Dever et al. 1998). Upon reaching the stop codon, the eIF1A-40S interaction tightens which leads to displacement of eIF1 and switches the complex to a closed conformation thereby stopping scanning (Jackson, Hellen et al. 2010). Displacement of eIF1 activates the GTPase activating protein eIF5 which then stimulates hydrolysis of eIF2 bound GTP and the release of all eIFs in the complex, barring eIF1A (Poulin and Sonenberg 2013). The 60S ribosomal subunit is then recruited to the 40S subunit, facilitated by the ribosome dependent GTPase eIF5B. Following

recruitment, GTP hydrolysis leads to eIF5B and eIF1A release, leaving the completed, elongation competent 80S ribosome (Pestova, Lomakin et al. 2000).

The alternative, cap-independent process of translation initiation plays a key role in gene regulation under stress conditions such as hypoxia and nutrient limitation, and is gaining increasing attention due to its involvement in a number of pathological conditions, particularly in the translation of viral mRNA (Komar and Hatzoglou 2011). This process bypasses the need for cap-dependent scanning via direct binding of the 40S ribosomal subunit to Internal Ribosome Entry Sites (IRESs) found in the 5' UTRs of some mRNAs. Direct binding to the mRNA reduces or, in some cases, completely abolishes the need for canonical eIFs, and allows for protein synthesis when Cap-dependent translation is compromised (Pisarev, Shirokikh et al. 2005). A number of oncogenes, such as Vascular Endothelial Growth Factor (VEGF) and c-Myc contain IRESs, making cap-independent translation an important process to consider when targeting translation in cancer (Nanbru, Lafon et al. 1997, Huez, Creancier et al. 1998).

In comparison to cap-dependent translation, cap-independent, IRES-mediated translation is poorly understood. There are no known common sequences or structures within mRNA that can predict the presence of an IRES and individual mRNAs must therefore be tested for the presence of an IRES element (Komar and Hatzoglou 2011). IRES location within an mRNA sequence can also vary; most are found directly upstream of the start codon, whereas some are located in the middle of the 5'UTR or even in the coding region, leading to the formation of truncated proteins. The location and structure of IRESs also lead to differing dependencies on the components of the translational machinery, including eIFs. Cap-independent translation requires a number of proteins known as IRES trans-activating factors (ITAFs) which act as chaperones to modify RNA structure and provide a bridge between the IRES RNA and the ribosome (Spriggs, Bushell et al. 2005). These ITAFs are RNA-binding proteins that often play roles in various cellular processes but are not necessary for cap-dependent initiation. There are a growing number of identified ITAFs and it is thought that each IRES has a distinct set of ITAF requirements. A study on the Myc family of IRESs identified three key ITAFs, G-rich RNA sequence binding factor (GRSF-1), Y-box binding protein 1 (YB-1) and polypyrimidine tract binding protein-associated splicing factor (PSF) (Cobbold, Spriggs et al. 2008). It is thought that YB-1 plays a particularly important role and could replace eIF4E in the translation of the Myc proteins. This data is supported by a separate study which looked at the canonical eIFs involved in internal initiation of the Myc family mRNAs and found that translation of MYCN and c-Myc is dependent on eIF4A and the C-terminus of eIF4G for activity, but doesn't require eIF4E (Spriggs, Cobbold et al. 2009). Indeed, cells put under stress conditions have been shown to efficiently

translate c-Myc, despite the down-regulation of eIF4E and decreased cap-dependent translation (Subkhankulova, Mitchell et al. 2001). A further study shows that eIF4A is not only essential for IRES-mediated c-Myc translation, but is the limiting step, as addition of exogenous eIF4A led to a 6 to 11 fold increase in translation (Thoma, Bergamini et al. 2004). Thus eIF4A may be a better target for cancer interventions than eIF4E in Myc driven cancers and MYCN amplified neuroblastoma.

1.3.2 Regulation of the eIF4F complex

Regulation of translation is important for adjustment of the proteome in response to environmental stimuli and cellular stresses. Because translation initiation is the most complex and rate limiting process in translation, the majority of regulation is thought to be carried out at this step (Bhat, Robichaud et al. 2015). A number of eIFs are linked to extracellular signalling pathways, allowing for rapid responses to environmental changes. Aberrant signalling and over activation of these pathways is common in cancers which can lead to increased protein synthesis, cell growth and division (Malina, Cencic et al. 2011). It is thought that these cancer cells can become 'addicted' to elevated protein synthesis.

The eIF4F complex is a central node of regulation that is controlled by a number of major extracellular signal transduction pathways. The binding of certain growth factors to receptor tyrosine kinases initiates a signalling cascade and activation of the PI3K/Akt pathway (Fresno Vara, Casado et al. 2004). Phosphorylation of Akt allows direct activation of mTORC1. As mentioned previously, mTORC1 is a key regulator of translation initiation that controls short term changes in translation by promoting eIF4F complex formation, as well as longer term changes in translation by promoting transcription of genes that encode ribosomal proteins and translation factors (Fonseca, Smith et al. 2014). Overexpression or constitutive activation of RTKs is a common feature of cancer cells, and this increased activity leads to higher rates of protein synthesis (Luo, Manning et al. 2003). mTORC1 regulates eIF4E activity by phosphorylation of the eIF4E binding proteins (4E-BPs) which inhibit the binding of eIF4E to eIF4G and interfere with eIF4F complex assembly (Jackson, Hellen et al. 2010). There are three known 4E-BPs which are all controlled by mTORC1; 4E-BP1, 2 and 3, and activation of mTOR signalling causes hyper-phosphorylation of these 4E-BPs which leads to dissociation from eIF4E allowing eIF4F complex formation (Bhat, Robichaud et al. 2015). mTORC1 can also increase translation initiation by phosphorylation and activation of the S6 kinases S6K1 and S6K2. These kinases can directly phosphorylate eIF4B, which enhances binding to eIF3 and 48S complex formation (Shahbazian, Roux et al. 2006). S6Ks can also phosphorylate Programmed Cell Death 4 (PDCD4) which initiates its proteasomal degradation by the E3 ubiquitin ligase β-transducin repeat-containing protein 1 (β-TrCP1) (Dorrello, Peschiaroli et al. 2006). PDCD4 is a translational

suppressor which binds to eIF4A and prevents eIF4F assembly (Yang, Jansen et al. 2003). Phosphorylation and subsequent degradation of PDCD4 therefore leads to an increase in translation. mTORC1 can change long term rates of protein synthesis by activation of the RNA polymerase I transcription factor (TIF1A) which causes increased ribosome biogenesis, or by suppression of the RNA polymerase III inhibitor MAF1 which leads to increased tRNA synthesis (Bhat, Robichaud et al. 2015). The PI3K/Akt/mTOR pathway is a key regulatory pathway that is upregulated in a number of cancers including MYCN amplified neuroblastoma (Johnsen, Segerstrom et al. 2008). A consequence of this is a large increase in global protein synthesis as well as translation of tumour-promoting mRNAs that are needed to maintain the high division and growth rates of cancer cells (Gao and Roux 2015).

The Ras (rat sarcoma)/MAPK/Mnk (MAPK-interacting kinases) pathway is also an important modulator of protein synthesis in response to extracellular signals (Bhat, Robichaud et al. 2015). MAPK phosphorylates a number of different proteins and acts as an on/off switch for the transcription of many genes involved in cell growth and proliferation. Aberrant MAPK signalling is a hallmark of a number of cancers and a key contributor to uncontrolled cell growth (Santarpia, Lippman et al. 2012). RTK activation can initiate a signalling cascade which phosphorylates and activates MAPK. MAPK exerts its effect on protein synthesis by phosphorylation of Mnk1 which directly phosphorylates eIF4E at a single residue, Ser^{209} (Waskiewicz, Johnson et al. 1999). Phosphorylation of eIF4E leads to enhanced translation, particularly of mRNAs involved in cell survival, growth and proliferation (Furic, Rong et al. 2010). How the phosphorylation status of eIF4E affects translation remains unclear. A study by Minich et al. showed that phosphorylation of eIF4E may lead to an increased affinity for the 5' cap, and therefore increase the rate of translation (Minich, Balasta et al. 1994). However, a number of more recent studies have shown that phosphorylation may actually attenuate its ability to bind the 5' cap (Scheper, van Kollenburg et al. 2002, Zuberek, Wyslouch-Cieszynska et al. 2003). Decreased affinity for the 5' cap may seem contradictory to the increase in protein translation following eIF4E phosphorylation. However, two models have been proposed to explain the observed effect on translation initiation (Scheper and Proud 2002). Phosphorylation of eIF4E may occur after 5' cap binding, which leads to dissociation of eIF4E from the cap and allows for more efficient scanning of the 5' UTR. Dissociation of eIF4E from the cap would also allow for the rapid loading of a new initiation complex and increase the translational rate of the mRNA. Alternatively, phosphorylation may occur after recognition of the start codon and enhance the dissociation of the eIFs from the complex, freeing the components to form a new initiation complex. MAPK can also regulate eIF4F complex formation by direct phosphorylation of S6Ks (Bhat, Robichaud et al. 2015). This leads to increased eIF4B and eIF4A

activity as is seen after S6K activation by mTORC1. Activating mutations in the Ras/MAPK pathway are frequently found in neuroblastoma, particularly in relapsed cells following chemotherapy (Eleveld, Oldridge et al. 2015).

Regulation of the eIF4F complex also occurs at a transcriptional level. Several transcription factors are known to increase the expression of eIF4E, including c-Myc, nuclear-factor-κB (NF-κB) and hypoxia-inducible factor 1 (AUF1) (Bhat, Robichaud et al. 2015). Both c-Myc and MYCN have also been shown to increase the expression of eIF4A, eIF4G and a number of proteins involved in ribosome and tRNA biogenesis (Boon, Caron et al. 2001, Riggelen, Yetil et al. 2010). This suggests that the Myc proteins are part of a feed-forward loop where Myc increases levels of the eIF4F complex, which in turn enhances the translation of Myc mRNA (Lin, Malina et al. 2009).

1.3.3 Inhibition of the eIF4F complex as a therapy in cancer

Targeted monotherapies that inhibit a specific signalling pathway or oncogenic-protein that the cancer cell depends on have been the subject of intense investigation as they can provide greater specificity and fewer side-effects than classic chemotherapeutic drugs. However, intra-tumour heterogeneity means that whilst these drugs may show high short-term efficacy, a small percentage of cells are likely to possess intrinsic resistance leading to relapse (Polyak 2014). Many different oncogenic signalling pathways converge on protein synthesis, particularly via eIF4F, to enhance tumorigenesis, making increased protein synthesis a key marker of the majority of cancers (Pelletier, Graff et al. 2015). This makes targeting translation an attractive option for cancer therapy as it can overcome this intra-tumour heterogeneity that limits the use of other targeted cancer therapies. For instance, one study that carried out immunohistochemical staining in breast cancer samples found heterogeneous distribution of key oncogenic proteins such as MAPK, Akt and HER2 within a tumour, whereas elevated levels of eIF4E were found homogeneously distributed throughout the tumour (Ramon, De Mattos-Arruda et al. 2014).

The importance of the eIF4F complex in regulation of protein synthesis means that it is commonly upregulated in cancer cells and has been the most intensely investigated process for therapeutic intervention (Pelletier, Graff et al. 2015). eIF4E is thought to be of particular importance because it is the least prevalent eIF in healthy cells and is upregulated in a number of different cancers, including malignancies of the prostate, breast, stomach, colon, lung, skin and hematopoietic system (Hsieh and Ruggero 2010). As mentioned previously, eIF4E is the cap-binding component of the eIF4F complex and is therefore necessary for all cap-dependent translation. However, it is thought that the dependence of mRNAs on eIF4E for efficient translation can vary dramatically, leading to a subset of

mRNAs which are referred to as ‘eIF4E-sensitive’ (Siddiqui and Sonenberg 2015). These mRNAs often have a long, highly structured, G/C-rich 5’ UTR and they are usually poorly translated under normal conditions which may be due to a reduced ability to associate with eIF4F (West, Stoneley et al. 1998). Furthermore, it has been shown that eIF4G suppresses the activity of eIF4A when unbound to eIF4E, and that eIF4E therefore acts as an indirect activator of eIF4A, increasing 5’ UTR secondary structure resolution (Feoktistova, Tuvshintogs et al. 2013). This provides a further explanation for why mRNAs with long, complex 5’UTRs have a high dependency on eIF4E. A number of proteins involved in proliferation and survival are encoded by eIF4E sensitive mRNAs, including the cell cycle regulators cyclin D1 and D2, Myc transcription factors, the p53 inhibitor MDM2, VEGF, and anti-apoptotic proteins such as Bcl-2 and survivin (Siddiqui and Sonenberg 2015). The relatively restricted translation of these mRNAs in healthy cells may act as a regulatory step to suppress transformation, and increased eIF4E activity helps to bypass this step. Indeed, overexpression of eIF4E increases translation of mRNAs with long 5’ UTRs and results in malignant transformation of rodent cells (Lazaris-Karatzas, Montine et al. 1990, Koromilas, Lazaris-Karatzas et al. 1992).

The obvious role eIF4E plays in cancer development and oncogenic signalling means that there has been considerable effort to target eIF4E for cancer therapy. One avenue is the indirect targeting of eIF4E by inhibition of upstream regulators in the PI3K/Akt/mTOR pathway which would lead to de-phosphorylation of the 4E-BPs and decrease eIF4E activity. As has been mentioned previously, a number of mTOR and PI3K inhibitors are currently in trials for various cancers, including neuroblastoma. The large number of cellular processes controlled by PI3K and mTOR signalling makes the contribution of eIF4E inhibition to therapeutic activity uncertain (Siddiqui and Sonenberg 2015). However, a number of studies have been carried out which highlight the importance of eIF4E inhibition for this approach. For instance, it has been shown that the ratio of eIF4E/4E-BP is a predictor of the efficacy of mTOR inhibition, in that a higher eIF4E/4E-BP ration leads to decreased efficacy (Alain, Morita et al. 2012). Downregulation of 4E-BP also leads to an acquired resistance to mTOR inhibition. Furthermore, resistance to the dual PI3K/mTOR inhibitor BEZ235 was shown to be a result of amplification of the Myc and eIF4E genes, and resistant cells displayed elevated cap-dependent protein translation (Ilic, Utermark et al. 2011). A drawback of this approach is that, because these are targeted therapies upstream of eIF4E, they may be rendered ineffective by intra-tumour heterogeneity. For instance, alternative activation of eIF4E by the MAPK/Mnk pathway may lead to cells that are insensitive to PI3K/mTOR inhibition. Moreover, upregulation of eIF4E appears to act as a mechanism of resistance to this approach.

A number of approaches have also been developed to directly inhibit eIF4E. One approach was the generation of an anti-sense oligonucleotide (ASO) against eIF4E (Graff, Konicek et al. 2007). ASOs function by binding to and inactivating mRNA, leading to inhibition of translation. The eIF4E ASO was found to cause a preferential decrease in translation of eIF4E sensitive mRNAs such as VEGF, cyclin D1, survivin and c-Myc (Graff, Konicek et al. 2007). Intravenous administration of the ASO in mice was also found to significantly reduce eIF4E in human xenografts and suppress tumour growth. The ASO also showed minimal side effects with no changes in body weight or organ weight, despite inhibition of around 80% of eIF4E activity in the liver (Graff, Konicek et al. 2007). This data shows that cancer cells are more sensitive to eIF4E inhibition than healthy cells, making eIF4E a bona fide cancer target. A phase I trial with the eIF4E ASO LY2275796 showed good tolerance up to 1000mg; however, despite decreases in tumour eIF4E expression, responses were limited with stable disease in 7 of 30 patients being the best response (Hong, Kurzrock et al. 2011).

Small molecule inhibitors of eIF4E are also in development. 4EGI-1 is a small molecule that functions by inhibiting the interaction between eIF4E and eIF4G, thereby inhibiting eIF4F complex formation. Use of 4EGI-1 has been shown to cause a preferential decrease in translation of oncogenic, eIF4E sensitive mRNAs and induce apoptosis *in vitro* (Moerke, Aktas et al. 2007). Importantly, 4EGI-1 was shown to induce apoptosis in acute myeloid leukaemia (AML) blast cells but showed limited toxicity in healthy hematopoietic progenitors (Tamburini, Green et al. 2009). This study also showed a role of the Pim-2 kinase in phosphorylation of 4E-BP1 and subsequent translational control in AML independent of mTORC1. This further highlights the importance of the eIF4F complex as a central node of transformation and its usefulness as a target in cancer therapy. Although highly promising, the use of 4EGI-1 has been limited as it is active in the micro-molar range, making it less likely to be considered for clinical use. Additionally, it has not been conclusively shown that 4EGI-1 specifically inhibits the interaction between eIF4E and eiF4G without inhibiting any other protein-protein interactions. 4EGI-1 has been shown to induce apoptosis via proteasome-mediated degradation of the anti-apoptotic c-FLIP, independently of eIF4E/eIF4G (Fan, Li et al. 2010). This calls into question the specificity of 4EGI-1 and suggests that it may elicit certain effects through general inhibition of oncogenic signalling pathways (Hsieh and Ruggero 2010). A second small molecule that can inhibit the activity of eIF4E is the anti-viral drug ribavirin which functions by mimicking the 5' cap structure and competes with mRNAs to bind eIF4E (Hsieh and Ruggero 2010). Ribavirin has been used as an anti-viral drug for around 40 years, although its anti-viral mechanism of action remains unclear (Borden and Culjkovic-Kraljacic 2010). A phase II trial with ribavirin in AML patients showed promise, with one complete remission, two partial remissions, two blast responses and four stable diseases out of 11 patients tested (Assouline, Culjkovic et al. 2009). However, eventual resistance to ribavirin

and subsequent relapse was a common feature of all patients. It is thought that this resistance may be due to upregulation of glioma-associated protein 1 (GLI1) and UDP glucuronosyltransferase (UGT1A) enzymes, which are dependent on the sonic hedgehog transcription factor (Zahreddine, Culjkovic-Kraljacic et al. 2014). UGT1A is activated by GLI1 and adds glucuronic acid to ribavirin, leading to its inactivation and acquisition of resistance. Work is now being carried out on overcoming resistance to ribavirin, as well as using ribavirin as an adjuvant in combination therapies (Borden and Culjkovic-Kraljacic 2010).

eIF4E has long been considered the key factor in tumorigenesis because it is the least abundant eIF at 0.26 copies per ribosome, whereas eIF4A is the most abundant eIF at three copies per ribosome (Malina, Cencic et al. 2011). However, this does not take into account the localisation of the eIFs or interactions with their respective inhibitors 4E-BP and PDCD4. Upregulation of eIF4A has been reported in a subset of cancers including melanoma, and hepatocellular carcinoma (Eberle, Krasagakis et al. 1997, Shuda, Kondoh et al. 2000). Inhibition of eIF4A is therefore gaining increasing attention in cancer therapy as an alternative mechanism for disrupting the eIF4F complex. eIF4A (also known as DDX2) is a DEAD-box (contains an Asp-Glu-Ala-Asp motif) helicase which exhibits RNA-dependent ATPase and ATP-dependent bidirectional helicase activities and is necessary for unwinding the secondary structure of the 5' UTR mRNA (Rogers, Komar et al. 2002). There are three main isoforms, eIF4AI, II and III. eIF4A I and II share 91% homology with indistinguishable function and are collectively referred to as eIF4A. eIF4AIII on the other hand only shares 65% homology and is thought to be needed for assembly of the exon junction complex following splicing (Parsyan, Svitkin et al. 2011). eIF4AIII is unable to substitute for eIF4AI and is therefore thought to be functionally distinct and not involved in translation initiation (Li, Imataka et al. 1999). The helicase activity of eIF4A is highly dependent upon its association within the eIF4F complex. When bound to eIF4G, eIF4A is locked in a compact conformation which increases its affinity for both ATP and RNA and causes an increase in helicase activity by around 20-fold when compared to unbound eIF4A (Chu and Pelletier 2015). The helicase activity of eIF4A is further increased by eIF4B and eIF4H which modulate the affinity of eIF4A for ATP (Marintchev, Edmonds et al. 2009). The length of the 5'UTR within an mRNA has been found to be directly proportional to its need for eIF4A activity (Chu and Pelletier 2015). This means that, as with eIF4E, mRNAs with long and highly structured 5' UTRs such as Cyclin D1 and Myc have a higher dependence on eIF4A and are therefore more affected by eIF4A inhibition. A recent study found that a 12-mer $(CGG)_4$ motif present in the 5' UTR of a subset of mRNAs was a hallmark of sensitivity to eIF4A inhibition by the small molecule silvestrol (Wolfe, Singh et al. 2014). These motifs correspond to a G-quadruplex (GQ) structure which forms by non-Watson-Crick interactions between paired guanine nucleotides that align parallel or anti-parallel

arrangements in different planes and are connected by at least one linker nucleotide (A or C). mRNAs that contained these GQs included a number of transcription factors and oncogenes including Myc, MYB, NOTCH, CDK6 and BCL2. This data suggests that eIF4A could be an effective target for cancer therapy.

Three small molecule inhibitors of eIF4A have been identified; silvestrol, pateamine A (PatA) and hippuristanol. Silvestrol is a rocaglate derived from *Aglaia foveolata* which appears to function by reversible over-stimulation of eIF4A helicase activity resulting in non-specific mRNA binding and sequestration of eIF4A from the eIF4F complex (Bordeleau, Robert et al. 2008). Silvestrol is the most commonly studied eIF4A inhibitor and has shown efficacy in breast and prostate cancer xenograft models, causing increased apoptosis, decreased proliferation and inhibition of angiogenesis (Cencic, Carrier et al. 2009). Efficacy has also been shown in hepatocellular cancers, where silvestrol prolonged survival in tumour-bearing mice and also acted synergistically with other therapeutic agents including rapamycin (Kogure, Kinghorn et al. 2013). As well as solid tumours, silvestrol has been shown to be highly effective as a single agent or synergistically in a number of models for leukaemia and lymphoma and shows selectivity for malignant B cells over healthy T cells (Lucas, Edwards et al. 2009, Alachkar, Santhanam et al. 2013, Wolfe, Singh et al. 2014). As mentioned previously, it is likely that silvestrol is preferentially toxic towards cancer cells due to downregulation of key oncogenic proteins containing G-quadruplexes in the 5' UTR of their mRNA (Wolfe, Singh et al. 2014). Silvestrol shows good bioavailability and stability *in vivo* following intra-peritoneal injection, although availability is greatly reduced following oral-administration. It is thought that this reduced bioavailability is because silvestrol is a substrate of permeability-glycoprotein (Pgp), also known as multidrug resistance protein 1, which is an efflux transporter that pumps the drug out of the cell (Gupta, Sass et al. 2011). Pgp is present on the intestinal mucosa lining and can inhibit efficient drug absorption (Murakami and Takano 2008). This may also pose problems for the future use of silvestrol as Pgp is often upregulated in cancer cells, conferring resistance to a number of small molecules. Work is currently being carried out to develop silvestrol analogues that have better drug-like properties but maintain efficacy (Liu, Nair et al. 2012).

PatA is a biologically active metabolite isolated from the sea sponge *Mycale* sp. which functions in the same way as silverstrol, by over-stimulation of eIF4A (Low, Dang et al. 2005). PatA has shown potent activity *in vitro*, causing cytotoxicity and an induction of apoptosis at nano-molar concentrations in cancer cell lines (Hood, West et al. 2001). The PatA analogue, des-methyl, des-amino pateamine a (DMDA-PatA) has also shown potent anticancer activities against a range of cell

lines, with limited activity in non-transformed cells (Kuznetsov, Xu et al. 2009). The same study also showed resistance to drug efflux by Pgp as well as efficacy in xenograft models.

Hippuristanol is a polyoxygenated steroid that was initially isolated from the gorgonian (closely related to coral) *Isis hippuris*. It functions as an allosteric inhibitor of eIF4A which locks it into an open, inactive conformation and therefore inhibits the RNA binding and helicase activities (Chu and Pelletier 2015). A study of the effects of hippuristanol on adult T-cell leukaemia showed that it caused a preferential decrease in levels of cyclins D1 and D2, CDK4, CDK6 and Bcl-x_L (Tsumuraya, Ishikawa et al. 2011). This led to the induction of apoptosis in different leukaemia cell lines with lower potency in healthy control cells. The same study also found that hippuristanol suppressed tumour growth in mouse xenografts. Hippuristanol has also been shown to re-sensitise chemo-resistant Eμ-Myc lymphomas to DNA damaging agents such as doxorubicin in mice (Cencic, Robert et al. 2013). Further use of hippuristanol *in vivo* has been limited by relatively low potency (IC50s range from 50-300nM) and low solubility (Chu and Pelletier 2015). However, work is ongoing to generate analogues that are more suitable for the clinic (Ravindar, Reddy et al. 2011).

In summary, there are a number of inhibitors of the eIF4F complex that are currently in development. Although many of them show a highly selective mechanism of action *in vitro* as well as in certain mice models, a lot of work remains to be carried out to further improve efficacy and bioavailability before these drugs can be used in a clinical setting.

1.3.4 Burkholderia lethal factor 1

Burkholderia lethal factor 1 (BLF1), which was recently renamed from BPSL1549, is a 24 kDa toxin produced by the Gram-negative bacterium *Burkholderia pseudomallei* (Cruz-Migoni, Hautbergue et al. 2011). *B. pseudomallei* is endemic in areas of Southeast Asia and northern Australia and is responsible for the disease melioidosis, which became known as the 'Vietnamese time-bomb' for its long incubation period and infection of US soldiers during the Vietnam war (Currie 2015). Melioidosis mimics many symptoms seen in tuberculosis, typhoid fever and malaria, including acute pneumonia, septicaemia, hyperthermia, skin and organ abscesses, and neurological lesions (Hautbergue and Wilson 2012). *B. pseudomallei* is an intracellular bacterium that is thought to spread between cells by increasing actin polymerisation and cell fusion (Kespichayawattana, Rattanachetkul et al. 2000). This can also give rise to very large multinucleated cells. Once inside the cell, the pathogen secretes a number of survival factors and exotoxins including proteases, lipases and haemolysins which cause tissue necrosis (Hautbergue and Wilson 2012). A comparison of proteomes between the pathogenic *B. pseudomallei* and the non-pathogenic, related strain *B.*

thailandensis identified 14 uncharacterised proteins that were expressed in the pathogenic strain (Wongtrakoongate, Mongkoldhumrongkul et al. 2007). This led to the characterisation of BLF1 as a key virulence factor and contributor to pathogenicity (Cruz-Migoni, Hautbergue et al. 2011).

BLF1 functions by deamidating a specific glutamine residue (Gln^{339}) on eIF4AI and eIF4AII, which abolishes helicase activity, but does not affect ATPase activity (Cruz-Migoni, Hautbergue et al. 2011). Gln^{339} modified eIF4A is thought to act as a dominant-negative mutant which inhibits protein synthesis and prevents eIF4A recycling. As mentioned previously, eIF4A is a key regulator of translation initiation, and small molecule inhibition of this enzyme has shown promising results as an anticancer therapy because it leads to a preferential down regulation of the translation of important oncogenic proteins (Wolfe, Singh et al. 2014). Interestingly, the Gln^{339} residue targeted by BLF1 maps precisely to the strongest chemical shifts induced in eIF4A after the addition of hippuristanol, which has been limited as a therapeutic agent by poor potency (Malina, Mills et al. 2012). The catalytic nature of BLF1 should make it highly potent, thereby overcoming issues faced with hippuristanol.

BLF1 is a monomeric enzyme which doesn't contain any cell binding or translocation properties (Cruz-Migoni, Hautbergue et al. 2011). This is likely because *B. pseudomallei* is an intracellular bacterium which secretes the toxin directly into the cytoplasm of the target cell. Fusion of BLF1 to targeting moieties could make a highly specific form of cancer therapy as it could combine dual specificity of the mechanism of cytotoxicity by inhibition of eIF4A, with targeting to cancer cells. This could help to overcome a number of side effects that have been seen with conventional targeted toxins such as VLS and hepatotoxicity. However, to be an appropriate toxin for targeted therapy, BLF1 would need to show a high level of potency in cancer cells, similar to that seen with RIPs and other bacterial translation inhibitors.

1.4 Botulinum neurotoxin proteases

Botulinum neurotoxins (BoNTs) are the most potent poisons known to man (Gill 1982). These toxins are gaining increasing attention due to their wide possible applications in medicine. Recent data suggests that they may be of use in the treatments of some cancers, particularly neuroblastoma (Arsenault, Cuijpers et al. 2013). Re-targeting of these toxins using a targeted toxin approach could offer an alternative payload, with a reduction in side effects when compared to classic translation inhibiting enzymes.

1.4.1 Botulinum neurotoxins

BoNTs are neurotoxins secreted by the Gram-positive bacterium *Clostridium botulinum.* This spore-forming, anaerobic bacterium mainly found in soil was first identified in 1895 and has since been shown to cause the deadly disease botulism (Mahajan and Brubaker 2007). Botulism occurs naturally in three forms; food-borne, wound and intestinal, and causes flaccid paralysis by blocking the release of acetylcholine to motor neurons (Coleman and Yergler 2002). There are seven main serotypes of neurotoxin, BoNT/A-G, which were identified by neutralisation of toxicity by specific polyclonal antibodies (Hill and Smith 2013). However, DNA sequencing has revealed at least 40 unique BoNTs, known as subtypes, although there is a lack of sufficient evidence to link these genetic variances with functional changes in the toxin (Maslanka, Luquez et al. 2016). It is thought that the different serotypes are responsible for causing the different types of botulism. For instance, BoNT/A, B, E and F cause foodborne botulism, whereas BoNT/C causes wound botulism in animals (Coleman and Yergler 2002). Infant botulism, a form of intestinal botulism, typically affects children under the age of one and is the most common form of botulism in the United States with between 70 and 100 cases annually (Rosow and Strober 2015). It occurs because a lack of competitive intestinal flora allows ingested *C. botulinum* spores to colonise the gastrointestinal tract (Long 2001). Release of the neurotoxin (usually types A and B) results in failed neurotransmission and flaccid paralysis. Symptoms include bulbar dysfunction, weakness and respiratory failure, and treatment of infant botulism usually involves intensive care, including mechanical ventilation and intravenous feeding, for a number of weeks until the neurological effects of the toxin wear off (Rosow and Strober 2015). A human derived antitoxin was also approved by the FDA for treatment of botulism in 2003 (Thompson, Filloux et al. 2005). This antitoxin contains botulism immunoglobulin (BIG) derived from pooled plasma of volunteer donors that have been immunised with pentavalent botulinum toxoid. Intravenous administration of BIG acts to neutralise circulating toxin and has been shown to reduce mean hospitalisation time from 5.7 to 2.6 weeks (Arnon, Schechter et al. 2006). The high potency of BoNTs (approximately 1µg/kg of BoNT/A if ingested in humans) can be attributed to the small number of synaptic junctions that need to be inhibited for paralysis to occur (Coleman and Yergler 2002).

As with DT and PE, these 150 kDa BoNTs contain three distinct domains which convey cell targeting, translocation and enzymatic activity. Structurally, BoNTs consist of a 100 kDa heavy chain, which contains a C-terminal cell binding domain and an N-terminal translocation domain, linked via a disulphide bond to a 50 kDa light chain which contains the enzymatic domain (Swaminathan 2011). Binding specificity of the different serotypes varies slightly, but all BoNTs are neuro-specific and

target gangliosides, which are sialylated glycosphingolipids involved in the development, function and maintenance of the nervous system (Verderio, Rossetto et al. 2006). The complex polysialogangliosides (PSGs) GT1b, GD1b and GQ1b are thought to be particularly important for BoNT binding, and a conserved peptide motif (H…SXWY…G) on BoNT/A and B was found to directly bind to GT1b (Schiavo, Matteoli et al. 2000, Rummel, Mahrhold et al. 2004). Another study found that GT1b and GD1b are necessary for BoNT/C binding, but that BoNT/D is able to bind cells in a PSG independent manner (Tsukamoto, Kohda et al. 2005). However, this study used a GM3 synthase KO mouse model that was unable to make a- and b-series PSGs but could still make o-series PSGs. A more recent study using a KO mouse that could not make any PSGs showed reduced BoNT/D mediated toxicity, suggesting that BoNT/D binding is dependent on PSG expression (Peng, Tepp et al. 2011). There is growing evidence that most BoNTs require a protein element for binding as well as PSGs, and that they are internalised in a double-receptor manner. BoNT/A has been found to interact directly with the luminal domain of synaptic vesicle protein 2 (SV2) (Dong, Yeh et al. 2006). SV2 is a glycoprotein expressed in secretory vesicles of neural cells and has three main isoforms, A, B and C, which are thought to have a role in regulating neurotransmitter release by preparing vesicles for fusion (Custer, Austin et al. 2006). It is thought that BoNT/A is able to interact with all three SV2 isoforms, but has the highest affinity for SV2C (Dong, Yeh et al. 2006). BoNT/D and E have also been found to utilise SV2 as well as PSGs for internalisation (Peng, Tepp et al. 2011, Mahrhold, Strotmeier et al. 2013). Protein receptor binding sites have been identified for BoNT/B and G, although these have been found to bind to synaptotagmins I and II (Rummel, Eichner et al. 2007). Synaptotagmins are evolutionarily conserved transmembrane vesicular proteins which act as calcium sensors in the regulation of neurotransmitter release (Sudhof 2013). The proposed model for BoNT binding is that initial binding to neurons occurs via interaction with PSGs, and during vesicle exocytosis they associate with the luminal domains of vesicle-associated proteins leading to internalisation. BoNT/C is thought to be unique in that it is internalised via dual PSG receptors, independently of other protein receptors, and is internalised as the result of an increase in vesicle endocytosis following membrane depolarisation (Karalewitz, Fu et al. 2012).

Upon internalisation into synaptic vesicles, a decrease in pH triggers a conformational change in the translocation domain, allowing it to penetrate into the vesicle membrane (Montal 2010). This forms a trans-membrane channel that chaperones the partially unfolded light chain into the cytosol. Reduction of the disulphide bond between the heavy and light chains occurs after translocation into the cytosol, releasing the light chain to carry out its enzymatic action (Rossetto, Pirazzini et al. 2014). The light chain is a sequence specific metalloprotease that cleaves neuronal soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), which are involved in

synaptic vesicle exocytosis and neurotransmitter release (Schiavo, Matteoli et al. 2000). The proteases within the different serotypes have different SNARE specificities. BoNT/B, D, F and G cleave the vesicle associated membrane protein (VAMP), also known as synaptobrevin; BoNT/A and E cleave synaptosomal-associated protein of 25 kDa (SNAP25); and BoNT/C cleaves both SNAP25 and the syntaxin (Verderio, Rossetto et al. 2006). These are the three main constituents of the SNARE complex and they are needed to bring the vesicular membrane in close enough proximity to the plasma membrane for membrane fusion to occur and neurotransmitter release (Schiavo, Matteoli et al. 2000). Cleavage of any of these three SNAREs by BoNTs results in the potent block of synaptic signalling that underlies flaccid paralysis and botulism.

1.4.2 Synaptic vesicle fusion

Intracellular membrane fusion is a fundamental process carried out universally in eukaryotic cells that is necessary for a diverse range of cellular processes including exocytosis and endocytosis, exchange of lipids, metabolites and proteins, and organelle biogenesis (Jahn, Lang et al. 2003). Successful fusion requires three basic components; Rab-GTPases which organise the fusion site; SNARE proteins which are necessary during the fusion process; and N-ethylmaleimide-sensitive factor (NSF) which is required for recycling of the fusion machinery (Ungermann and Langosch 2005). Vesicle tethering is the earliest event in membrane fusion which precedes SNARE complex formation and involves an initial association between the vesicle and target membrane with links that extend over distances of around half the diameter of the vesicle (>25nm) (Pfeffer 1999). These links are generated by the recruitment of a large fibrous protein complex by the action of Rab-GTPases (Rizo and Sudhof 2002). Rab-GTPases are a large family of membrane bound enzymes which are important in a number of secretory and endocytic pathways (Schimmoller, Simon et al. 1998). Cycling between an active GTP-bound form and an inactive GDP-bound form alters the conformation of Rab proteins which regulates tethering factor recruitment (Waters and Hughson 2000). Tethering factors vary depending on the fusion event and can consist of long coiled coil tethers (such as p115 which is required for trafficking between the ER and Golgi) or multi-protein complexes (such as the exocyst which is an octameric complex that tethers secretory vesicles to the plasma membrane) (Hutagalung and Novick 2011).

Following tethering, formation of the SNARE complex brings the vesicle into close enough proximity to the plasma membrane for membrane fusion to occur. There are over 35 different SNARE proteins in mammalian cells and, although their structures are diverse, they all share a common SNARE motif of approximately 60 amino acids necessary for complex formation (Weimbs, Low et al. 1997). SNAREs can be broadly split into two categories; SNAREs located on the plasma, or target membrane

(t-SNAREs); and SNAREs located on the vesicular membrane (v-SNAREs) (Söllner, Bennett et al. 1993). The unique intracellular localisations of these SNARE proteins led to the SNARE hypothesis, which proposes that the formation of specific SNARE complexes is the main mediator of specificity in vesicle targeting (Söllner, Whiteheart et al. 1993). The SNAREs involved in synaptic vesicle fusion are the t-SNAREs SNAP25 and syntaxin 1, and the v-SNARE VAMP1 or 2. The high specificity of BoNTs for these proteins has made them valuable tools for dissecting the mechanism behind membrane fusion (Rizo and Sudhof 2002). During fusion these SNAREs associate to form what is known as the core complex. This complex consists of four SNARE motifs, two from SNAP25 and one each from syntaxin 1 and VAMP, which form a parallel four-helix bundle on assembly (Rizo and Sudhof 2002). Of the four helices involved, three must contain a central Gln (Q) residue and one must contain an Arg (R) residue in a buried, hydrophobic layer. Different SNAREs contribute different helices which has led to an alternative nomenclature of Gln containing Q-SNAREs (SNAP25 and syntaxin 1) and Arg containing R-SNAREs (VAMP) (Montal 2010). The complex formed is resistant to both BoNT cleavage and denaturation by sodium dodecyl sulphate (SDS), suggesting that it is an incredibly stable complex (Hayashi, McMahon et al. 1994). SNARE complex formation is initiated by N to C terminal zippering of the *trans*-SNARE complex which bridges the gap between the vesicular and target membranes (Südhof and Rizo 2011). This zippering stresses the membranes leading to fusion-pore opening. Complex formation is also thought to release the energy necessary to overcome the energy barrier of membrane fusion (Rizo and Sudhof 2002). Following fusion, the opening of the fusion-pore converts the *trans*-SNARE complex to a *cis*-SNARE complex leading to disassembly by the ATPase activity of NSF which binds to the complex via soluble NSF attachment proteins (SNAPs, no relation to SNAP25) (Söllner, Bennett et al. 1993).

Sec1/Munc18-like (SM) proteins are hydrophilic 60-70 kDa polypeptides that are essential for membrane fusion. Mammalian uncoordinated-18-1 (Munc18-1) was the first SM protein to be linked to membrane fusion in mammalian cells and is of particular importance in synaptic vesicle exocytosis (Hata, Slaughter et al. 1993). The role of Munc18-1 in vesicle fusion remains enigmatic. Early studies found that it binds to the closed conformation of syntaxin 1 and hinders SNARE complex assembly (Dulubova, Sugita et al. 1999). However, knockout of Munc18-1 in mice leads to a complete block of neurotransmitter release, which is contradictory to a solely inhibitory role of Munc18-1 (Verhage, Maia et al. 2000). Levels of syntaxin 1 are reduced by around 70% in Munc18-1 knockout mice and it has therefore been proposed that Munc18-1 acts as a chaperone that stabilises syntaxin 1 and directs the protein to the active zone of exocytosis (Toonen, de Vries et al. 2005). The essential role of Munc18-1 remains unclear, but it has been suggested that, upon reaching the active zone, a conformational change in syntaxin 1 to an open state allows translocation of Munc18-1 to the

SNARE motifs where it facilitates assembly of the four-helix bundle and may act to prevent premature dissociation of the SNARE complex by NSF (Rizo and Südhof 2012). This model of Munc18-1 function is supported by the finding Munc13, which is present at the active zone, plays a key role in the conformational transition of syntaxin 1 (Rizo and Sudhof 2002). The activity of Munc13 appears to be dependent upon interaction with Rab3 interacting molecule (RIM), a protein localised to the active zone which has a possible role in vesicle tethering (Betz, Thakur et al. 2001).

Synaptic vesicle fusion is particularly complex as it requires a high level of spatial and temporal precision. When an action potential reaches the nerve terminal, an inflow of Ca^{2+} stimulates vesicle exocytosis which occurs in the sub-millisecond range (Sudhof 2013). Docking of the vesicles at the synapse followed by vesicle priming leads to a readily releasable pool of fusion competent synaptic vesicles that can rapidly fuse upon Ca^{2+} stimulation (Imig, Min et al. 2014). Recent data suggests that tethering of the vesicle to the membrane leads to clustering of syntaxin 1 and Munc18-1 at the docking site (Gandasi and Barg 2014). These clusters interact with Rab3 on the vesicle membrane to stabilise the docked state of the vesicle and prevent dissociation from the target membrane. Subsequent recruitment of Munc13, RIMs, SNAP25 and VAMP leads to the formation of a partially assembled *trans*-SNARE complex and vesicle priming. Calcium channels are also recruited to the docked vesicle via RIMs which form a complex with Munc13 and Rab3; this allows for localised calcium influx to the active zone (Wang, Okamoto et al. 1997). Stimulation by Ca^{2+} then triggers tight SNARE complex formation and membrane fusion. The influx of Ca^{2+} influences the SNARE complex via the synaptotagmin 1 and complexin proteins (Rizo and Xu 2015). Synaptotagmin 1 is a transmembrane protein that acts as a calcium sensor for Ca^{2+} mediated membrane fusion. The protein contains two C_2 domains in its cytoplasmic region which interact with Ca^{2+} causing a conformational change that leads to an increased affinity for the SNARE complex and binding to phospholipids in the target membrane (Sudhof 2013). This pulls the membranes closer together, facilitating SNARE action and membrane fusion. Complexins are small soluble proteins that insert into a groove of the SNARE complex which play both active and inhibitory roles in neurotransmitter release (Sudhof 2013). It has been proposed that complexins simultaneously promote the assembly of SNARE complexes and block completion of the fusion reaction, leading to a metastable SNARE state and rapid fusion following Ca^{2+} influx (Tang, Maximov et al. 2006). Activation of synaptotagmin 1 displaces complexin which drives SNARE complex mediated membrane fusion. Therefore, in periods of low Ca^{2+} when synaptotagmin 1 is inactive, complexin acts as an inhibitor of membrane fusion.

1.4.3 Current uses of botulinum neurotoxins in medicine

Synaptic vesicle fusion is a highly complex and exquisitely regulated process which coordinates nerve firing with neurotransmitter release. BoNTs have developed evolutionarily over millions of years to target this release with a high level of specificity, by both targeting to neurons and cleaving neuronal SNAREs. This potent activity has been exploited for use in the treatment of a number of neurological conditions as well as cosmetically to reduce facial wrinkles.

BoNTs primarily act by blocking acetylcholine release at neuromuscular junctions which leads to the muscles becoming clinically weak and atrophic (Munchau and Bhatia 2000). The blockage of neurotransmitter release is irreversible, and function is only recovered by the sprouting of nerve terminals and formation of new junctions which can take 2-3 months (de Paiva, Meunier et al. 1999). The BoNT that is most often used in medicine is BoNT/A, of which various commercial preparations are available. OnabotulinumtoxinA (commercially known as BOTOX) is the most commonly used preparation and contains a number of non-toxic accessory proteins which act to stabilise the toxin and protect it from degradation (Brin 2009). Local injection is reasonably well tolerated as the toxin stays concentrated in the target area with a decrease in effect as it diffuses away from the injection site (Munchau and Bhatia 2000). The minute amount of toxin injected also means that immunogenicity rates are very low when compared to other protein biologics, making repeat injections possible (Naumann, Boo et al. 2013). BoNT/A is used to treat a wide variety of disorders caused by muscle over activity such as strabismus and dystonia. Strabismus is a condition that interferes with binocular vision by misalignment of the eyes and an inability to direct both eyes simultaneously to the same focal point. Local BOTOX injection into ocular muscles can relax specific muscles, allowing for realignment of the eyes by shortening of the non-injected antagonist muscle (Munchau and Bhatia 2000). Dystonia occurs as a result of sustained muscle contractions which lead to twitching and spasming of the affected muscle. BoNT/A is the treatment of choice for focal dystonia, which affects specific areas of the body with little spread. The most common forms of focal dystonia are cervical dystonia (abnormal head twisting), blepharospasm (involuntary closure of the eyelids), oromandibular dystonia (forceful contractions of the face and jaw), laryngeal dystonia (vocal fold spasms which impair verbal communication) and limb dystonia (abnormal limb movement) (Truong 2012). Treatment of dystonia with BoNT/A involves local intramuscular injection into the affected area and careful dosing so that the overactive muscle activity is abrogated without impairment of normal muscle function.

Early uses of BoNT/A in medicine focused on the inhibition of transmitter release from neuromuscular junctions and the somatic nervous system. Findings that BoNT/A can also target

autonomic nerve terminals has led to introduction of BoNT/A in the treatment of a widening number of conditions involving autonomic innervation. BoNT/A now has a use in the treatment of hypersecretory disorders such as sialorrhea (excessive drooling) and focal hyperhidrosis (excessive sweating). Sialorrhea is a complication of a number of different neurological conditions including amyotrophic lateral sclerosis and Parkinson's disease, and poses choking risks as well as affecting patient quality of life. BoNT/A functions by blocking acetylcholine release from autonomic neurons which innervate the salivary gland (Bhidayasiri and Truong 2005). Focal hyperhidrosis usually affects the palms and soles of the feet, armpits and the face. Mild hyperhidrosis can be treated by administration of topical antiperspirants, but BoNT/A has been shown to be effective in the treatment of severe cases, leading to a decrease in perspiration that lasts between 4 and 12 months post injection (Bhidayasiri and Truong 2005). The exact mechanism by which BoNT/A functions is unclear, but one study demonstrated that the toxin leads to a decrease in lumen diameter within the sweat gland which suggests denervation (Swartling, Naver et al. 2004). Chronic pain relief is another area of medicine that is seeing an increasing use of the BoNT/A toxin. Chronic migraine is a debilitating headache that has a number of causative factors and is characterised by headaches that occur 15 or more days a month (Gooriah and Ahmed 2015). Chronic migraine arises as a gradual progression from episodic migraine due to an increase in atypical pain processing, central and peripheral sensitisation, cortical hyperexcitability and neurogenic inflammation (Gooriah and Ahmed 2015). BOTOX has been approved for use in the treatment of chronic migraine after a series of clinical trials showed a reduction in headache days and an increase in quality of life following BOTOX injection (Aoki and Francis 2011). However, its exact mechanism of action and overall efficacy remains unclear. A number of pre-clinical studies have shown a direct anti-nociceptive role of BoNT/A by inhibition of the release of pain neuromodulators, such as substance P and calcitonin gene-related peptide (CGRP), from peripheral nociceptive neurons (Aoki and Francis 2011). Indeed BOTOX has recently been show to decrease CGRP plasma levels in chronic migraine patients (Cernuda-Morollon, Ramon et al. 2015). This reduction in peripheral sensitisation likely results in the indirect inhibition of central sensitisation (Gooriah and Ahmed 2015).

A commercial BoNT/B preparation, MYOBLOC, gained FDA approval in 2000 for the treatment of cervical dystonia and is now used as an alternative to BoNT/A in a wide range of disorders (Bhidayasiri and Truong 2005). BoNT/B has a different substrate specificity to BoNT/A (cleavage of VAMP instead of SNAP25) but exhibits similar effects in patients. Main differences observed appear to be a more rapid onset of action, but shorter duration (11 week duration for MYOBLOC versus 17 weeks for BOTOX in the treatment of laryngeal dystonia) (Blitzer 2005). The similar effects observed with BoNT/B make it particularly useful for the treatment of patients that have developed resistance

to the BoNT/A toxin (Figgitt and Noble 2002). BoNT/E and F have also been investigated but show a much shorter duration of action (Carruthers 2003). This may be of use in future treatments where short-term nerve inhibition is desirable, such as during facial surgery or after trauma (Sunil, Babu et al. 2015).

Development of novel BoNTs that target specific neuronal subpopulations or non-neuronal cells is ongoing to expand the possible therapeutic uses of BoNTs. This is generally done by replacing the targeting domain of the BoNT whilst maintaining the translocation and catalytic domains. For instance, a novel toxin containing the light chain (catalytic domain) and translocation domain of BoNT/C (LcTd/C) fused to EGF was able to bind and internalise into a human respiratory epithelial cell line, causing a block of mucin secretion by cleavage of non-neuronal syntaxin proteins (Foster, Adams et al. 2006). This could have future uses in the treatment of hyper-secretion dependent conditions such as chronic obstructive pulmonary disease and asthma. Fusion of a modified growth hormone-releasing hormone to LcTd/D led to VAMP2 cleavage in somatotropic cells in rats (Somm, Bonnet et al. 2012). This caused a decrease in insulin growth factor 1 (IGF1) production and a subsequent decrease in body weight and length. This study demonstrates that retargeted BoNTs can be effectively used to selectively inhibit non-neuronal secretion *in* vivo. A further study utilised a novel stapling technique to attach various different ligands to LcTd/A, which allowed for specific targeting of different neuroendocrine cells as well as a number of neuronal sub-populations (Arsenault, Ferrari et al. 2013). This is encouraging as it could be used to inhibit the release of specific neurotransmitters, such as CGRP release by nociceptor neurons, and bypass the paralytic effects of the toxin.

1.4.4 Cytotoxic botulinum toxins

The majority of BoNT serotypes cause a block of neurotransmitter release with no effect on the viability of the neuron. However, BoNT/C and more recently BoNT/E have been shown to induce neurodegeneration and apoptosis in neurons. Treatment of mature mouse spinal cord neurons with BoNT/C led to enlargement of synaptic terminals followed by degeneration of axons, dendrites and cell bodies (Williamson and Neale 1998). This effect was neuron specific as co-cultured glial cells were unaffected. The same process of neurodegeneration and cell death following BoNT/C treatment was observed in a separate study using cultured rat hippocampal neurons (Osen-Sand, Staple et al. 1996). Both of these studies carried out a direct comparison with BoNT/A which showed no effect on neuron viability. A more detailed investigation into the effects of BoNT/C showed that initial neurodegenerative effects were due to an active mechanism, as evidenced by the ability of fragmented processes to maintain membrane integrity and the presence of functional mitochondria

along the neurites (Berliocchi, Fava et al. 2005). This initial process was also shown to occur in a caspase-independent manner and preceded the caspase-dependent apoptotic demise of the cell bodies, suggesting the initiation of two distinct programs of neurodegeneration and apoptosis by BoNT/C. A study investigating the role of SNARE cleavage in the cytotoxic effects of BoNT/C suggested that the simultaneous cleavage of both SNAP25 and syntaxin 1 causes a block of plasma membrane recycling processes, independently of synaptic vesicle exocytosis, that are necessary for cell viability (Peng, Liu et al. 2013). This was demonstrated by the exogenous expression of non-neuronal SNAP23 and syntaxins 2, 3 and 4 which are resistant to BoNT/C cleavage and were able to rescue the cells from the cytotoxic effect of BoNT/C. These non-neuronal SNAREs are unable to carry out the rapid Ca^{2+} regulated vesicle fusion needed for neurotransmitter release, suggesting a separate process is disrupted. It was also shown that BoNT/C does not cause a block of constitutive exocytosis, but likely exerts its effects by blocking of membrane recycling exocytosis, as evidenced by an attenuation of BoNT/C cytotoxicity following general inhibition of endocytosis. This study suggests that mature neurons do not express the non-neuronal SNAREs such as SNAP23 and syntaxins 2-4 which carry out important roles in cell trafficking in non-neuronal cells. Neurons instead utilise SNAP25 and syntaxin 1 for these processes, which makes them susceptible to the dual cleavage of SNAP25 and syntaxin 1 by BoNT/C. The importance of SNAP23 in cell viability has recently been demonstrated in a study which showed that knockdown of SNAP23 leads to cell death in a number of cell models (Kaul, Mittal et al. 2015). The same study by Peng et al. showed for the first time that BoNT/E is also cytotoxic to neurons (Peng, Liu et al. 2013). BoNT/E, like the non-cytotoxic BoNT/A, cleaves only SNAP25. The difference in activity of the two toxins is explained by the fact that BoNT/E cleaves 26 amino acids from the C-terminal of SNAP25 whereas BoNT/A only cleaves 9 amino acids. It was therefore postulated that SNAP25 cleavage by BoNT/A attenuates the function of SNAP25 without completely abolishing its activity. Indeed a recent study shows that BoNT/E cleaved SNAP25 is completely unable to form the SNARE complex, but BoNT/A cleaved SNAP25 can still form a SNARE complex, although its ability to form fusion pores is decreased (Lu 2015). As with BoNT/C, neuron viability was completely restored by the exogenous expression of the non-neuronal SNARE homologue, in this case SNAP23 (Peng, Liu et al. 2013).

The selective neurotoxic effects of BoNT/C and E may be of use in the treatment of neuroblastoma, as these cells are of neuronal origin and express neuronal SNAREs. Indeed, a recent study showed that co-delivery of the BoNT/C and D proteases into neuroblastoma cells led to a potent induction of apoptosis (Arsenault, Cuijpers et al. 2013). It was suggested that this effect was due to the cleavage of all three SNAREs, leading to the generation of toxic SNARE fragments and interruption of important cell trafficking events. Administration of BoNT/C alone was found to be non-toxic in the

mouse neuroblastoma cell line N2a, and this is likely due to the expression of non-neuronal SNAREs that are able to compensate for SNAP25 and syntaxin 1 cleavage (Peng, Liu et al. 2013). However, as mentioned previously, current treatment of neuroblastoma involves the use of high-dose retinoic acid therapy which induces neuronal differentiation *in vivo* (Reynolds, Matthay et al. 2003). It would be interesting, therefore, to investigate in closer detail the effect of BoNT/C on neuroblastoma cells following neuronal differentiation. It has been demonstrated that BoNTs can be retargeted to specific cell types in a targeted toxin approach (Somm, Bonnet et al. 2012, Arsenault, Ferrari et al. 2013). Re-targeting to differentiated neuroblastoma cells may help to prevent relapse in advanced stage neuroblastoma and show a high level of specificity not seen with current targeted toxins in cancer therapy.

1.5 Aims of this thesis

Despite a number of advances in the generation of immunotoxins for cancer therapy, three main problems persist that have limited their clinical use. These are immunogenicity, efficient delivery into the cytosol of target cells, and dose-limiting side effects caused by off-target toxicity. This thesis aims to address the issue of off-target toxicity by investigating the action of two novel enzymatic payloads that have an alternative and potentially more selective cytotoxic mechanism than currently used enzymes, particularly in neuroblastoma.

Large polypeptides such as enzymes are unable to enter the cell unaided due to the impermeable nature of the cell membrane. Chapter 3 aims to further investigate previous findings that DNA lipofection reagents can be used to deliver protein into cells by testing the ability of a number of different transfection reagents to deliver enzymes into different cell lines. This will allow for the delivery of the novel payloads into cells without the need to generate a targeted fusion protein.

In chapter 4, the effect of BLF1 on cell proliferation and viability of neuroblastoma cells will be investigated. Previous studies using small molecule inhibitors of eIF4A have shown high potency in Myc-driven tumours. Whether this is true for MYCN driven neuroblastoma will be tested by the use of cell lines that have different MYCN amplification status. The SHEP-TET21N MYCN inducible cell line will also be used to analyse the direct effect of MYCN expression on enzyme potency.

Neuronal differentiation of neuroblastoma cells with retinoic acid is part of the treatment regimen for advanced stage disease. This differentiation may make cells susceptible to the cytotoxic effects of the BoNT/C protease as has been observed in primary neurons. This will be investigated in chapter 5 by differentiating neuroblastoma cells with retinoic acid *in vitro* and assaying the effect of BoNT/C

and its protease on cell viability. Potency will be directly compared to traditional immunotoxin payloads to confirm the validity of this protease in future immunotoxin development

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell lines

All cell lines used for experiments are listed below:

Cell line	Species	Disease	Cell type	Supplier
A549	Human	Carcinoma	Epithelial	Gift from Lynda Partridge
Caco-2	Human	Colorectal adenocarcinoma	Epithelial	Gift from Kai Erdmann
IMR-32	Human	Neuroblastoma	Neuroblast	Gift from Peter Andrews
J774.2	Mouse	Reticulum cell sarcoma	Macrophage	Gift from Lynda Partridge
LA-N-5	Human	Neuroblastoma	Neuroblast	Children's Oncology Group
LA-N-6	Human	Neuroblastoma	Neuroblast	Children's Oncology Group
N2a	Mouse	Neuroblastoma	Neuroblast	ATCC
QGP-1	Human	Islet cell carcinoma	Islet-cell	JCRB
RAW 264.7	Mouse	Abelson murine leukaemia	Macrophage	Gift from Lynda Partridge
SH-SY5Y	Human	Neuroblastoma	Neuroblast	Sigma
SHEP-TET21N	Human	Neuroblastoma	Neuroblast	Gift from Manfred Schwab
SiMa	Human	Neuroblastoma	Neuroblast	DSMZ
SK-N-BE(2)	Human	Neuroblastoma	Neuroblast	Children's Oncology Group
SK-N-FI	Human	Neuroblastoma	Neuroblast	Children's Oncology Group
SK-N-SH	Human	Neuroblastoma	Neuroblast	Gift from Peter Andrews

Table 2.1 Details of cell lines used

2.1.2 Enzymes and proteins

Enzymes and proteins used to treat cells are listed in the table below:

Protein	Source	Supplier	Reference
BLF1	Recombinant	Gift from David Rice	(Cruz-Migoni, Hautbergue et al. 2011)
mChe-BLF1	Recombinant	Gift from Guillaume Hautbergue	(Rust, Hassan et al. 2015)
mChe-BLF1 C94S	Recombinant	Gift from Guillaume Hautbergue	(Rust, Hassan et al. 2015)
Diphtheria enzyme	Recombinant	Generated by Dhevahi Niranjan (Davletov lab)	(Rust, Hassan et al. 2015)
Ricin enzyme	Recombinant	Generated by Dhevahi Niranjan (Davletov lab)	(Rust, Hassan et al. 2015)
BoNT/A	Recombinant	Gift from Thomas Binz	(Strotmeier, Institut für Toxikologie et al. 2016)
BoNT/C	Recombinant	Gift from Thomas Binz	(Strotmeier, Institut für Toxikologie et al. 2016)
BoNT/C Cy3-Rbd	Recombinant	Generated by Charlotte Leese (Davletov lab)	(Rust, Leese et al. 2016)
C Protease	Recombinant	Gift from Thomas Binz	(Arsenault, Cuijpers et al. 2013)
D Protease	Recombinant	Gift from Thomas Binz	(Arsenault, Cuijpers et al. 2013)
Gelonin	Extracted from *Gelonium Isoform*	Enzo Life Sciences	
Saporin	Extracted from *Saponaria Officinalis* seeds	Sigma	

Table 2.2 Details of proteins and enzymes used

2.1.3 Antibodies

All primary antibodies used for Western blotting (WB) and immunocytochemistry (ICC) are listed below:

Primary Antibody	Species	Dilutions	Supplier
CDK4	Rabbit	1:1000 (WB)	Abcam
Cyclin D1	Mouse	1:500 (WB)	Abcam
eIF4A I	Rabbit	1:1000 (WB)	Cell Signalling
GAPDH	Rabbit	1:5000 (WB)	Thermo-Scientific
GT1b	Rabbit	1:500 (ICC)	Millipore
MYCN	Mouse	1:500 (WB)	Santa Cruz
SNAP23	Rabbit	1:4000 (WB)	Synaptic Systems
SNAP25	Rabbit	1:4000 (WB), 1:500 (ICC)	In-house
Syntaxin 1	Rabbit	1:1000 (WB)	In-house
Syntaxin 2	Rabbit	1:1000 (WB)	Sigma
Syntaxin 3	Rabbit	1:1000 (WB)	Synaptic Systems
Syntaxin 4	Rabbit	1:1000 (WB)	Synaptic Systems
VAMP3	Rabbit	1:2000 (WB)	Gift from Andrew Peden
SV2A	Rabbit	1:2000 (WB)	Synaptic Systems
Tau	Rabbit	1:1000 (WB)	Sigma
α-tubulin	Mouse	1:6000 (WB)	Sigma
β-III-tubulin	Mouse	1:2000 (WB), 1:1000 (ICC)	R&D Systems

Table 2.3 Details of primary antibodies used

All secondary antibodies used for WB and ICC are listed below:

Secondary Antibody	Dilutions	Supplier
Sheep anti-mouse (HRP tag)	1:12000 (WB)	Amersham
Donkey anti-rabbit (HRP tag)	1:24000 (WB)	Amersham
Goat anti-mouse (Alexa Fluor 488 tag)	1:2000 (ICC)	Life Technologies
Goat anti-rabbit (Alexa Fluor 488 tag)	1:2000 (ICC)	Life Technologies

Table 2.4 Details of secondary antibodies used

2.2 Cell culture

2.2.1 Cell line cultures

All cell lines were cultured in a 37 °C, 5 % CO_2 incubator and passaged twice a week. A549, IMR-32, J774.2, N2a and RAW 264.7 cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies) supplemented with 10 % foetal bovine serum (FBS) (Life Technologies). LA-N-5, LA-N-6, QGP-1, SHEP-TET21N, SiMa, SK-N-BE(2) and SK-N-FI cell lines were cultured in RPMI 1640 (Life Technologies) with 10 % FBS. Caco-2 and SK-N-SH cell lines were cultured in minimum essential media (MEM) (Life Technologies) with 10 % FBS and the SH-SY5Y cell line was cultured in a 50:50 ratio of MEM and F12 (Life Technologies) with 1 % non-essential amino acids (NEAA) (Life Technologies) and 15 % FBS.

All cell lines were passaged using 0.25 % trypsin-EDTA (Sigma), apart from the J774.2 and RAW 264.7 cell lines which were passaged via cell scraping. For viable cell counting, cells were diluted 1:1 in trypan blue dye (Biorad) and counted using an automated cell counter (Biorad).

Unless otherwise indicated, A549, N2a, J774.2, RAW 264.7 and Caco-2 cells were seeded at a density of 5 $x10^3$ cells per well in 96-well plates, and 2 $x10^4$ cells per well in 48-well plates. LA-N-5, LA-N-6, QGP-1, SH-SY5Y, IMR-32, SK-N-BE(2), SK-N-FI, SK-N-SH and SiMa cells were seeded at a density of 1 $x10^4$ cells per well in 96-well plates and 5 $x10^4$ cells per well in 48-well plates.

2.2.2 Protein transduction

After seeding, cells were left for at least 5 hours to attach. Proteins were pre-incubated at 10 times the final concentration for 20 minutes at 20 ^oC in Optimem (Life Technologies) with or without transfection reagents at dilutions recommended by the manufacturer: Lipofectamine 3000 (Life Technologies), 1:50; Lipofectamine LTX (Life Technologies), 1:40; Lipofectamine 2000 (Life Technologies), 1:50; Metafectene (Biontex), 1:40; and Proteofectene (Biontex), 1:40. Following the 20 minute incubation, 10 µl (for 96-well) or 30 µl (for 48-well) of protein was added to the wells. Cells were incubated at 37 ^oC and 5% CO_2 with the indicated proteins and transfection reagents for 72 h before being assayed.

2.2.3 Neuroblastoma cell line differentiation

For laminin coating, culture plates were pre-coated with 10 µg/ml laminin (Sigma) in phosphate buffered saline (PBS) and left for at least 1 hour at 37 °C. Wells were washed twice PBS immediately before cell seeding. For collagen coating, Type 1 Collagen solution (Sigma) was diluted 100 times in PBS before adding to plates and allowing to bind for 3 hours at 37°C. Excess solution was then

removed and plates were left at 20°C overnight to dry. For Poly-L-lysine, a 0.1% solution (Sigma) was diluted to 0.01% in PBS and added to wells. After 30 minutes, the wells were washed three times with PBS before leaving to dry overnight at 20°C.

After cell counting, cells were centrifuged at 800 xg for 5 minutes and the pellet was re-suspended in the required amount of differentiation media. The differentiation media consists of RPMI with 1x B27 supplement (Life Technologies), 1 % NEAA, 1 % HEPES (Fisher) and 10 µM all-trans-retinoic acid (Sigma). Cells were plated at a density of 1 $x10^4$ cells per well in a 96-well plate or 2 $x10^4$ cells per well in a 48-well plate. SiMa cells were left for 72 hours and SH-SY5Y cells were left for 144 hours to differentiate.

2.2.4 Primary mouse fibroblast culture

C57BL/6 mice were sacrificed using a humane method as listed in Schedule 1 of the Animal (Scientific procedure) Act 1986. Small segments of tissue (approximately 3 mm^2) were taken from the ear using scissors and placed in 500 µl Hank's balanced salt solution (HBSS) (Life Technologies) in a 1.5 ml tube. The tissue was then transferred to a 6 cm dish and diced into small pieces using razor blades. The tissue was placed back in the 1.5 ml tube and 500 µl of 4000 U/ml collagenase XI (Sigma) was added to give a final concentration of 2000 U/ml. The sample was incubated for 25 minutes at 37 °C and then centrifuged at 800 xg for 5 minutes. The supernatant was carefully discarded and the sample was washed with 1 ml HBSS by mixing and centrifuging. The HBSS was discarded and 500 µl of 0.05 % trypsin was added, followed by a 20 minute incubation at 37 °C. The supernatant was discarded and the tissue was re-suspended in 500 µl fibroblast media (DMEM + 1 % NEAA + 1 % penicillin/streptomycin (P/S) (Sigma) + 10 % FBS), followed by trituration to break up cell aggregates. The suspension was plated in a 3 cm dish and a further 2 ml of fibroblast media was added followed by incubation at 37 °C for 3 to 4 days to allow cell growth. Cells were expanded to a 25 cm^2 flask followed by a 75 cm^2 flask. Sub-culturing was carried out every 3-4 days when spent media was replaced or cells were passaged.

For viability studies, cells were seeded in a 96-well plate and grown for 3-4 days until fully confluent. The media was then changed to low serum (0.1% FBS) and cells were cultured for a further 1-2 days before treatment with proteins.

2.3 SDS-Page and Western blotting

2.3.1 Cell protein extraction: 4x SDS sample buffer

Media was removed and cells were washed once with PBS. The 4x SDS sample buffer (224 mM SDS, 250 mM Tris-HCl pH 6.8, 6.4 mM EDTA, 25 % glycerol and traces of bromophenol blue) was diluted to 1x in distilled water and supplemented with 1 mM $MgCl_2$ and 0.1 % benzonase (Sigma). For 48-well plates, 60 µl of sample buffer was added directly on to the cells followed by shaking at room temperature, 650 rpm for 10 minutes to lyse cells. Lysates were collected in 1.5 ml tubes, boiled for 3 to 4 minutes at 90 °C and centrifuged at 16 000 xg for 1 minute. Lysates were loaded directly onto a gel or stored at -20 °C until needed.

2.3.2 Cell protein extraction: RIPA buffer

In chapter 4, cell lysates were collected on ice using RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 % SDS, 0.5 % sodium deoxycholate, 1 % NP-40). Media was removed and cells were washed once with PBS. For 24-well plates, 60 µl of RIPA buffer was added to the wells and cells were detached via scraping and transferred to 1.5 ml tubes. They were incubated for 20 minutes on ice and vortexed every 2-3 minutes, before being centrifuged at 4 °C, 16 000 xg for 15 minutes. The supernatants were transferred to new tubes and the pellets were discarded. Lysates were stored at -20 °C until needed. Before loading on a gel, lysates were mixed with 4x SDS sample buffer to give a 1x concentration and boiled for 3-4 minutes at 90 °C.

2.3.3 Lysate protein quantification

Protein concentration of lysates was measured using the detergent compatible (DC) assay (Biorad) according to manufacturer's instructions. This assay is based on the Lowry assay and relies on the reaction of protein with copper in an alkaline solution followed by reduction of Folin reagent by the copper-treated protein. Reduction of the Folin reagent leads to a change in colour that can be measured by absorbance. A standard curve was prepared by diluting bovine serum albumin (BSA) (Sigma) in the required sample buffer at the following concentrations: 1.5 mg/ml, 0.75 mg/ml, 0.325 mg/ml, 0.1625 mg/ml and 0 mg/ml. 5 µl of standards and samples were pipetted into a 96-well plate. Reagent S (surfactant solution) was diluted 1:50 in Reagent A (alkaline copper tartrate solution) to make Reagent A[1] and 25 µl of this was added to the standards or samples. 200 µl of Reagent B (Folin reagent) was added to each well and the plate was incubated in the dark at room

temperature for 15 minutes. Absorbance was read on a plate reader (Biorad) at 750 nm and concentrations of lysate were calculated using the standard curve.

2.3.4 SDS-PAGE and gel staining

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), NuPAGE 12 % Bis-Tris pre-cast gels (Life Technologies) were placed in an XCell SureLock system (Life Technologies) which was filled with 20x NuPAGE MES SDS running buffer (Life Technologies) diluted to 1x in distilled water. One lane of the gel was loaded with 5µl of Dual Colour protein ladder (Biorad) and 14µl of lysate or protein was loaded per lane. The gel was run at a constant voltage of 180 V for 60 minutes or until the dye reached the end of the gel. For investigating SNAP25 cleavage, the gel was run for 90 minutes before replacing the running buffer and running for a further 60 minutes.

Gels were stained using Coomassie blue dye (200ml methanol, 1 tip of Coomassie brilliant blue (Biorad), 700ml dH_2O and 100ml glacial acetic acid) heated for 1 minute in a microwave and left on a rocker at room temperature for 20 minutes. The gel was then de-stained in distilled water for 24-48 hours before bands were observed. Images were taken on an XRS imaging system (Biorad).

2.3.5 Western blotting

Western blotting of unstained gels was carried out in a Mini Trans Blot Cell (Biorad) containing transfer buffer (distilled water, 20 % methanol and 10 % 10x transfer buffer (Fluka)) and a cooling unit to prevent over-heating. The transfer cassette was set up using a foam pad followed by Whatmann paper, the gel, an Immun-Blot PVDF membrane (Biorad) pre-wetted in methanol, Whatmann paper and finally a second foam pad. All components were soaked in transfer buffer before assembly. The transfer was run at a constant current of 250 mA for 60 min and transfer efficiency was assessed by transfer of the protein ladder onto the membrane.

Blocking solution was prepared by diluting skim milk powder (Sigma) to 5 % in PBS-T (PBS with 0.1 % Tween 20 (Fisher)). After protein transfer the membrane was incubated for 30 minutes in blocking solution before adding the primary antibodies (for dilutions, see table 2.3). The membrane was incubated with the primary antibody for 60 minutes at room temperature or overnight at 4 °C. The membrane was washed (3x 5 minutes) in PBS-T to remove unbound antibody and was then incubated in secondary antibody (for dilutions, see table 2.4), diluted in blocking solution, for 30 minutes at room temperature. After a further 3x 5 minute washes, bound secondary antibody was detected by enhanced chemiluminescence using the SuperSignal West Dura extended duration substrate (Thermo Scientific). Luminescence was detected on X-ray film (Amersham) and developed

using an Optimax 2010 X-ray developer (Protec). Full blots for all developed films are shown in Appendix Figure 1.

2.4 Cell count and viability assays

2.4.1 Automated cell counts

To directly count cells grown in 96-well plates, an automated cell counter was used (Biorad). Before counting, media was removed and cells were washed with 40µl PBS which was collected in a 0.5ml tube. 40µl of trypsin was then placed onto the cells and left for 2 to 3 minutes at 37°C or until cells had detached. The trypsin was then added to the PBS and 10µl was transferred to a disposable slide for counting.

2.4.2 CCK-8 assay

The Cell Counting Kit-8 (CCK-8) (Sigma) was used to ascertain cell numbers after treatment with enzymes. The kit uses a tetrazolium-based salt that produces a formazan dye in the reducing conditions of the cytoplasm of viable cells. Cells were incubated for 72 hours with treatments before the CCK-8 reagent was added in a 1:10 dilution and left for 1 to 2 hours depending on cell type. Absorbance was then measured on a plate reader (Biorad) at 450nm. Readings observed in the absence of any treatment were taken as 100% after normalisation to a blank control (cell-free medium with CCK-8 reagent).

2.4.3 alamarBlue

The alamarBlue reagent (Life Technologies) contains resazurin which becomes fluorescent in the reducing conditions of the cytoplasm of viable cells. It was used as an alternative to the CCK-8 reagent in some experiments to measure cell numbers. Following 72 hour treatment of cells, the alamarBlue reagent was added to cells in a 1:10 dilution followed by a 4 hour incubation at 37°C. Fluorescence was then measured at 560nm excitation/590nm emission using a Fluoroskan Ascent plate reader (Labsystems). Readings observed in the absence of any treatment were taken as 100% after normalisation to a blank control (cell-free medium with alamarBlue reagent).

2.4.4 Hoechst/PI viability assay

Hoechst 33342 (Life Technologies) and propidium iodide (PI) (Life Technologies) stains were used to investigate cell viability. Hoechst dye is cell permeable and stains the DNA of all cells, whereas PI is cell impermeable and only stains the DNA of dead cells. Cells were seeded into µClear 96-well plates

(Greiner) to allow for clearer visualisation via microscopy and treated in the same way as in the CCK-8 assay. After 72 hours, 1 μg/ml Hoechst dye and 0.5 μg/ml PI were added and cells were incubated at 37 °C for 15 minutes before imaging using an epifluorescence microscope (DMIRB, Leica). 3-4 images were taken per condition, using a 40x objective, and total viable and dead cells were counted using ImageJ. Cells that were undergoing early apoptosis were unstained by PI but were made apparent by brighter Hoechst staining; these were also counted as non-viable cells. Cell viability was calculated as a percentage of dead to live cells. At least 4 images were taken per well and experiments were performed in duplicate.

2.4.5 Caspase 3/7 activation assay

Cells were seeded into μClear 96-well plates and treated for 72 hours before Caspase 3/7 activation was measured using the CellEvent Caspase 3/7 Green detection kit (Life Technologies). The CellEvent reagent was added to cells at a final concentration of 5μM and cells were incubated at 37°C for 15 minutes. Hoechst 33342 (1μg/ml) was then added and the cells were incubated for a further 15 minutes before imaging using a 40x magnification. The number of apoptotic cells was then calculated as a percentage of total cell number. At least 4 images were taken per well and experiments were performed in duplicate.

2.5 Fluorescence microscopy

2.5.1 Fluorescent BoNT/C Rbd binding

Cells were seeded in μClear 96-well plates and left to grow for 48 hours. They were then incubated with 60 ng/ml Cy3-tagged BoNT/C Rbd for 45 minutes at 37 °C. Hoechst was added at a concentration of 1 μg/ml and the cells were incubated for a further 15 minutes. The wells were then washed 3 times with PBS before imaging via fluorescence microscopy using a 40x magnification.

2.5.2 Macropinocytosis inhibition

J774.2 macrophage cells were seeded in μClear 96-well plates and left to grow for 48 hours. Cells were pre-treated for 30 minutes with 1 mM amiloride (Sigma) or 50 μM LY-294,002 (Sigma), before a 4 hour incubation with 1μM mCheC94S. After a 15 minute incubation with 1 μg/ml Hoechst, cells were washed 3 times with PBS and imaged. For quantification, five images were taken per well using a 60x magnification, and the fluorescence intensity of the cytoplasm was measured using ImageJ. Representative images were taken using a 40x magnification.

2.5.3 Dextran co-localisation

J774.2 cells were seeded in µClear 96-well plates and left to grow for 48 hours. The cells were treated with 1 µM mCheC94S and 1µM FITC-Dextran (Life Technologies) and incubated for 4 hours. Cells were than washed 3 times with PBS before imaging at a 60x magnification. Five images were taken per well and colocalisation was quantified using ImageJ and the Just another colocalization plugin (JACoP).

2.5.4 Immunocytochemistry

Cells were seeded in µClear 96-well plates for indicated times. Cells were washed once for 5 minutes with ice cold PBS and fixed on ice for 10 minutes with 4 % paraformaldehyde. Cells were then permeabilised for 5 minutes in PBS-0.1 % triton and washed once for 5 minutes with PBS. The samples were saturated for at least 1 hour in blocking solution (8 g BSA, 8 ml fish gelatine, 0.4 ml Tween-20, 40 ml 10x PBS, 0.2 g sodium azide and 8 ml normal goat serum, to 400ml in dH_2O) before incubation with the primary antibody (for dilutions, see table 2.3) for 2 hours at room temperature or overnight at 4 °C. The cells were washed in PBS (3x 5 minutes) and were then incubated with secondary antibodies (for dilutions, see table 2.4) and DAPI (Sigma) for 45 minutes at room temperature and protected from light. Cells were washed a further 3 times in PBS before imaging via fluorescence microscopy at a 40x magnification.

2.6 Recombinant protein purification

2.6.1 Generation of recombinant eIF4A Q339E

Rosetta pLys S bacteria (Novagen) containing the His-tagged eIF4A Q339E insert (encoding amino-acid 20-406) cloned into a pET9A (Novagen) plasmid (Cruz-Migoni, Hautbergue et al. 2011) were grown overnight at 37°C in TB media (120 g tryptone, 240 g yeast extract, 23.1 g KH_2PO_4, 125.4 g K_2HPO_4 and 40 ml glycerol to 10 l in distilled water), in the presence of the selection antibiotics chloramphenicol (Sigma; 34 ug/ml) and kanamycin (Sigma; 50 ug/ml). 1.5 ml of the overnight culture was then added to 750 ml media (with kanamycin only) and cultures were grown on a shaker at 37 °C for 2.5 hours or until an OD ~ 0.7 was reached. 300 µl of 1 M IPTG was then added and cultures were incubated for approximately 3 hours at 37 °C. Cultures were transferred to 0.5 l centrifuge bottles and centrifuged for 12 minutes at 6371 xg. Pellets were resuspended in 25 ml of loading buffer (50 mM Tris, 1 M NaCl, 0.5 % triton X100) supplemented with 150 µl complete protease inhibitor (Roche) and 1 mM PMSF. The bacteria were then lysed in ice/water bath using a sonicator

(Soniprep) at 99 % amplitude for 5x 30 seconds with 30 seconds off in between pulses. The lysate was then centrifuged at 48384 xg for 20 minutes at 4 °C. During centrifugation, TALON cobalt-coated beads (Clontech) were prepared by mixing 2 ml of beads with 10 ml dH_2O and allowing the beads to settle. The water was then removed and the beads were washed in 10 ml 1 M cobalt loading buffer (50 mM Tris pH 8.0, 1 M NaCl pH8, 0.5 % triton X100). Following lysate centrifugation, the supernatant was poured onto the beads and incubated at 4 °C with rotation for 30 minutes to allow binding. The beads were then poured onto a gravity flow column and washed once with loading buffer and twice with wash buffer (50 mM Tris pH 8.0, 1 M NaCl and 5 mM Imidazole). Following washes, 2.5 ml of elution buffer (20 mM Tris pH 8.0, 500 mM NaCl and 200 mM Imidazole) was added to the beads twice and two fractions of 2.5 ml were collected. Bradford reagent (Biorad) was used to measure the yield of eluted proteins. Fractions were pooled and the eluate (5 ml) was subjected to gel filtration chromatography on a S200 16/600 column (GE Healthcare) in PBS. Fractions corresponding to the monomeric protein were pooled and concentrated on Amicon (Millipore) prior to flash freezing in liquid nitrogen and storage at -80 ˚C.

2.7 Statistical analysis

Data were expressed as mean ± SD unless otherwise indicated and specific statistical tests are indicated in the figure legends. Statistical analysis was carried out using Graphpad Prism 6, apart from normal probability plots that were carried out using Minitab 17. A P-value < 0.05 was considered statistically significant. For single comparisons, unpaired t-tests were used. For single comparisons to a normalised control the one-sample t-test was used. For multiple comparisons, data was analysed by either one-way ANOVA followed by the Dunnet's method for comparison to a single control sample, or by two-way ANOVA followed by the Tukey range test. Normal probability plots were carried out using pooled data from these tests to assess normal distribution. All tested data fit this linear regression model (App. Fig. 2).

Chapter 3: Two complementary approaches for the intracellular delivery of exogenous enzymes

3.1 Introduction

Biologicals are macromolecules derived from biological sources which include vaccines, ribonucleic acids, antibodies and enzymes. As small molecule drug pipelines are decreasing, the use of these biologicals for therapeutic applications is becoming increasingly attractive, and in 2011 one third of all newly licenced therapeutic agents were biological (Kling 2012). Enzymes are of interest because their catalytic nature conveys high specificity and potency not possible with small molecule drugs (Vellard 2003). Most enzymes currently used in medicine target extracellular substrates. For instance, asparaginases are used in the treatment of leukaemia to decrease serum levels of asparagine, and DNases are used to break down extracellular DNA in the treatment of cystic fibrosis (Jones and Wallis 2010, Rizzari, Conter et al. 2013). The use of enzymes with intracellular substrates has been limited because the cell membrane acts as a barrier to macromolecules, making delivery into the cytosol, both therapeutically and experimentally, a challenge (Conner and Schmid 2003). Simple and economical techniques to deliver enzymes into cells will be of great benefit for the assessment of novel, intracellularly active, enzymes.

Macromolecules can be non-selectively taken up into cells via fluid phase endocytosis (Conner and Schmid 2003). However, the majority of material that is internalised will be degraded via the endo-lysosomal pathway. This means that high concentrations are needed which can increase production costs and cause problems with solubility. A range of techniques to assist in protein delivery have therefore been developed. These include physical techniques such as microinjection and electroporation, chemical techniques using protein transfection reagents, and biological techniques via fusion with cell penetrating or endosome disrupting peptides (King 2004, Torchilin 2006). However, these techniques suffer from issues of cytotoxicity, low efficiency, complexity and cost. Efficient delivery of proteins into cells remains a bottle neck for assaying intracellular function and limits the flexibility of experimental design.

We have previously shown that the DNA transfection reagent lipofectamine LTX can be used to deliver BoNT proteases (light chains only) into neuroblastoma cells (Arsenault, Cuijpers et al. 2013). In this chapter, different transfection reagents were tested to find the most efficient reagent for the delivery of enzymes into a range of cells lines. The newly available DNA transfection reagent lipofectamine 3000 (LF3000), was able to deliver botulinum proteases and translation inhibiting enzymes into different cells with high efficiency and low toxicity. Testing the efficiency of LF3000 in

different cell lines led to the surprising observation that the mouse macrophage cell line J774.2 demonstrated high sensitivity to enzymes even in the absence of LF3000. This opens up possibilities for the use of this cell line for high-throughput screening and quality control in biotechnology.

3.2 Results

3.2.1 Lipofectamine 3000 can efficiently deliver saporin into N2a cells

Saporin is a highly potent RIP that has been used extensively for targeted killing of different cell populations, including cancer cells and neurons (Polito, Bortolotti et al. 2013). It is a 30 kDa, monomeric protein that is unable to enter the cell unaided at low nanomolar concentrations and was therefore used as a positive reporter for testing intracellular delivery. It has previously been shown that botulinum proteases can be delivered into neuroblastoma cells using the DNA transfection reagent lipofectamine LTX (Arsenault, Cuijpers et al. 2013). A range of DNA transfection reagents including lipofectamine LTX, lipofectamine 2000, lipofectamine 3000 and metafectene, as well as proteofectene, a bona fide protein delivery reagent, were tested at recommended dilutions in N2a cells to find the most efficient and least cytotoxic delivery agent. Cell counting using the CCK8 assay shows that 72 hour incubation of 30nM saporin alone is not significantly cytotoxic compared to the control (Fig. 3.1A). Of the reagents tested, LF3000 and LF2000 were the most efficient at delivering saporin into cells, however, LF2000 showed some cytotoxicity even without saporin. LF3000 was used in future experiments as it showed high efficiency with no observed toxicity. Titration experiments showed an increase in potency of saporin by around 1000-fold in the presence of LF3000 (Fig. 3.1B). Delivery of BSA into N2a cells with LF3000 showed no toxicity, even up to concentrations of 300µM (Fig. 3.1C). This confirms that cytotoxicity is due to the enzymatic action of saporin and not bulk delivery of protein into the cell.

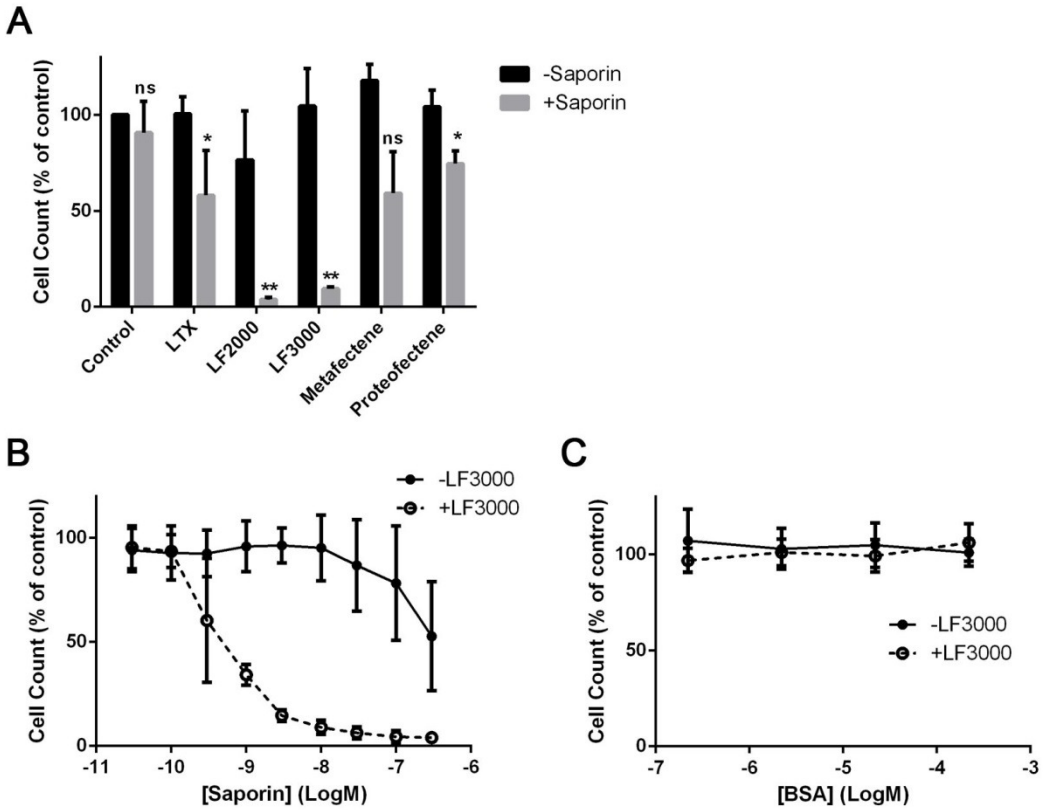

Figure 3.1: A, CCK-8 assay showing the effect of 30nM saporin on N2a proliferation following protein delivery using different transfection reagents (n=3, ± SD). Data was analysed by multiple unpaired t-tests with *P < 0.05 and **P < 0.01. **B,** CCK-8 assay following titration with saporin in N2a cells shows that LF3000 increases potency of saporin by approximately 1000-fold (n=3, ± SD). **C,** CCK-8 assay following titration with BSA shows that bulk protein uptake does not affect cell growth (n=3, ± SD).

3.2.2 LF3000 can deliver structurally and functionally distinct enzymes into a range of cell lines

Having confirmed that LF3000 can deliver saporin into cells, the ability of LF3000 to deliver structurally and functionally distinct enzymes was tested. BLF1 is a 24kDa enzyme that inhibits translation initiation and, like saporin, is unable to enter cells unaided (Cruz-Migoni, Hautbergue et al. 2011). Incubation of N2a cells with 30nM BLF1 for 72 hours had no effect on cell number, however, a significant drop was observed in the presence of LF3000 (Fig. 3.2A). The BoNT/C protease cleaves the SNARE proteins SNAP25 and Syntaxin 1 (Montecucco and Schiavo 1994). Removal of the receptor binding and translocation domains means that this 50kDa enzyme is unable to enter cells. Immunoblotting reveals that incubation of 30nM BoNT/C protease with N2a cells leads to near total cleavage of SNAP25 in the presence of LF3000 (Fig. 3.2B). This is demonstrated by a shift in band size

due to the cleavage of 8 amino acid residues from the C-terminus of the protein. A small amount of SNAP25 cleavage is also seen in the absence of LF3000 which is likely due to low level, passive uptake of the protease. These data show that LF3000 can be used to deliver enzymes of different sizes and functions into N2a cells.

To be a useful tool to study intracellular enzymatic action, LF3000 needs to be able to deliver enzymes into different cell types. Delivery of saporin was therefore tested in a range of different cell lines, including the human neuroblastoma SH-SY5Y, lung adenocarcinoma A549, colorectal adenocarcinoma Caco-2, pancreatic carcinoma QGP-1 and mouse macrophage J774.2 cell lines. LF3000 was able to deliver saporin into all the different cell lines, although different efficiencies were observed (Fig. 3.2C). This is comparable to the different efficiencies seen when transfecting cells with DNA. Interestingly saporin showed a high level of potency in the J774.2 cell line even in the absence of LF3000.

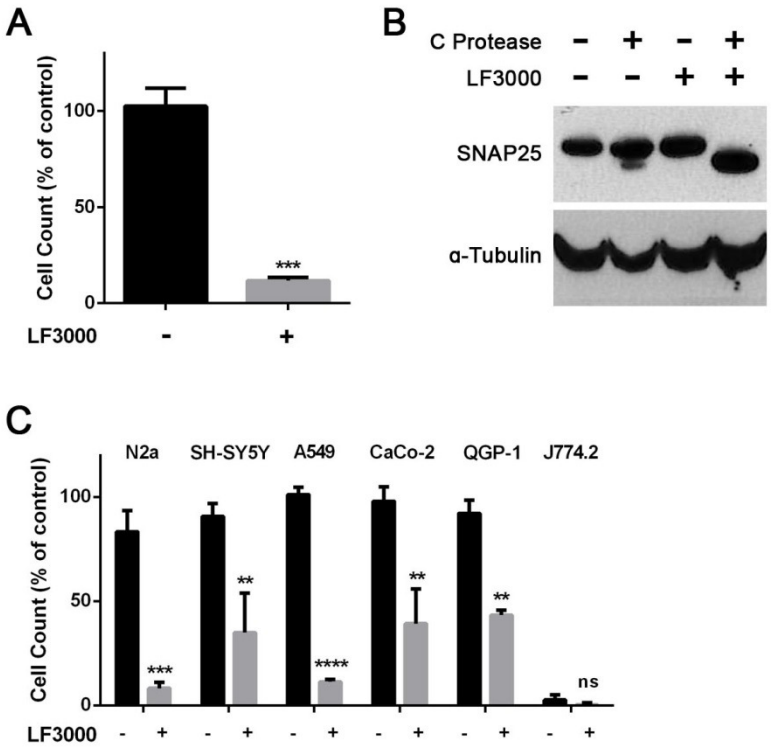

Figure 3.2: A, CCK-8 assay showing that 30nM BLF1 inhibits proliferation only in the presence of LF3000 (n=3, ± SD). Data was analysed by an unpaired t-test with ***P < 0.001. **B,** Immunoblot shows that LF3000 greatly increases SNAP25 cleavage by the BoNT/C protease. **C,** CCK-8 assay showing that LF3000 can efficiently deliver 30nM saporin into a range of different cell lines, and that J774.2 shows sensitivity to saporin even in the absence of LF3000 (n=3, ± SD). Data was analysed by multiple unpaired t-tests with **P < 0.01, ***P < 0.001 and ****P < 0.0001.

To assess how enzymes are delivered into cells in greater detail, a mCherry-tagged BLF1 enzyme and a non-functioning mutant (C94S) were generated. Coomassie staining shows the native BLF1 enzyme at 24kDa, and the modified enzymes with the 27kDa mCherry tag at approximately 50kDa (Fig. 3.3A). Additional, smaller bands are also present which is likely due to low-level degradation of the protein. The C94S mutant has a point mutation, changing a cysteine residue to serine, which attenuates its enzymatic activity (Cruz-Migoni, Hautbergue et al. 2011). This can be seen in titration experiments which show that the C94S has around 100 times less activity than the native BLF1 enzyme and the mCherry-tagged enzyme (Fig. 3.3B). Despite a loss of enzymatic action, C94S is still able to bind and inhibit eIF4A, meaning that it still has anti-proliferative action at higher concentrations. Live imaging of the N2a cells 24 hours after protein transduction shows the majority of protein is present in small vesicle like structures (Fig.3.3C). This suggests that, although LF3000 is able to deliver a large volume of protein into cells, entry into the cytosol is a limiting factor. However, enough protein is passing into the cytosol to give an observable effect, meaning that this is still a useful approach for the intracellular delivery of enzymes.

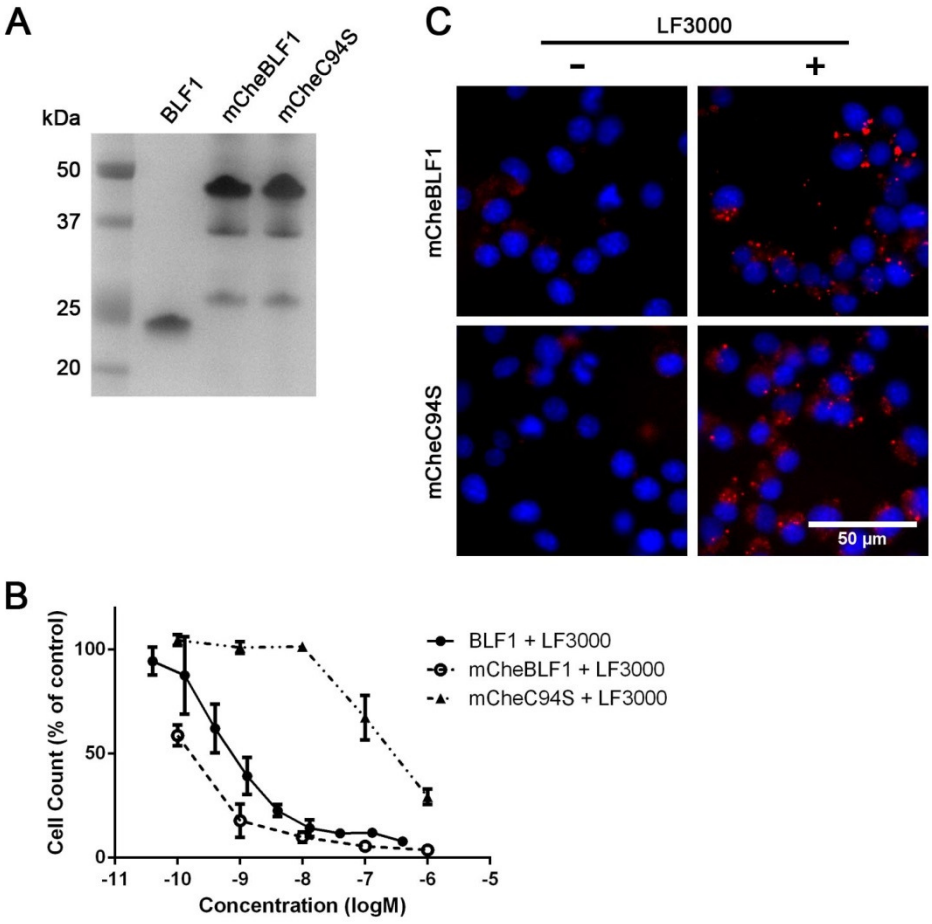

Figure 3.3: A, Coomassie stain showing the relative sizes of BLF1, mCherry-tagged BLF1 and the mCherry-tagged BLF1 mutant C94S. **B,** CCK-8 assay following titration with BLF1, mCheBLF1 and mCheC94S in the presence of LF3000 in N2a cells shows greatly reduced anti-proliferative activity of the C94S mutant (n=3, ± SD). **C,** Fluorescence microscopy shows mCheBLF1 and mCheC94S (red) to be present in vesicles following 24 hour incubation with 100nM protein in the presence of LF3000. Nuclei are stained using Hoechst 33342 (blue).

3.2.3 J774.2 cells take up enzymes without the need for delivery agents

The sensitivity of the J774.2 cell line to saporin could make it a useful model for testing enzymes without the need for other delivery techniques. The sensitivity of J774.2 cells to different enzymes was tested. A titration of saporin and BLF1 in the absence of LF3000 revealed that the J774.2 cell line is around 100 times more sensitive than N2a and A549 cells to both enzymes (Fig. 3.4A). This suggests that J774.2 cells are actively taking up the enzyme into the cytosol. Unlike N2a cells, J774.2 cells are not of neural origin and therefore do not express SNAP25 or Syntaxin 1. This meant that the BoNT/C protease could not be tested in this cell line. The BoNT/D protease is structurally similar to BoNT/C, but it cleaves the vesicle-associated membrane proteins (VAMPs) VAMP2 and VAMP3 (Yamamoto, Ida et al. 2012). VAMP3 is a non-neuronal SNARE protein involved in vesicle trafficking and is expressed ubiquitously in all cells (McMahon, Ushkaryov et al. 1993). J774.2 and N2a cells were incubated with increasing concentrations of BoNT/D protease for 72 hours and VAMP3 cleavage was assessed. BoNT/D cleaves 57 amino acid residues from the C-terminus of VAMP3 and the cleavage products are quickly targeted for degradation. VAMP3 cleavage is therefore detected by disappearance of the full protein. Immunoblotting showed cleavage of VAMP3 in J774.2 cells, with total cleavage (absence of protein) seen at 100nM (Fig. 3.4B). No cleavage was observed in the N2a cells at any concentration. Interestingly, cleavage of VAMP3 in J774.2 cells led to a dramatic change in cell morphology, with cells adopting a stellate-phenotype (Fig. 3.4C). This change in phenotype has been observed in previous studies that knocked down VAMP3 using siRNA, and highlights a possible role of VAMP3 in cell spreading, adhesion and migration (Veale, Offenhauser et al. 2011).

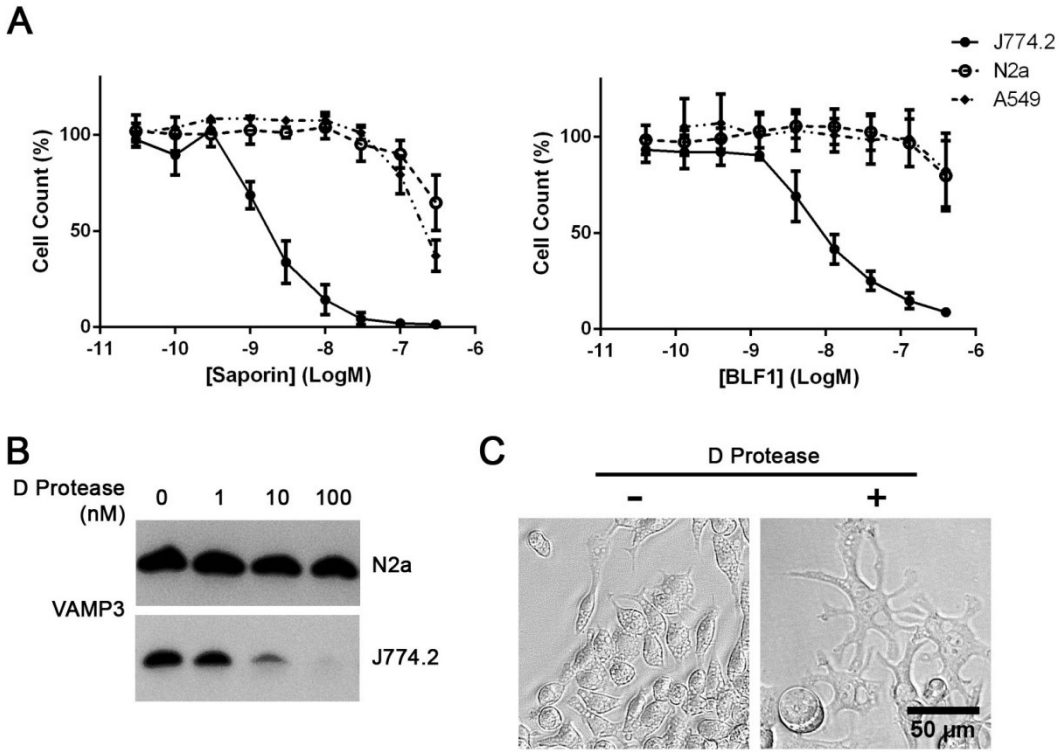

Figure 3.4: A, CCK-8 assay following titrations with BLF1 and saporin shows that J774.1 cells are around 100 times more sensitive than N2a or A549 cells (n=3, ± SD). **B,** Immunoblotting showing cleavage of VAMP3 in J774.2 cells only following treatment with BoNT/D protease. **C,** Light microscopy images showing stellate phenotype of J774.2 cells following VAMP3 cleavage by BoNT/D protease.

3.2.4 Uptake of BLF1 into J774.2 cells occurs via macropinocytosis

To further investigate the ability of J774.2 cells to non-specifically take up enzymes into the cytosol, the mCherry-tagged BLF1 enzyme and C94S mutant were used. Titration experiments demonstrate a similar potency of mChe-BLF1 to the wild type (Fig. 3.5A). Interestingly, the C94S mutant had no effect on cell number, suggesting that a lower amount of protein is reaching the cytosol than is seen with the LF3000 based approach. This data also shows that uptake of enzyme into the cytosol has a degree of size-independence as the mCherry tag is 27kDa and doubles the size of the molecule, but this increase in size has no impact on efficacy. Flow cytometry analysis showed that the mCherry-tagged proteins are taken up gradually, with all cells taking up the protein after 24 hours (Fig. 3.5B). This slow process is indicative of a non-specific, fluid-phase uptake such as macropinocytosis. Live imaging via fluorescence microscopy shows that the majority of protein is present in large vesicle-like structures, rather than in the cytoplasm (Fig. 3.5C).

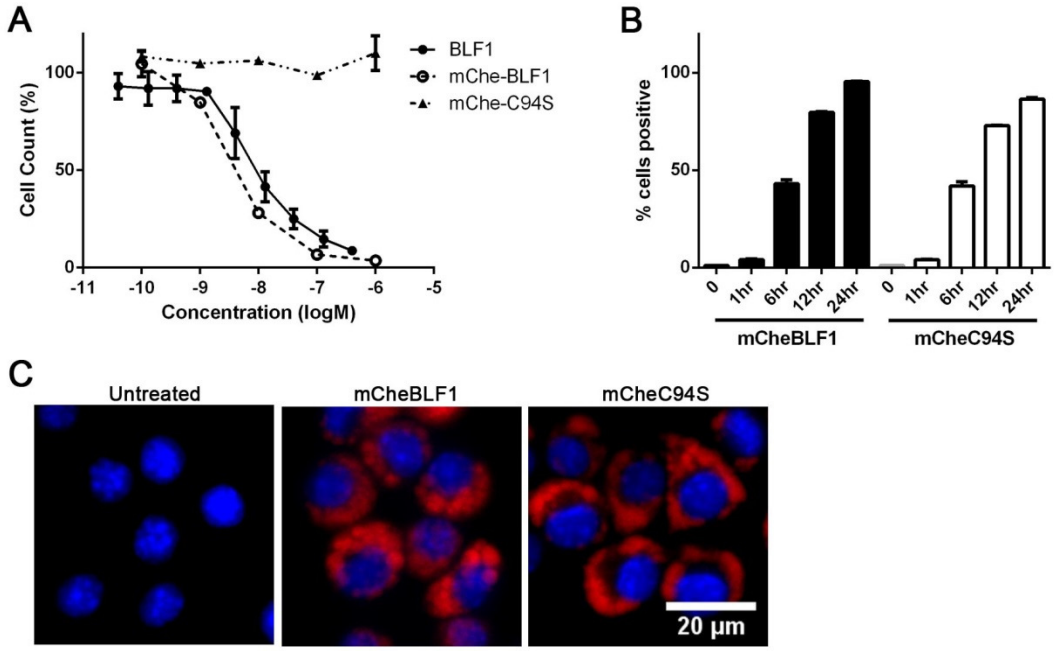

Figure 3.5: A, CCK-8 assay following titration with BLF1, mCheBLF1 and mCheC94S in J774.2 cells shows that mCheC94S has no effect on cell proliferation at concentrations used (n=3, ± SD). **B,** Flow cytometry showing gradual uptake of 100nM mCherry-tagged protein over 24 hours. **C,** Fluorescence microscopy images showing that the majority of mCherry-tagged protein (red) is localised to large, vesicle-like structures. Nuclei are stained using Hoechst 33342 (blue).

Macrophages are known to exhibit high levels of macropinocytosis, a form of non-specific bulk uptake of extracellular fluid involved in antigen presentation (Lim and Gleeson 2011). To assess the contribution of macropinocytosis to enzyme uptake, J774.2 cells were pre-treated with two different known macropinocytosis inhibitors, amiloride and L-Y294,002. It is thought that amiloride functions by inhibiting sodium channels on the cell membrane leading to a decrease of submembranous pH and inhibition of Rac1 and Cdc42 signalling (Koivusalo, Welch et al. 2010). Rac1 and Cdc42 are downstream targets of PI3K and are necessary for actin polymerisation and membrane ruffling. LY294,002 is a PI3K inhibitor, which also inhibits Rac1 and Cdc42. When added to J774.2 cells, both amiloride and LY294,002 caused a dramatic decrease in mChe-C94S uptake after a 4 hour incubation (Fig. 3.6A). Quantification of fluorescence intensity shows that amiloride treatment led to a decrease in uptake of 50%, and LY294,002 led to a decrease of 70% (Fig. 3.6B). Neither inhibitor had a dramatic effect on cell viability, as demonstrated by a CCK8 assay (Fig. 3.6C). It would have been useful to test the effect of macropinocytosis inhibition on sensitivity to BLF1, but due to toxicity of the macropinocytosis inhibitors in long term incubations, this was not possible. To confirm that macropinocytosis is involved in protein uptake, mChe-C94S was co-incubated with FITC-tagged

dextran (70kDa), a classical marker of fluid-phase uptake (Falcone, Cocucci et al. 2006). Fluorescence microscopy revealed almost complete co-localisation between mChe-C94S and FITC-dextran (Fig. 3.7A). Assessment of the level of dextran uptake in different cell lines was also carried out using flow cytometry. This showed that J774.2 cells internalised around four times more dextran than the next most active cell lines HeLa and A549 (Fig. 3.7B). Taken together, these data demonstrate the major role macropinocytosis plays in uptake of proteins by J774.2 cells.

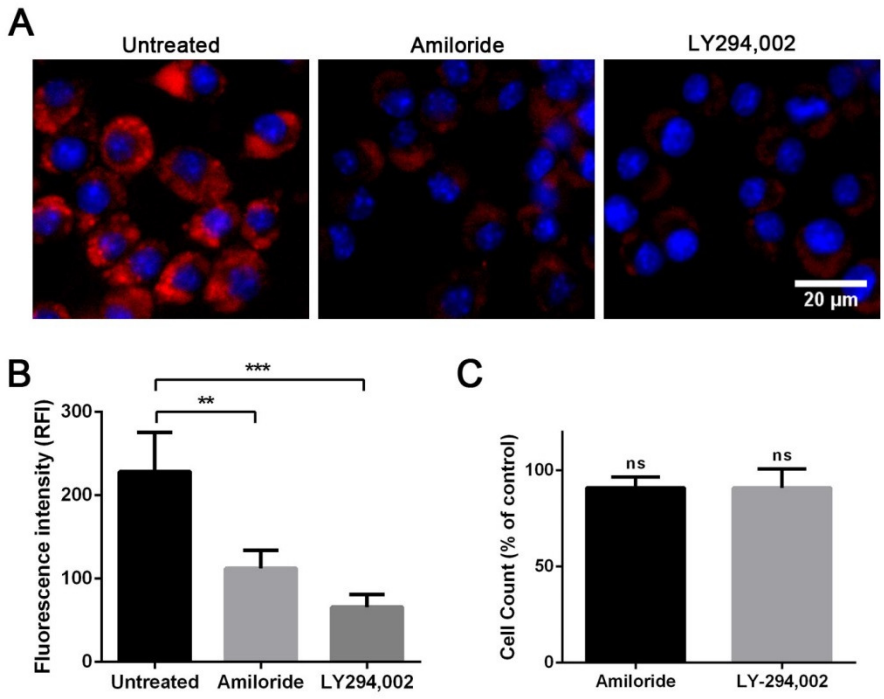

Figure 3.6: A, Representative fluorescence microscopy images showing decreased mCheC94S (red) uptake following a 30 minute pre-incubation with the macropinocytosis inhibitors amiloride (1mM) and LY294,002 (50µM). Nuclei are stained using Hoechst 33342 (blue). **B,** Quantification of mCheC94S uptake seen in A (n=3, ± SD). Data was analysed by one-way ANOVA and Dunnet's multiple comparisons test with **P < 0.01 and ***P < 0.001. **C,** CCK-8 assay showing that amiloride and LY294,002 do not significantly affect cell viability. Data was analysed by multiple one-sample t-tests.

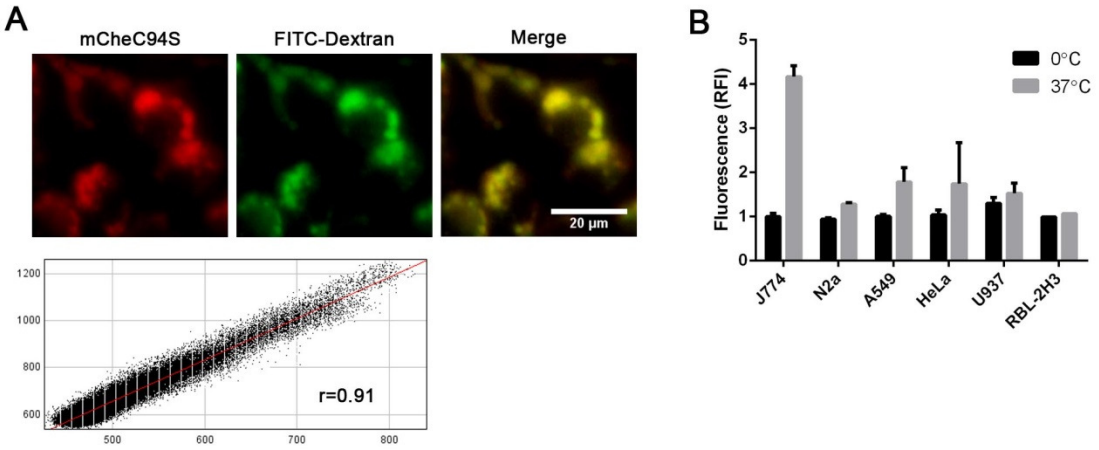

Figure 3.7: A, Representative fluorescence microscopy images and cytofluorogram showing strong colocalization of mCheC94S (red) and FITC-dextran (green) following a 4 hour incubation (n=3, Pearson correlation coefficient *r*=0.91, SD: ± 0.006). **B,** Quantification of FITC-dextran uptake in different cell lines analysed by flow cytometry shows around four times higher uptake in J774.2 cells compared to the next most active cell line (n=3, ± SD).

As mentioned previously, active macropinocytosis is a characteristic of macrophages. This means that other macrophage cell lines may display a similar sensitivity to enzymatic activity. To investigate this, a second mouse macrophage cell line, RAW 264.7, was used. Live imaging showed that uptake of mChe-C94S by RAW 264.7 cells was similar to J774.2 cells (Fig. 3.8A), and this was confirmed by quantification (Fig. 3.8B). Despite this, J774.2 cells were still almost 100-times more sensitive to BLF1 than RAW 264.7 cells (Fig. 3.8C). This suggests that although macropinocytosis is involved in the uptake of protein, a second process absent in RAW 264.7 cells is necessary for cytosolic entry of the enzyme.

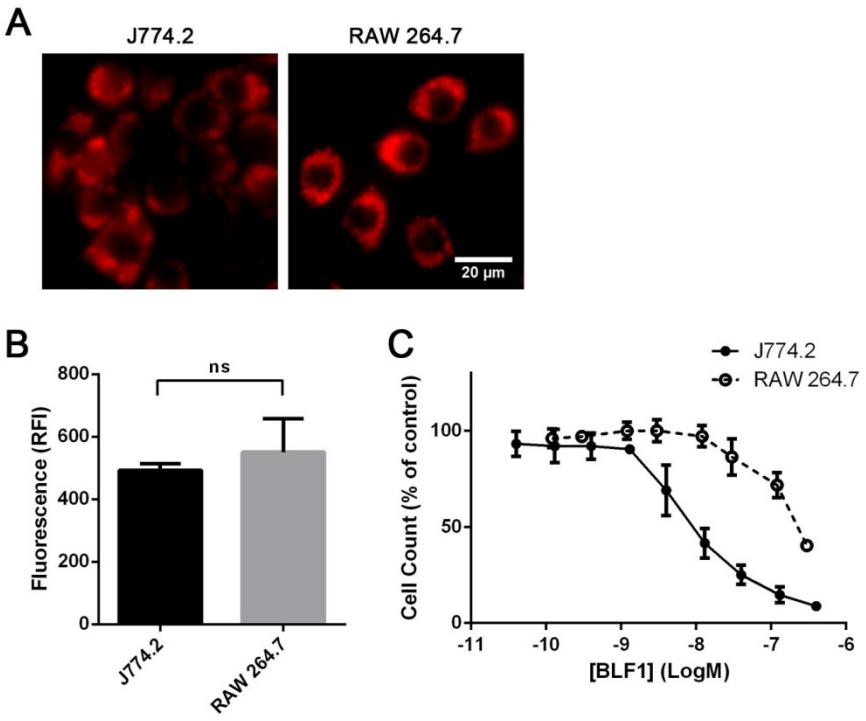

Figure 3.8: A, Representative fluorescence microscopy images showing similar levels of mCheC94S (red) uptake in J774.2 and RAW 264.7 cells. **B,** Quantification of mCheC94S uptake seen in A (n=2, ± SD). Data was analysed by an unpaired t-test. **C,** CCK-8 assay following a titration with BLF1 shows that J774.2 cells are around 100 times more sensitive than RAW 264.7 cells (n=3, ± SD).

3.2.5 Intracellular delivery of non-enzymatic proteins

The two approaches demonstrated in this chapter show sensitivity in the pico-molar to low nano-molar range when using potent toxic enzymes. However, microscopy demonstrates that the majority of internalised material remains trapped in membrane bound structures, suggesting that only a small percentage of protein is reaching the cytosol. This may limit the use of these techniques when investigating less potent, non-catalytic proteins. Additionally, all proteins currently used are derived from toxins and therefore show good stability. BLF1 functions by deamidating the Gln^{339} residue on eIF4A, which then acts as a dominant-negative mutant that inhibits translation (Cruz-Migoni, Hautbergue et al. 2011). A dominant-negative eIF4A mutant, Q339E, was generated recombinantly by substitution of glutamine with glutamic acid. Coomassie staining shows the protein purification steps with the purified Q339E protein (48kDa) present in fraction 52 following gel filtration (Fig. 3.9A). Numerous smaller bands are also present which is likely due to instability and degradation of the protein. Delivery of Q339E into N2a cells using LF3000 dramatically increased potency with an

IC50 of 3.5nM (Fig. 3.9B). The fold-increase could not be calculated as little effect on cell count was seen without LF3000, even at a 1µM concentration. Delivery of BLF1 with LF3000 showed higher potency, with an IC50 of 550pM (Fig. 3.9B). The high level of sensitivity seen with Q339E following delivery with LF3000 suggests that this delivery system is suitable even for non-catalytic proteins. Analysis of cell numbers following treatment of J774.2 cells with Q339E and BLF1 shows IC50s of 180nM and 4nM respectively (Fig. 3.9C). Sensitivity of the J774.2 cell line therefore appears to be lower than delivery with LF3000, as was also seen with addition of mCheC94S, which should be taken into account when testing protein function.

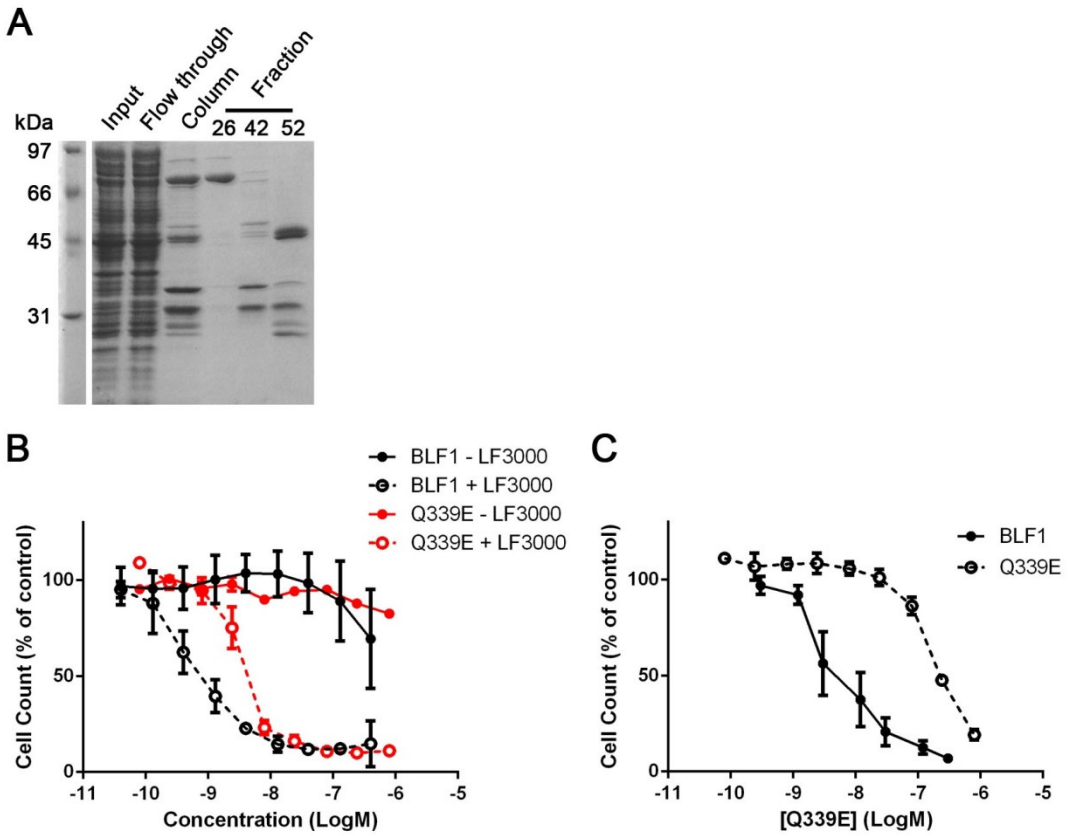

Figure 3.9: A, Coomassie stained SDS-PAGE gel showing the purification of the eIF4A Q339E mutant including: whole bacterial lysate (input), flow through from cobalt-coated bead purification, eluate following bead-binding (Column), and gel purification fractions 26, 42 and 52. **B,** CCK-8 assay following titrations of BLF1 and Q339E with or without LF3000 in N2a cells shows the higher potency of BLF1 (n=3 technical repeats, ± SD). **C,** CCK-8 assay following titrations of BLF1 and Q339E without LF3000 in J774.2 cells shows higher potency of BLF1 (n=3 technical repeats, ± SD).

3.3 Discussion

This chapter highlights the use of two novel and complementary approaches for the delivery of exogenous enzymes into cells. The first approach involves the use of LF3000, which allows the delivery of enzymes into different cell lines, and the second approach is the J774.2 cell line which allows the direct comparison of different enzymes without the need of additional delivery techniques.

Lipofection is a common technique for the delivery of DNA into cells and involves a cationic liposome formulation that functions by complexing with negatively charged DNA molecules, allowing them to overcome the electrostatic repulsion of the negatively charged cell membrane (Felgner, Gadek et al. 1987). More modern formulations also contain a neutral co-lipid that promotes fusion of the lipoplex with the cell membrane (Dalby, Cates et al. 2004). Interestingly, the activity of LF3000 for delivery of protein appears to be charge independent, as is highlighted by the efficient delivery of saporin into cells, despite its net positive charge at physiological pH (Korennykh, Correll et al. 2007). The reason for this remains unclear, but it may mean that the neutral co-lipid is sufficient for protein delivery. Nevertheless, this charge-independent mechanism greatly increases the possible uses of LF3000 for protein delivery. Microscopy using fluorescently tagged BLF1 and C94S reveals that the majority of protein remains trapped in vesicle-like structures. However, enough material is able to enter the cytosol to show efficacy at low nano-molar concentrations, even when the protein is non-catalytic.

The J774.2 cell line shows high sensitivity to intracellularly active enzymes, even in the absence of delivery reagents. Co-localisation with dextran and inhibition of macropinocytosis demonstrates that this is partly due to high macropinocytic activity. Indeed, a recent study demonstrated that induction of macropinocytosis is an efficient way to deliver protein into cells (D'Astolfo, Pagliero et al. 2015). However, macropinocytosis alone is not enough to convey sensitivity, as is shown by a comparison with the macrophage cell line RAW 264.7, which exhibits around 100-fold lower sensitivity to BLF1 despite similar levels of macropinocytosis and protein uptake. This suggests that a second mechanism is involved that likely causes an increase in endosome escape and entry into the cytosol. This may occur during cross-presentation, a process by which antigens are presented on major histocompatibility complex (MHC) molecules, which involves export of polypeptides from endocytic-like compartments into the cytosol for degradation (Mintern, Macri et al. 2015). Despite intense research over decades, mechanistic details of how internalised proteins are transported into the cytoplasm during cross-presentation are still under investigation.

Efficient and economical ways of delivering proteins into cells for research and therapeutic purposes are in high demand, particularly as the use of macromolecules in medicine increases. The two approaches developed in this chapter are both simple and efficient ways to greatly increase efficacy of protein delivery by around 100-1000 fold, allowing for analysis of action at therapeutically relevant, nano-molar concentrations.

Chapter 4: Inactivation of eIF4A by Burkholderia lethal factor 1 highlights translation initiation as a novel therapeutic target for MYCN-amplified neuroblastoma

4.1 Introduction

MYCN is a Myc family transcription factor and has been identified as a key marker of poor prognosis in neuroblastoma (Brodeur, Seeger et al. 1984). As with c-Myc, MYCN has long been considered 'undruggable' as it is a nuclear transcription factor with no active site amenable to binding by conventional small molecule drugs (Huang and Weiss 2013). Identifying novel pathways to down-regulate this protein is therefore of high importance for future therapies.

Translation initiation is the rate limiting step of translation and up-regulation of this process is considered a hallmark of cancer. The increased rate of protein synthesis contributes to proliferation, survival, altered immune response and angiogenesis (Bhat, Robichaud et al. 2015). The eIF4F complex is made up of the mRNA cap binding protein eIF4E, the RNA-helicase eIF4A and the scaffold protein eIF4G, and is necessary for recruitment of the small ribosomal subunit to the 5' cap of the mRNA and scanning of the 5' UTR to the translation start sight (Jackson, Hellen et al. 2010). This complex is of particular importance for the regulation of translation and acts as a central node upon which a number of oncogenic signalling pathways (e.g. Ras, PI3K/AKT/TOR and Myc) converge (Pelletier, Graff et al. 2015). Inhibition of the eIF4F complex is therefore gaining interest as a potential therapeutic target for a number of cancers. The RNA-helicase eIF4A is required during mRNA scanning for the resolution of secondary structures found in the 5' UTR of many mRNAs (Jackson, Hellen et al. 2010). It has recently been shown that a subset of mRNAs with long and complex 5'UTRs that contain G-quadruplex secondary structures have an increased dependence on eIF4A activity (Wolfe, Singh et al. 2014). These mRNAs encode a number of oncogenic proteins including transcription factors (c-Myc and c-Myb), cell cycle regulators (cyclins and CDKs) and survival proteins (Bcl2). Inhibition of eIF4A leads to the preferential down-regulation of these proteins, triggering growth arrest and cell death. Indeed, a number of small molecule inhibitors of eIF4A such as silvestrol and hippuristanol have shown anti-cancer activity both *in vitro* and *in vivo* (Lucas, Edwards et al. 2009, Tsumuraya, Ishikawa et al. 2011). Inhibition of eIF4A may therefore be a useful approach for treatment of neuroblastoma, particularly in MYCN-amplified cells.

Burkholderia Lethal Factor 1 (BLF1) is an enzyme produced by the bacterium *Burkholderia pseudomallei* which inactivates eIF4A (Cruz-Migoni, Hautbergue et al. 2011). This chapter demonstrates that BLF1 can down-regulate eIF4A-dependent proteins leading to growth arrest in a

number of human neuroblastoma cell lines and the induction of apoptosis only in MYCN-amplified cells. This makes translation initiation a promising novel therapeutic target for the treatment of MYCN-amplified neuroblastoma and highlights BLF1 as a potential payload for future targeted toxin based therapies.

4.2 Results

4.2.1 BLF1 shows cytostatic activity in J774.2 cells

The J774.2 cell line was used to compare the activity of different commonly used translation inhibiting enzymes with BLF1. As mentioned previously, J774.2 is able to take up enzymes without the need of any delivery reagents. BLF1 was compared to the type I RIP saporin and the enzymatic region of the type II RIP ricin. Both of these toxins function by permanently disrupting ribosome function (de Virgilio, Lombardi et al. 2010). As well as RIPs, the enzymatic region of DT was used. This enzyme disrupts the elongation phase of translation via inhibition of eEF2 (Li, Vallera et al. 2013). The relative sizes and concentrations of the different enzymes were visualised via Coomassie staining (Fig. 4.1A). A titration using the CCK-8 assay after 72 hours incubation showed that BLF1 had an almost identical efficacy to ricin and diphtheria, and saporin showed around five times higher efficacy than the other enzymes (Fig. 4.1B). Analysis of nuclear morphology by Hoechst 33342 staining revealed that saporin, diphtheria and ricin all caused nuclear condensation indicative of apoptosis, whereas BLF1 had no effect on morphology (Fig. 4.1C). This suggests that BLF1 is not cytotoxic to the cells, unlike the other toxins. This was confirmed using a Caspase 3/7 activation assay which showed induction of apoptosis after incubation with saporin, but no effect after incubation with BLF1 (Fig. 4.1D and E). The lack of toxicity of BLF1 has interesting implications for a targeted therapy, as off-target toxicity is a common problem of currently used toxins.

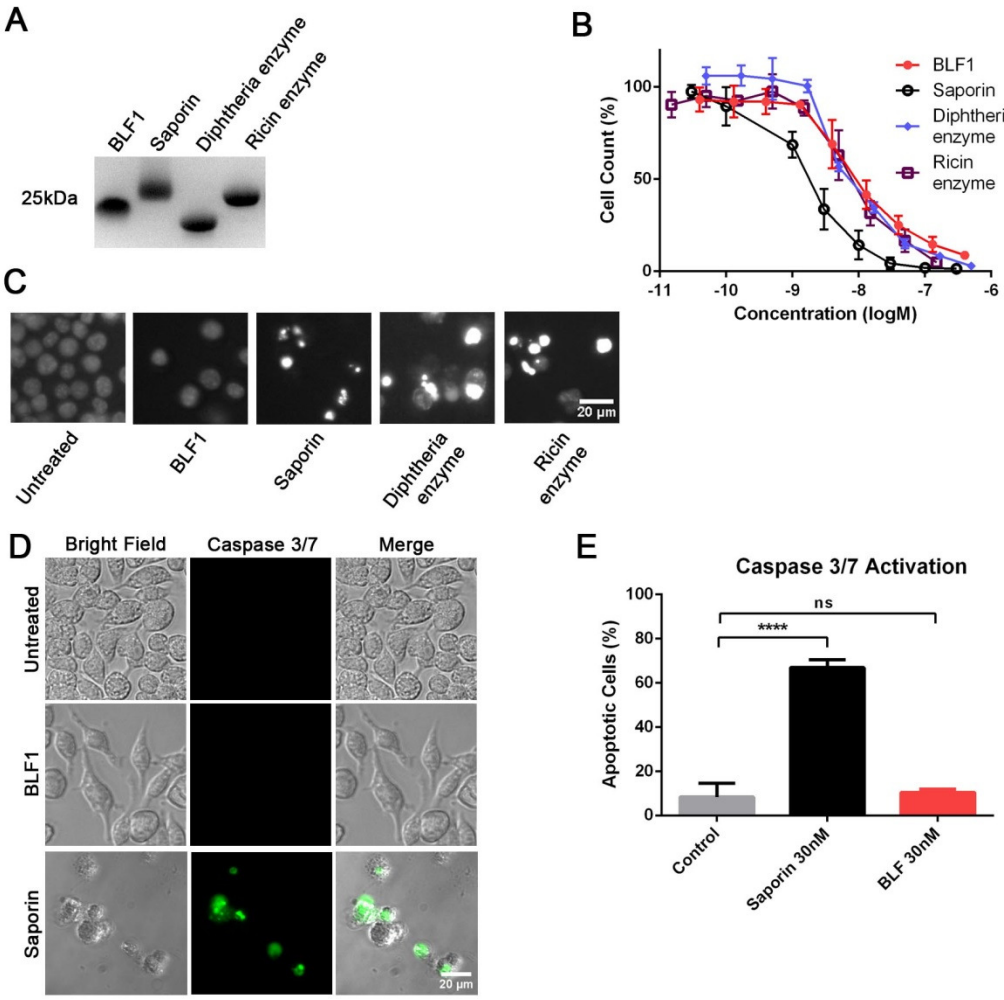

Figure 4.1: A, Coomassie stained SDS-PAGE gel showing the different translation inhibiting enzymes. **B,** CCK-8 assay following titrations of the different enzymes in J774.2 cells, shows saporin has the highest efficacy (n=3, ± SD). **C,** Fluorescence microscopy images after Hoechst 33342 staining shows saporin, diphtheria and ricin all cause nuclear condensation whereas BLF1 has no effect on cell morphology. **D,** Fluorescence microscopy images after caspase 3/7 assay shows caspase 3/7 activation only after treatment with saporin. **E,** Quantification of D (n=3, ± SD). Data was analysed by one-way ANOVA and Dunnet's multiple comparisons test with ****P < 0.0001.

4.2.2 BLF1 shows potent anti-proliferative activity in a range of neuroblastoma cell lines

The ability of BLF1 to inhibit proliferation was further tested in eight different neuroblastoma cell lines of which four were MYCN-amplified (IMR-32, SK-N-BE(2), SiMa and LA-N-5), and four were non-MYCN-amplified (SH-SY5Y, LA-N-6, SK-N-FI and SK-N-SH). MYCN expression in the different cell lines was confirmed by immunoblotting which showed substantially higher MYCN levels in the MYCN-amplified cell lines (Fig. 4.2A). BLF1 was delivered into cells using LF3000 and its effect on cell growth

following a 72 hour incubation was compared with saporin. BLF1 and saporin show similar levels of potency and inhibit 50% of cell growth (GI50) in the low nano-molar range (Fig. 4.2B). A comparison of GI50s shows that BLF1 may be more potent in MYCN-amplified cells than non-MYCN-amplified cells whereas saporin exhibits similar efficacy (Fig. 4.2C). However, this was not statistically significant with the number of cell lines tested. This suggests that inactivation of eIF4A is more toxic in MYCN-amplified cells, whereas general inhibition of protein synthesis by ribosome inactivation is not selective.

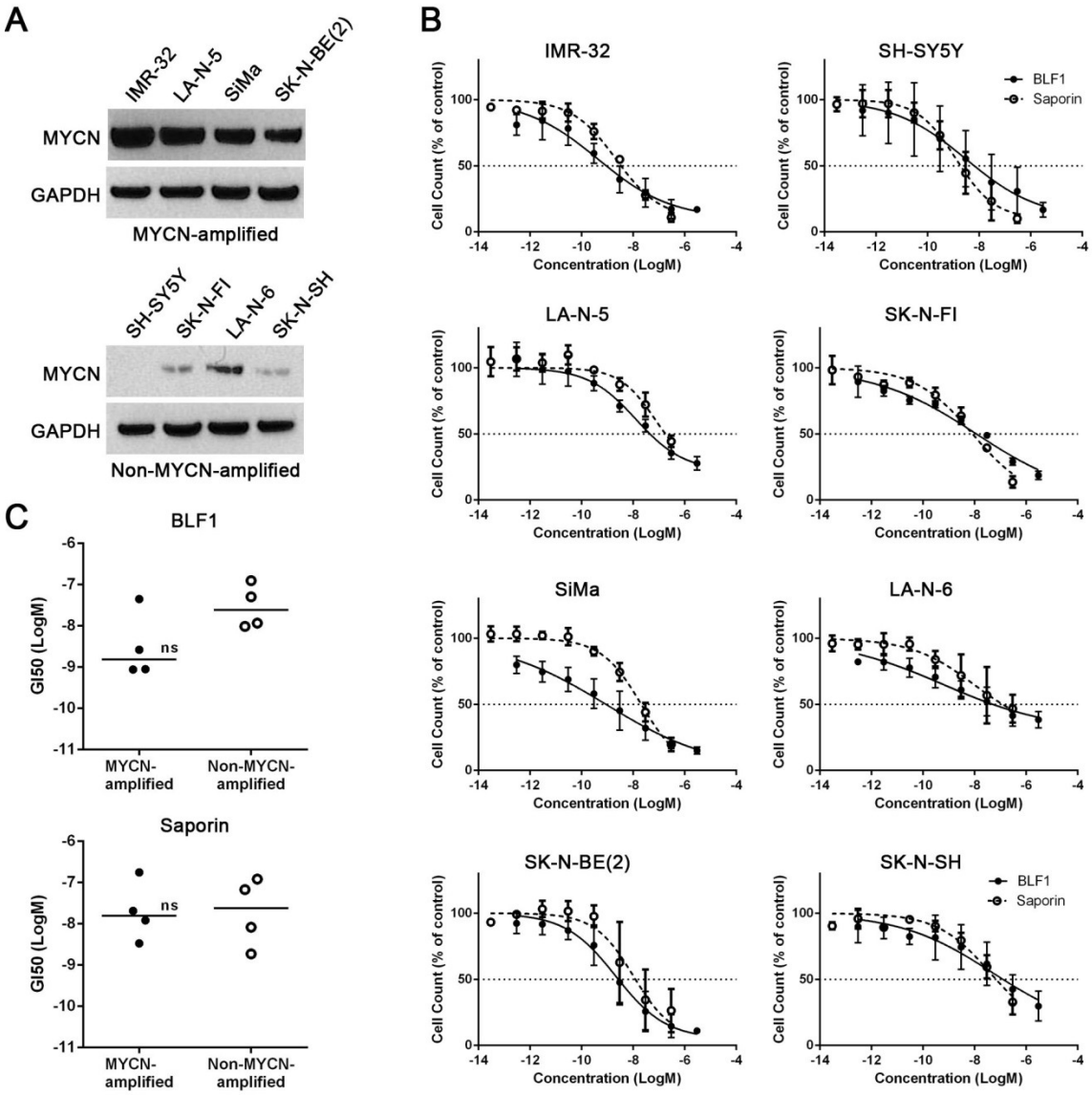

Figure 4.2: A, Immunostaining showing levels of MYCN compared to GAPDH in the MYCN-amplified and the non-MYCN-amplified cell lines. **B,** alamarBlue assay used to identify the GI50s following titrations of BLF1 and saporin in the presence of LF3000 in the different cell lines (n≥3, ± SD). **C,** Comparison of GI50s obtained from B. Data was analysed by unpaired t-tests, lines show median values.

4.2.3 BLF1 induces apoptosis in neuroblastoma cells in a MYCN-dependent manner

The alamarBlue assay is useful for assessing the effect of BLF1 on cell growth. However, when seeding at low cell density, it is unable to distinguish between cytostatic and cytotoxic effects. To directly observe the effect of BLF1 on cell viability, co-staining of cells with Hoechst 33342 and propidium iodide DNA dyes was carried out followed by fluorescence imaging. The Hoechst dye is cell permeable and stains the DNA of all cells, whereas propidium iodide is cell impermeable and only stains the DNA of dead cells. Comparison of the two stains allows assessment of cytotoxicity of BLF1. The MYCN amplified cell lines IMR-32 and SK-N-BE(2), and the non-MYCN-amplified cell lines SH-SY5Y and LA-N-6 were incubated for 72 hours with 300nM BLF1 or saporin followed by co-staining with Hoechst 33342 and propidium iodide. In MYCN-amplified cells, BLF1 caused cell death at a similar level to saporin (Fig. 4.3A and B). However, in MYCN-non-amplified cells, BLF1 had very little effect on cell viability as evidenced by the lack of propidium iodide staining and normal nuclear morphology, whereas saporin still caused cell death (Fig. 4.3A and B). This suggests a cytostatic effect of BLF1 in non-MYCN-amplified cell lines similar to that seen in J774.2 cells. Analysis of the mechanism of cell killing in the IMR-32 and SK-N-BE(2) MYCN-amplified cells revealed that both BLF1 and saporin cause caspase 3/7 activation and induce apoptosis (Fig. 4.3C). SK-N-BE(2) cells have a non-functional p53 mutation which suggests that the induction of apoptosis occurs independently of p53. This is of benefit because inactivating p53 mutations are often found in refractory tumours following relapse (Tweddle, Pearson et al. 2003).

To confirm that cytotoxicity caused by BLF1 is MYCN dependent, the SHEP-TET21N cell line was used. This cell line constitutively expresses high levels of MYCN which can be repressed by the addition of tetracycline. Down-regulation of MYCN after a 72 hour incubation with tetracycline was confirmed via immunoblotting (Fig. 4.4A). Down-regulation of MYCN also caused a decrease in levels of eIF4A, suggesting a regulatory role of MYCN in eIF4A expression (Fig. 4.4A). This is in agreement with a previous study which showed that MYCN up-regulates eIF4A as well as a number of other proteins involved in ribosome biogenesis and protein synthesis (Boon, Caron et al. 2001). Down regulation of MYCN also significantly decreased the growth rate of cells which further demonstrated the importance of MYCN in cell growth and division (Fig. 4.4B). Analysis of growth inhibition showed that BLF1 efficacy was decreased by the addition of tetracycline and down-regulation of MYCN (GI50: 1nM without tetracycline and 42nM with tetracycline) (Fig. 4.4C). Additionally, Hoechst 33342 and propidium iodide staining showed that 300nM BLF1 was only cytotoxic to the cells expressing high levels of MYCN (Fig. 4.4D and E). Taken together, these data strongly suggest a cytotoxic mechanism of action that is enhanced by MYCN amplification.

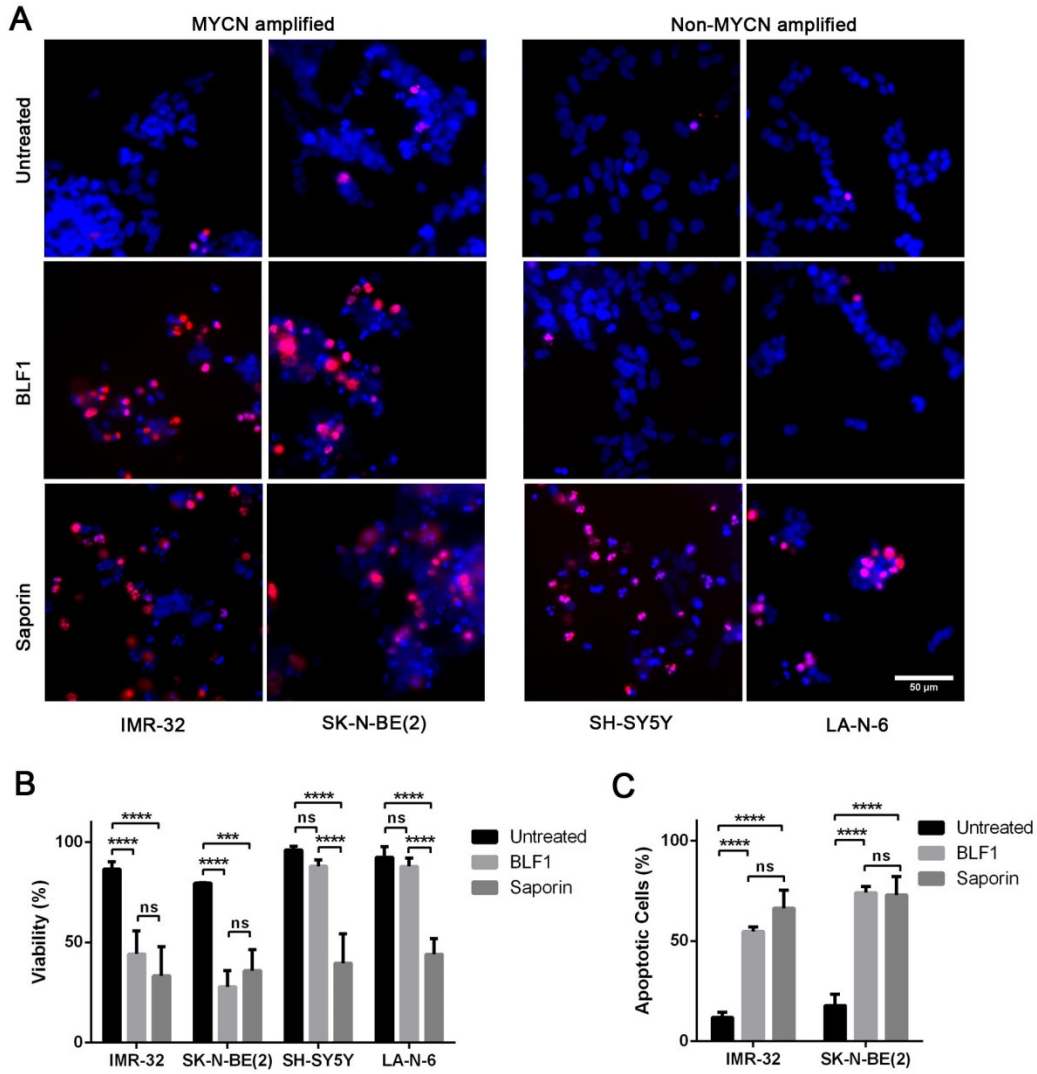

Figure 4.3: A, Fluorescence microscopy images showing Hoechst 33342 (blue) and propidium iodide (red) staining following treatment of neuroblastoma cell lines with 300nM BLF1 or saporin in the presence of LF3000. All cells are stained blue, but only dead cells are stained red. **B,** Quantification of A shows that BLF1 only kills MYCN-amplified cells, whereas saporin kills all cell types (n=3, ± SD). **C,** Caspase 3/7 assay showing that treatment of MYCN-amplified cells with 300nM BLF1 or saporin in the presence of LF3000 induces apoptosis (n=3, ± SD). Data was analysed by two-way ANOVA and Tukey's multiple comparisons test with ***P < 0.001 and ****P < 0.0001.

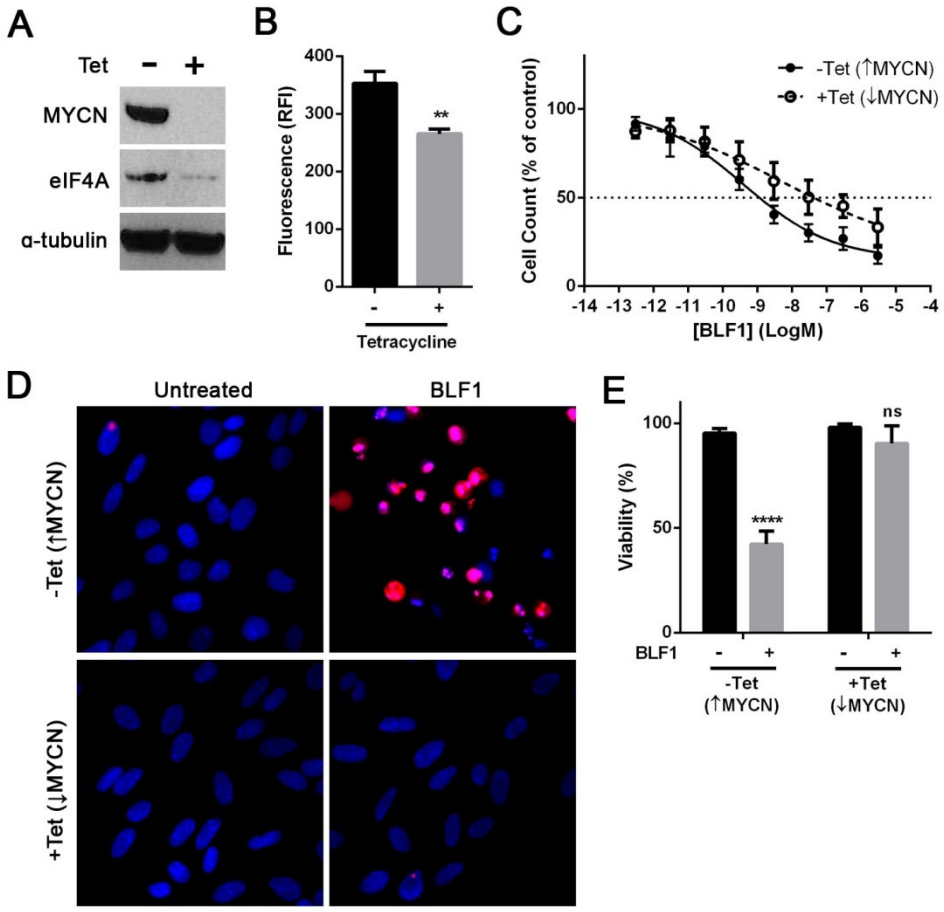

Figure 4.4: A, Immunoblot showing that 72 hour incubation with 1 µg/ml tetracycline in the SHEP-TET21N cell line leads to a down-regulation of MYCN and eIF4A but has no effect on the housekeeping protein α-tubulin. **B,** alamarBlue assay showing how the addition of tetracycline and down-regulation of MYCN affects cell growth. **C,** alamarBlue assay following titration with BLF1 in the presence of LF3000 shows that down-regulation of MYCN decreases sensitivity to BLF1 (n=3, ± SD). **C,** Fluorescence microscopy images showing Hoechst 33342 (blue) and propidium iodide (red) staining following treatment of SHEP-TET21N cells with 300nM BLF1 in the presence of LF3000. **D,** Quantification of C shows that BLF1 only kills cells when MYCN is highly expressed (n=3, ± SD). Data was analysed by multiple unpaired t-tests with ****P < 0.0001.

4.2.4 BLF1 down-regulates MYCN and other oncogenic proteins

Inactivation of eIF4A using the small molecule inhibitor silvestrol has been shown to down-regulate a number of oncogenic proteins, including c-Myc (Wolfe, Singh et al. 2014). MYCN is closely related to c-Myc and therefore may also be down-regulated by eIF4A inhibition. MYCN protein levels were therefore examined in IMR-32 cells by immunoblotting following 48-hour incubation with 30nM BLF1 (Fig. 4.5A). A dramatic decrease in MYCN protein was observed, only in the presence of LF3000,

which aids the delivery of BLF1 into the cytosol of the cell, whereas no effect was seen on levels of the house-keeping protein GAPDH. Quantification of the MYCN immunoblot confirmed BLF1 decreased MYCN levels by 60% (Fig. 4.5B). This suggests that, as with c-Myc, MYCN translation is dependent on a high level of eIF4A activity. Wolfe et al. (2014) also showed that silvestrol can down-regulate the cell cycle regulators cyclin D1 and CDK4. Both of these proteins have been shown to contribute to proliferation and an undifferentiated phenotype in neuroblastoma cells (Molenaar, Ebus et al. 2008). Inactivation of eIF4A with BLF1 led to a decrease in levels of both of these proteins, but did not affect the housekeeping protein α-tubulin (Fig. 4.5C). Preferential down-regulation of proteins that are important for cell proliferation and survival gives a mechanistic insight into the cytotoxic activity of BLF1.

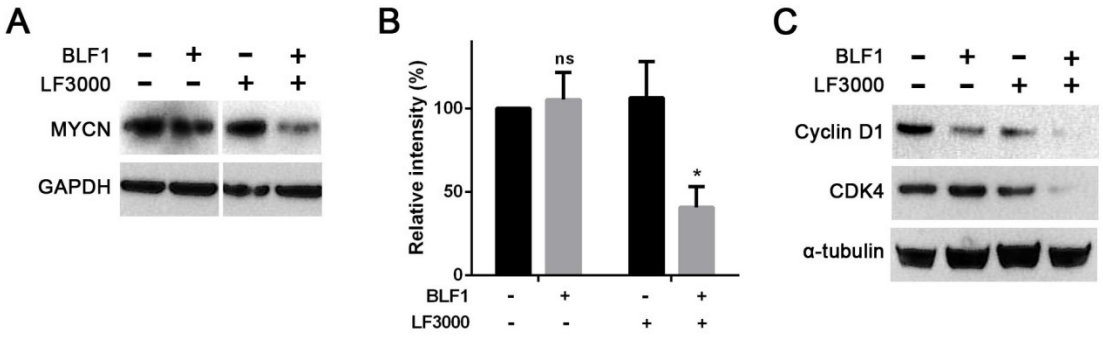

Figure 4.5: A, Immunoblot showing down-regulation of MYCN in IMR-32 cells following a 48 hour incubation with 30nM BLF1 only in the presence of LF3000. No effect on the housekeeping protein GAPDH was observed. **B,** Quantification of A shows that MYCN is down-regulated by approximately 60 % after treatment with BLF1 and LF3000 (n=4, ± SD). Data was analysed by multiple unpaired t-tests with *P < 0.05. **C,** Immunoblot showing down-regulation of Cyclin D1 and CDK4 in IMR-32 cells following a 48 hour incubation with BLF1 in the presence of LF3000. No effect was seen on the housekeeping protein α-tubulin.

4.2.5 BLF1 has no effect on primary fibroblasts

BLF1 shows cytostatic activity in non-MYCN-amplified cells which suggests that it may also have reduced efficacy in non-dividing healthy cells. This is an important characteristic when considering viable therapies for cancer treatment. To test this, primary fibroblasts were cultured from the mouse ear, grown until fully confluent and cultured in low serum media, which has previously been shown to inhibit cell division (Kuznetsov, Xu et al. 2009). Analysis of cell numbers using the alamarBlue assay showed that BLF1 had no effect on viability, whereas saporin was still able to potently induce

cell death (Fig. 4.6A). This was confirmed by Hoechst 33342 and propidium iodide staining which showed that 300nM saporin caused nuclear condensation characteristic of apoptosis, whereas 300nM of BLF1 had no effect on nuclear morphology (Fig. 4.6B and C). The lack of efficacy of BLF1 in non-dividing cells suggests a wide therapeutic window that will be of benefit for future therapy development.

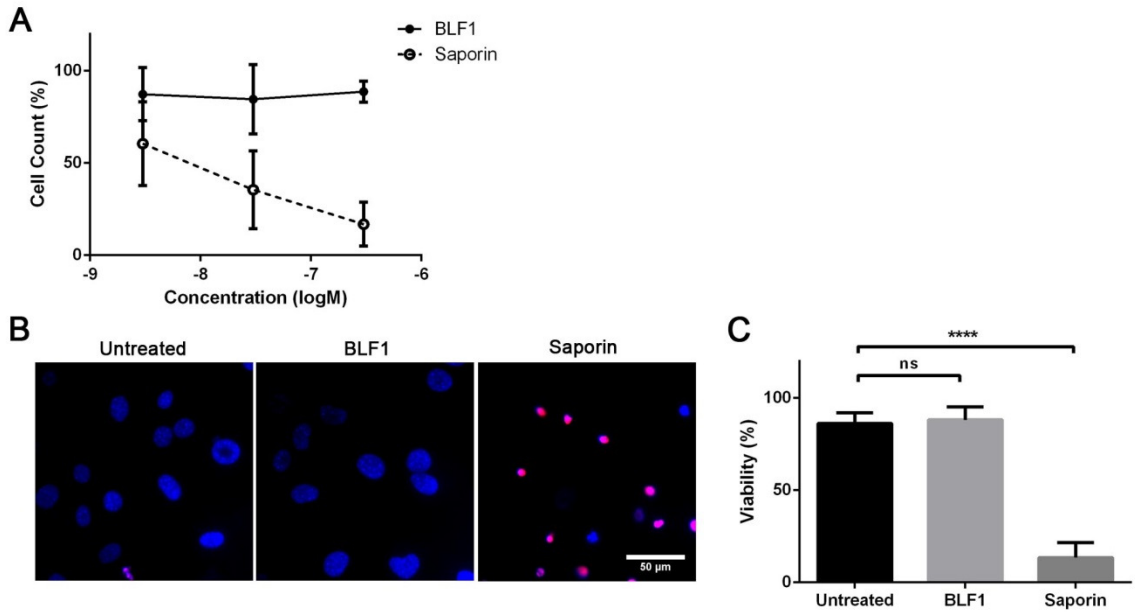

Figure 4.6: A, alamarBlue assay following titrations of BLF1 and saporin in the presence of LF3000 in quiescent mouse fibroblasts shows that BLF1 has no effect on cell numbers whereas saporin causes a large reduction (n=3, ± SD). **B,** Fluorescence microscopy images showing Hoechst 33342 (blue) and propidium iodide (red) staining following treatment of mouse fibroblasts with 300nM BLF1 or saporin in the presence of LF3000. **C,** Quantification of B shows that BLF1 has no effect on cells whereas saporin causes a significant amount of cell death (n=3, ± SD). Data was analysed by one-way ANOVA and Dunnet's multiple comparisons test with ****P < 0.0001.

4.2.6 The eIF4A mutant Q339E shows MYCN dependent cytotoxicity in SHEP-TET21N cells

In chapter 3 (Fig. 3.9) it was shown that the dominant-negative eIF4A mutant Q339E inhibits growth of J774.2 and N2a cells. To see how Q339E efficacy is affected by MYCN expression, the SHEP-TET21N cell line was used and Q339E was delivered to cells using LF3000. Hoechst and propidium iodide staining showed that 1µM of Q339E caused high levels of cytotoxicity in MYCN expressing cells, but had no significant effect on viability following down-regulation of MYCN with tetracycline (Fig. 4.7A and B). This further confirms the selective cytotoxicity of eIF4A inhibition towards cells expressing high levels of MYCN.

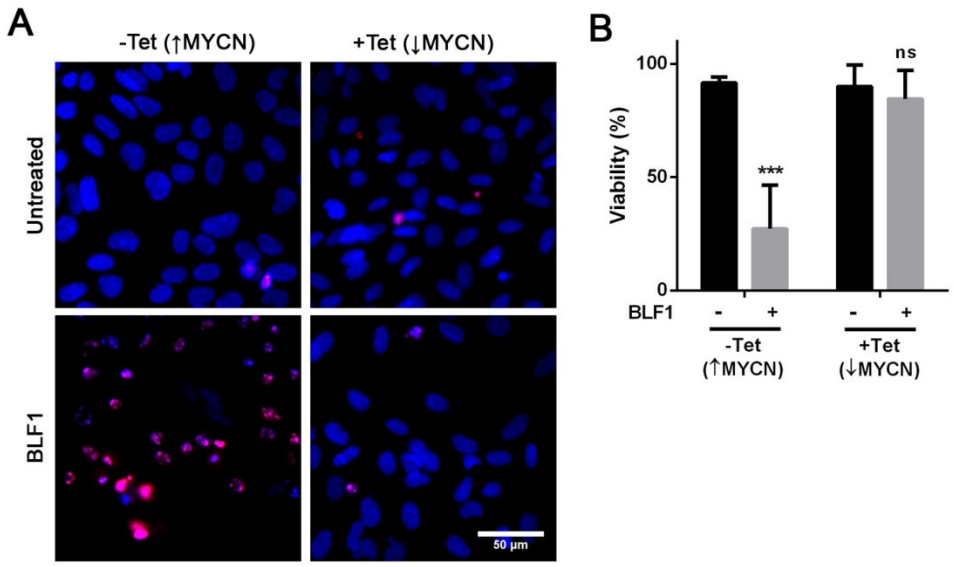

Figure 4.7: A, Fluorescence microscopy images showing Hoechst 33342 (blue) and propidium iodide (red) staining following treatment of SHEP-TET21N cells with 1µM Q339E in the presence of LF3000. **B,** Quantification of A shows that BLF1 causes a significant amount of cell death when cells are expressing high amounts of MYCN but has no effect on cell viability when MYCN expression is low (n=2, ± SD). Data was analysed by multiple unpaired t-tests with ***P < 0.001.

4.3 Discussion

This chapter has shown that inactivation of eIF4A by BLF1 inhibits the growth of neuroblastoma cells with high potency and exhibits increased efficacy and cytotoxic action in MYCN amplified cells. The c-Myc protein has previously been shown to be downregulated following small molecule inhibition of eIF4A with silvestrol (Wolfe, Singh et al. 2014). Similarly, inactivation of eIF4A by BLF1 causes the preferential downregulation of MYCN over housekeeping genes such as GAPDH and α-tubulin. Downregulation of MYCN via siRNA has previously been shown to induce caspase 3 activation and apoptosis in MYCN amplified neuroblastoma (Kang, Rychahou et al. 2006). Caspase 3 activation and induction of apoptosis in a MYCN dependent manner was similarly observed following BLF1 treatment, making the cytotoxic mechanism of action of this toxin selective towards MYCN amplified neuroblastoma cell lines. BLF1 also caused a down-regulation of the cell cycle regulators cyclin D1 and CDK4, which are important for maintaining an undifferentiated phenotype in neuroblastoma cell lines (Molenaar, Ebus et al. 2008). This study demonstrated that cyclin D1 and CDK4 knockdown caused a reduction in proliferation, G(1)-phase cell cycle arrest and extensive neuronal differentiation. Inactivation of eIF4A by BLF1 appears to concomitantly target multiple pathways that

are important for proliferation and cell survival of MYCN amplified neuroblastoma, making this a highly attractive target for the treatment of MYCN-amplified tumours.

As well as highlighting eIF4A as a novel target, BLF1 may be of direct use for future targeted toxin based therapies. Current toxins, including saporin, function via the global inhibition of protein synthesis which leads to cell death in both healthy and transformed cells. Off target toxicity due to low level target antigen expression in healthy cells or non-specific uptake of toxin causes dose-limiting side effects such as vascular-leak syndrome and hepatotoxicity (Alewine, Hassan et al. 2015). BLF1 induced cell death in MYCN-amplified neuroblastoma with high potency similar to saporin, but had little effect on non-dividing fibroblasts. This suggests that BLF1 has a more selective cytotoxic mechanism and a much wider therapeutic window than saporin, which would be of benefit to reduce the side-effects of an immunotoxin based therapy. Further investigation of BLF1 as an immunotoxin and targeting of this enzyme to MYCN-amplified neuroblastoma or other Myc driven cancers will therefore be of high interest. The cytotoxicity of Q339E may also be of interest as it is of human origin making it less immunogenic than bacterial or plant based toxins. However, more research into its efficacy is necessary as only a small amount of targeted toxin will reach the cytosol of target cells so high potency is needed.

MYCN-amplified neuroblastoma is a high risk disease with very few treatment options and poor outcome. A common feature of these cancers is the acquisition of chemo-resistance and relapse making identification of novel targets for treatment of this disease of high importance (Buechner and Einvik 2012). These results suggest that eIF4A inhibitors have potential as future therapeutics in the treatment of neuroblastomas exhibiting MYCN amplification. Furthermore, this data highlights BLF1 as a novel cytotoxic enzyme for targeted toxin development.

Chapter 5: Differentiation of neuroblastoma sensitises cells to BoNT/C protease mediated toxicity

5.1 Introduction

Current treatment of high risk neuroblastoma involves surgery and radiotherapy followed by myeloablative chemotherapy and haematopoietic stem cell rescue (Hara 2012). Treatment is completed by courses of high-dose retinoic acid and anti-GD2 monoclonal antibody therapy in combination with immuno-stimulatory cytokines, to help prevent relapse (Dhillon 2015). Despite this intensive, multimodal treatment, the long term survival rate is poor meaning there is high demand for new forms of therapy that can be incorporated into the treatment regimen.

The use of Dinutuximab, an anti-GD2 monoclonal antibody, has helped to increase long-term survival of high risk patients (Yang and Sondel 2010). This antibody functions by binding to the GD2 ganglioside which is highly expressed on the surface of neuroblastoma cells and eliciting an immune response which leads to cell death by ADCC or CDC. However, efficacy requires a functional immune system, which is often compromised following chemotherapy, and although the co-administration of the immuno-stimulatory cytokines IL-2 and GM-CSF has helped improve this, responses remain limited (Yu, Gilman et al. 2010). Attachment of a cytotoxic cargo, such as a toxin, to the anti-GD2 antibody or another neuroblastoma targeting moiety may help to improve the treatment, particularly in immunocompromised patients.

BoNTs are neurotoxins that function by cleavage of neuronal SNARE proteins necessary for synaptic vesicle fusion and neurotransmitter release (Schiavo, Matteoli et al. 2000). They are widely used in medicine for the treatment of a number of neurological disorders (Munchau and Bhatia 2000). Most of the BoNT serotypes are non-toxic, but BoNT/C and more recently BoNT/E have both been shown to cause neurite degeneration and apoptosis in primary neurons (Peng, Liu et al. 2013). Neuroblastoma cells express neuronal SNAREs which may make them susceptible to BoNT/C treatment, opening up opportunities for a targeted toxin approach. Indeed, a recent study in the Davletov lab has shown that co-delivery of the BoNT/C and BoNT/D proteases leads to apoptosis in mouse neuroblastoma N2a cells (Arsenault, Cuijpers et al. 2013). This is likely due to the production of non-functional SNARE fragments that inhibit SNARE complex formation and disrupt important vesicle trafficking events necessary for cell survival. However, the need to deliver two BoNT toxins makes this a less than ideal therapeutic strategy. The current treatment of neuroblastoma involves retinoic acid which functions by differentiating residual cells into a mature neuronal phenotype (Reynolds, Matthay et al. 2003). This inhibits growth and helps to prevent relapse following

chemotherapy. The effect of BoNT/C on human neuroblastoma cell lines following neuronal differentiation with AT-RA was investigated in this chapter.

5.2 Results

5.2.1 BoNT/C has no effect on the viability of undifferentiated neuroblastoma cells

The effect of BoNT/C on cells was initially tested on the undifferentiated SH-SY5Y and BoNT/A sensitive SiMa cell lines (Pahlman, Hoehner et al. 1995, Fernandez-Salas, Wang et al. 2012). BoNT/C binds to gangliosides that are usually only present on mature neurons, so to ensure a high level of cell entry and SNARE cleavage, 100nM of BoNT/C was initially used. Immunoblotting after a 72 hour incubation with BoNT/C showed complete cleavage of syntaxin 1 and near total cleavage of SNAP25 in both the SiMa and SH-SY5Y cell lines (Fig. 5.1A). This is evidenced by a shift in band size of the SNAP25 protein due to cleavage, and disappearance of syntaxin 1 protein due to degradation of the cleaved product. Despite this high level of SNARE cleavage, no effect was seen on cell viability, as shown by a CCK-8 assay (Fig. 5.1B). This suggests that BoNT/C is not cytotoxic in undifferentiated neuroblastoma cells and that SNAP25 and syntaxin 1 are not required for cell survival.

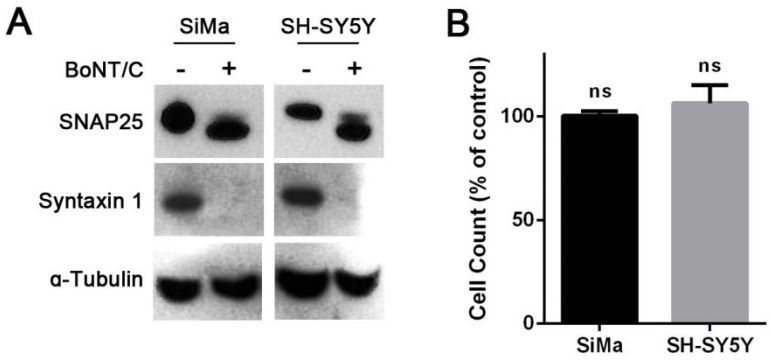

Figure 5.1: A, Immunoblot showing cleavage of SNAP25 and syntaxin 1 following a 72 hour incubation with 100nM BoNT/C. **B,** CCK-8 assay showing that 100nM BoNT/C does not affect cell growth (n=3, ± SD). Data was analysed by multiple one-sample t-tests.

5.2.2 Retinoic acid and serum removal robustly differentiates SiMa cells to a neuronal phenotype

Neuronal differentiation of SiMa cells has previously been shown to dramatically increase sensitivity to SNAP25 cleavage by BoNT/A (Fernandez-Salas, Wang et al. 2012). To investigate whether this is also true for BoNT/C, a differentiation protocol was developed to robustly differentiate SiMa cells into a neuronal phenotype. Optimisation of this protocol was initially carried out by observing morphological changes such as neurite outgrowth. This is a common method for initial assessment of neuronal differentiation of neuroblastoma cells (Schneider, Giordano et al. 2011, Dwane, Durack et al. 2013). SiMa cells were grown on different surface substrates including laminin, collagen and poly-L-lysine for 72 hours in the presence or absence of serum and changes in morphology were observed by microscopy (Fig. 5.2A). Of the substrates tested, laminin induced the highest level of neurite growth, both in the presence and absence of serum. However, the neuronal morphology was more pronounced after serum removal. *In vitro* studies demonstrate that retinoic acid contributes to the differentiation of cells into a neuronal phenotype, leading to up-regulation of neuronal markers and decreased cell division (Thiele, Reynolds et al. 1985). Addition of 10µM AT-RA to serum free cells led to a dramatic increase in neurite outgrowth, suggesting a more mature neuronal phenotype (Fig. 5.2B). Counting of cells over ten days in culture also showed that AT-RA attenuates cell division (Fig. 5.2C). SiMa cells grown in normal media will henceforth be referred to as undifferentiated, and SiMa cells grown on laminin in serum free media with 10µM AT-RA will be referred to as differentiated.

Analysis of cell morphology and division show that the protocol causes a large increase in neurite outgrowth. The extent of neuronal differentiation was then analysed in more detail at a molecular level. Immunocytochemistry was carried out after 72 hours of differentiation and showed an increase in the neuronal marker β-III-tubulin as well as the ganglioside GT1b which is a binding target of BoNT/C and usually only expressed on mature neurons (Fig. 5.3A). The increase in GT1b was confirmed by quantification of the percentage of cells that stained for GT1b (Fig. 5.3B). No difference in expression of SNAP25 was observed. The increase in β-III-tubulin levels was confirmed via immunoblotting, which also showed increases in expression of Tau proteins and synaptic vesicle protein 2A (SV2A), a binding target of BoNT/A (Fig. 5.3C). Quantification of band density showed a 50 % increase in β-III-tubulin, a two-fold increase in SV2A, and a three-fold increase in Tau (Fig. 5.3D). Again, no change in SNAP25 levels was observed. Taken together, these data show that SiMa cells can be robustly differentiated into a neuronal phenotype, exhibiting extensive neurite outgrowth, decreased cell division and increased expression of neuronal marker proteins.

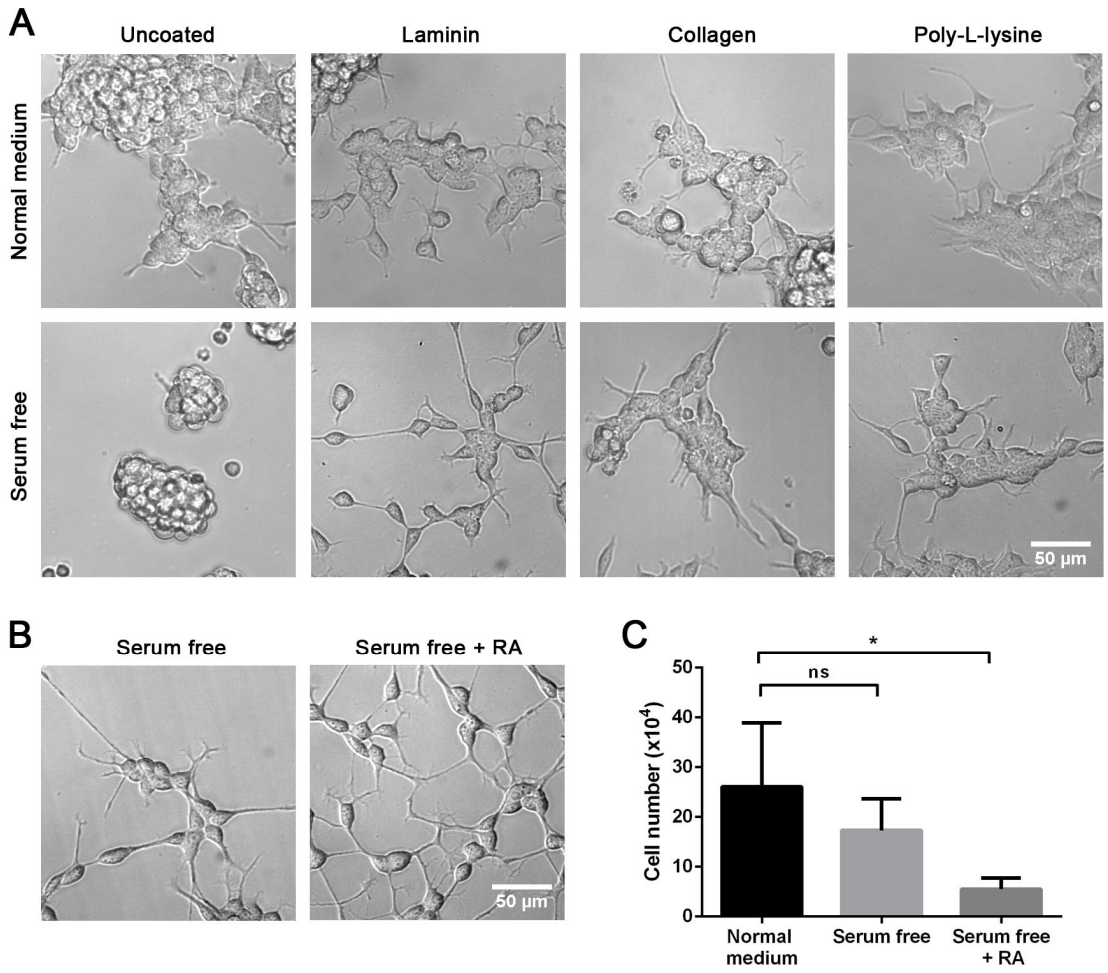

Figure 5.2: A, Light microscopy images showing morphology of SiMa cells in normal or serum free media using different surface coatings. **B,** Light microscopy images showing morphology of SiMa cells grown in serum free media on laminin with or without 10µM AT-RA. Neurite outgrowth is increased in the presence of AT-RA. **C,** Automated cell counts after growing SiMa cells for 10 days in normal media, serum free media and serum free media containing AT-RA (n=3, ± SD). Data was analysed by one-way ANOVA and Dunnet's multiple comparisons test with *P < 0.05.

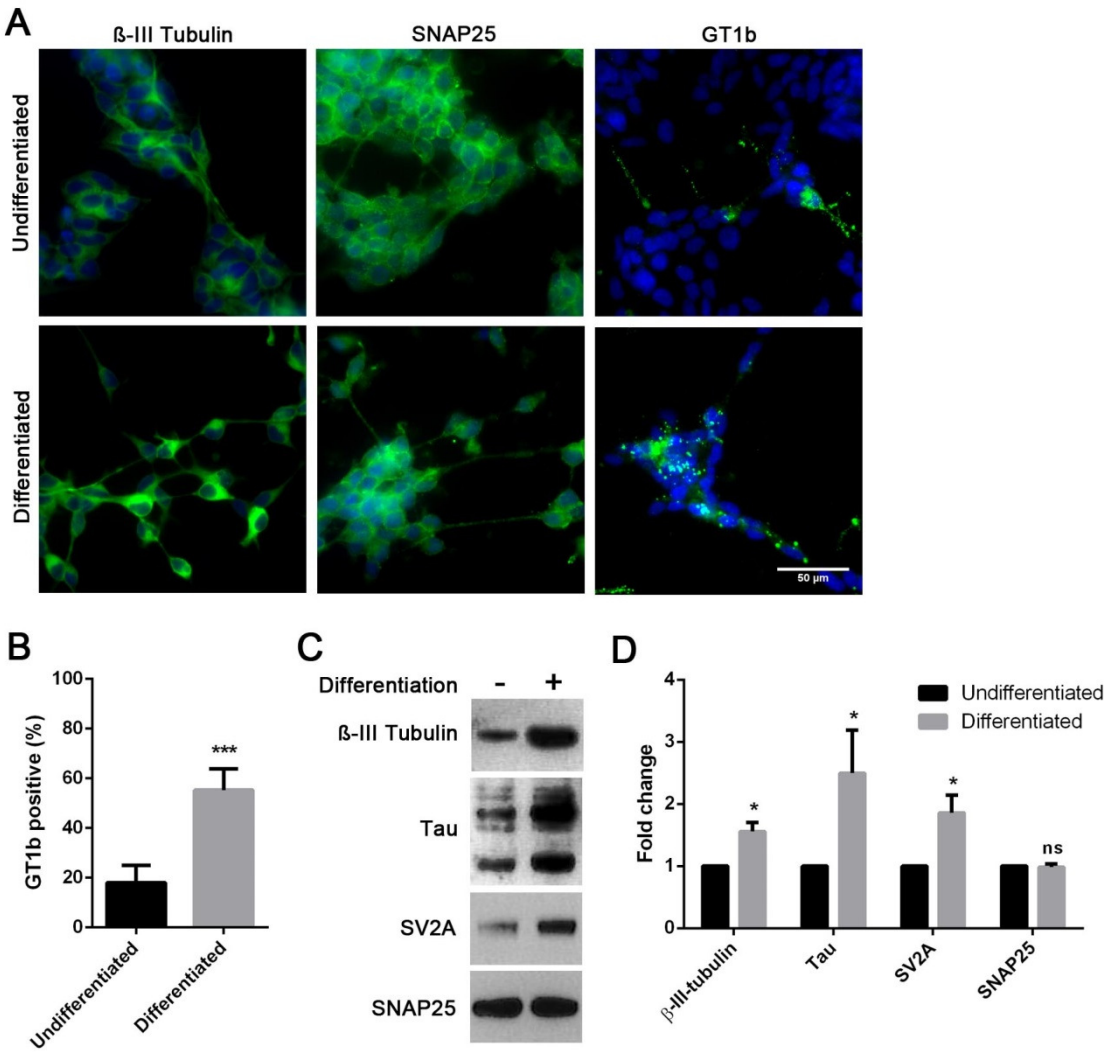

Figure 5.3: A, Immunocytochemistry images showing increased expression of the neuronal marker β-III Tubulin and the ganglioside GT1B following differentiation of SiMa cells. No change was seen in levels of the BoNT/C substrate SNAP25. Nuclei are stained using DAPI (blue). **B,** Quantification of the percentage of GT1b positive cells shown in A (n=4, ± SD). Data was analysed by an unpaired t-test with P*** < 0.001. **C,** Immunoblot showing increased levels of the neuronal markers β-III tubulin, Tau and SV2 following differentiation of SiMa cells. Again, no change was seen in levels of SNAP25. **D,** Quantification of B confirms increases in neuronal markers (n=3, ± SD). Data was analysed by multiple one-sample t-tests with *P < 0.05.

5.2.3 Neuronal differentiation of SiMa cells increases BoNT/C activity and sensitises cells to BoNT/C mediated cytotoxicity

Differentiation of SiMa cells leads to an increase in levels of GT1b, the binding target for BoNT/C, which suggests that it would also lead to increased binding of BoNT/C. To confirm this, the receptor

binding domain (Rbd) of BoNT/C (Fig. 5.4A) was recombinantly expressed with a Cy3-tag. Live imaging following a 1 hour incubation with BoNT/C Rbd showed a marked increase in binding in the differentiated cells when compared to the undifferentiated cells (Fig. 5.4B). Immunoblotting of SNAP25 and syntaxin 1 cleavage also showed an increase in sensitivity to BoNT/C enzymatic activity (Fig. 5.4C). Quantification of the blotting confirmed this and showed an increase in sensitivity of around 10-fold (Fig. 5.4D). This increased SNARE cleavage is likely due to increased binding and internalisation of BoNT/C.

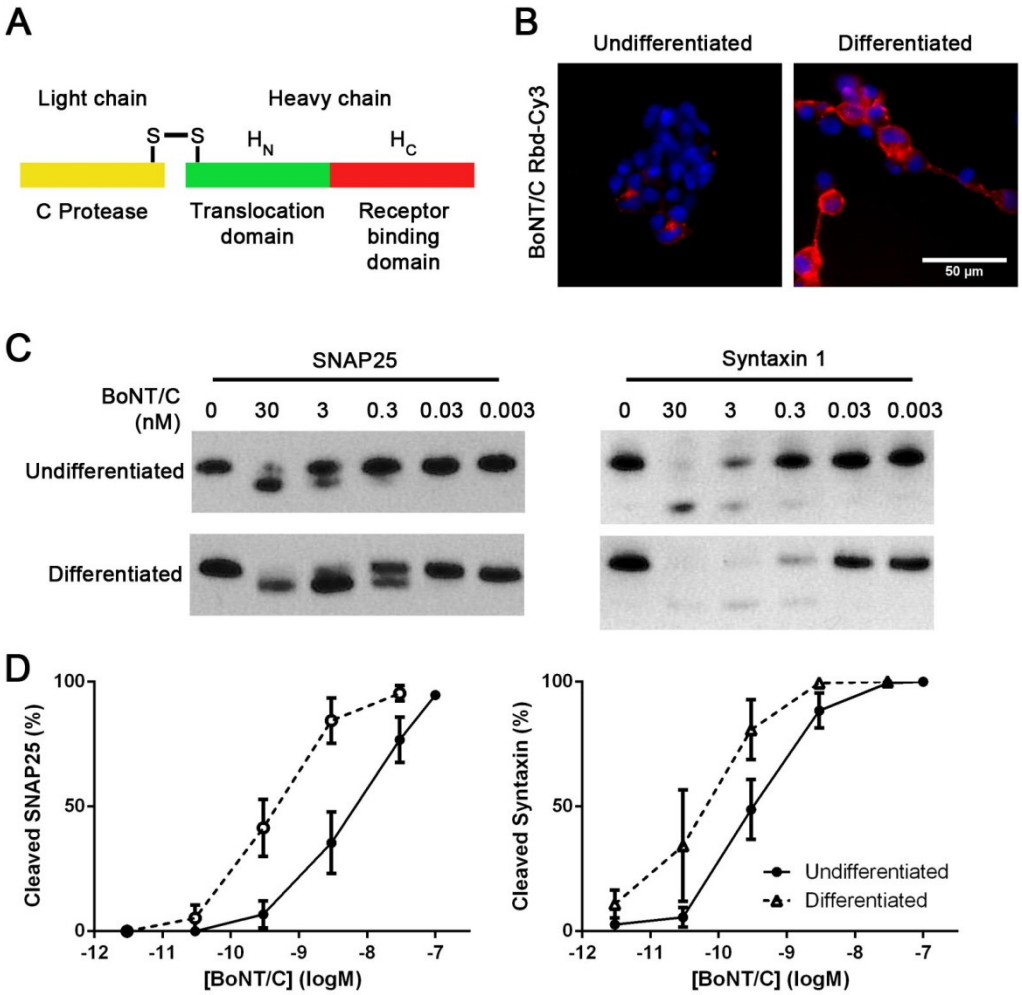

Figure 5.4: A, Schematic of the BoNT/C toxin showing the light chain protease (yellow) joined to the heavy chain via a disulphide bond. The heavy chain is further divided into a receptor binding domain (red) and a translocation domain (green). **B,** Fluorescence microscopy images showing that binding of Cy3-tagged BoNT/C Rbd is increased following differentiation of SiMa cells. **C,** Immunoblots demonstrate increased sensitivity to cleavage of SNAP25 and syntaxin 1 by BoNT/C following differentiation of SiMa cells. **D,** Quantification of C shows that differentiated SiMa cells are around 10 times more sensitive to SNARE cleavage by BoNT/C (n=3, ± SD).

Next, the effect of BoNT/C SNARE cleavage on cell viability was investigated. Analysis of cell morphology via light microscopy revealed that incubation of cells with 30nM BoNT/C for 72 hours caused neurite loss and rounding of differentiated cells, indicating cytotoxicity (Fig. 5.5A). No effect was seen on the morphology of the undifferentiated cells. This cytotoxicity was confirmed via a CCK-8 assay which showed an IC50 of approximately 10nM in the differentiated cells (Fig. 5.5B). BoNT/C had no effect on the number of undifferentiated cells, even at 100nM. This strongly suggests that differentiation of SiMa cells sensitises them to BoNT/C mediated cytotoxicity.

The mechanism of cell killing was then investigated in more detail. Incubation with BoNT/C caused nuclear condensation indicative of apoptosis in differentiated cells, whereas it had no effect on the nuclear morphology of undifferentiated cells, as shown by Hoechst 33342 staining (Fig. 5.5C). A caspase 3/7 assay was then carried out which showed caspase activation in almost all of the differentiated cells (Fig. 5.5D). This mechanism of killing by BoNT/C has been observed in previous studies which showed that BoNT/C induces apoptosis in a caspase 3/7 dependent manner in primary neurons (Berliocchi, Fava et al. 2005).

To confirm that cytotoxicity of differentiated SiMa cells is a characteristic of BoNT/C and not BoNT toxins in general, SNARE cleavage and cell viability was investigated following incubation with 30nM BoNT/A. Immunoblotting showed that differentiation leads to an increase in SNAP25 cleavage after a 72 hour incubation with 30nM BoNT/A (Fig. 5.6A). Despite almost total cleavage of SNAP25, no effect was seen on cell morphology as shown by light microscopy, or cell number, as shown by CCK-8 assay (Fig. 5.6B and C).

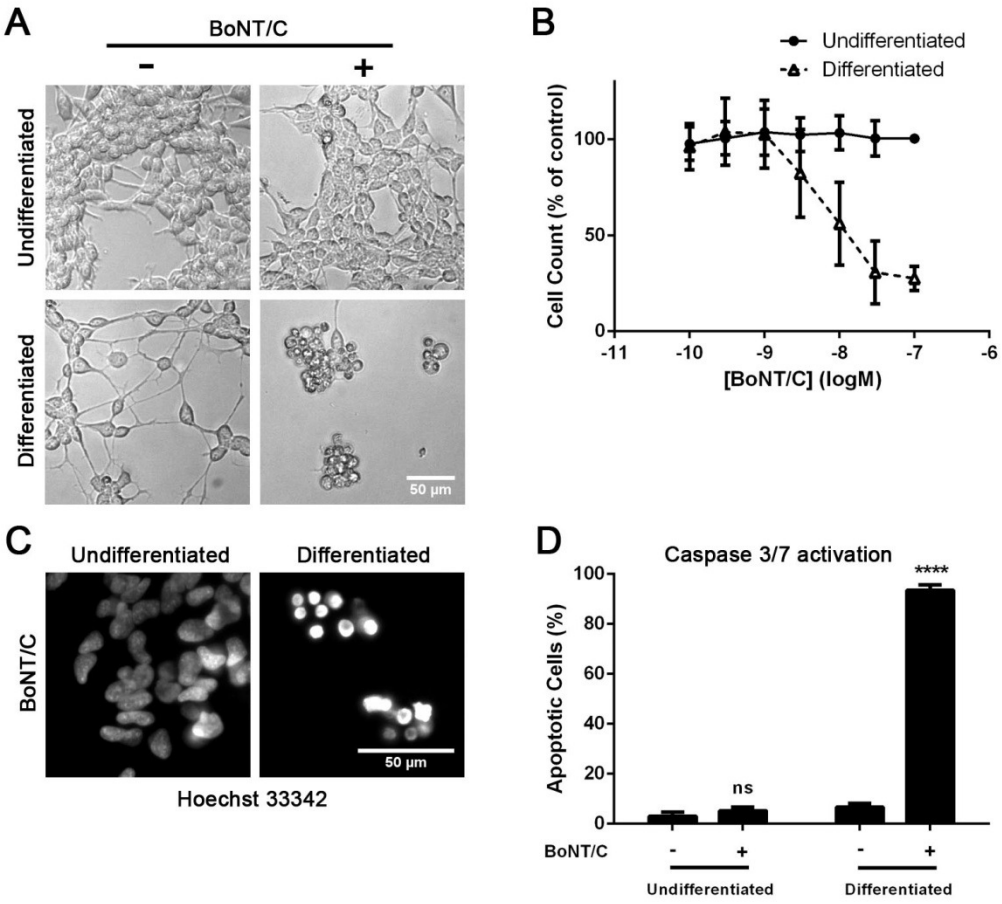

Figure 5.5: A, Light microscopy images show cell death of differentiated SiMa cells following incubation with 30 nM BoNT/C as characterised by cell rounding. No effect was observed on undifferentiated cells. **B,** CCK-8 assay following a titration with BoNT/C shows decreasing cell numbers after differentiation but no effect on undifferentiated cells (n=3, ± SD). **C,** Fluorescence microscopy images of Hoechst 33342 stained cells following treatment with 30nM BoNT/C. Shows nuclear condensation indicative of apoptosis only after differentiation. Caspase 3/7 assay shows high level of caspase 3/7 activation after treatment with 30nM BoNT/C in differentiated cells (n=3, ± SD). Data was analysed by multiple unpaired t-tests with ****P < 0.0001.

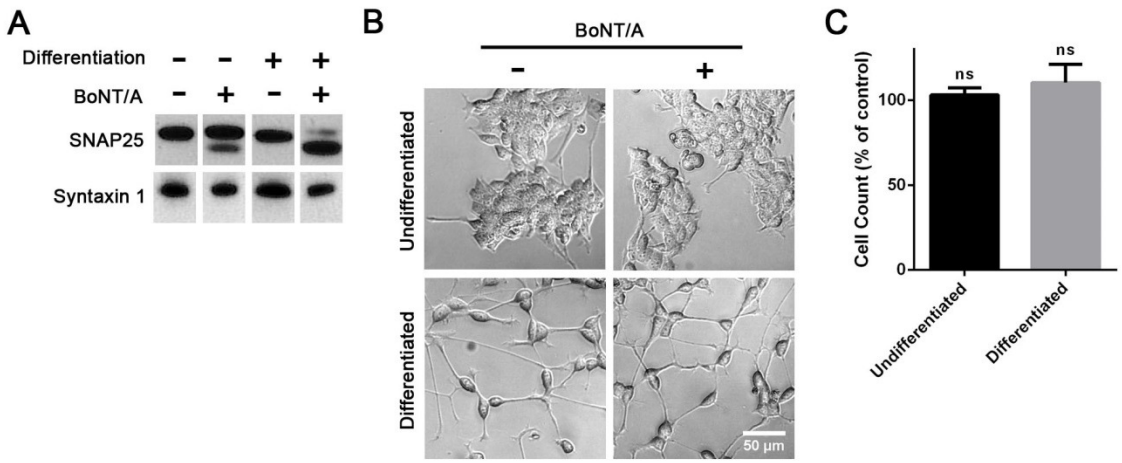

Figure 5.6: A, Immunoblot showing high levels of SNAP25 cleavage in differentiated SiMa cells following treatment with 30nM BoNT/A. **B,** Light microscopy images showing that BoNT/A has no effect on cell morphology. **C,** CCK-8 assay showing that BoNT/A has no effect on cell numbers (n=3, ± SD). Data was analysed by multiple one-sample t-tests.

5.2.4 Differentiation of SiMa cells down-regulates SNAP23

Exogenous expression of the non-neuronal SNAREs SNAP23 and syntaxins 2-4 has been shown to rescue mature neurons from BoNT/C mediated toxicity (Peng, Liu et al. 2013). Expression of these SNAREs in SiMa cells before and after differentiation was therefore investigated. Immunoblotting revealed that SiMa cells express high levels of syntaxin 1 and lower levels of the non-neuronal homologues syntaxins 2-4, and that levels were unaffected by differentiation (Fig. 5.7A). Interestingly, despite no effect on SNAP25, SNAP23 expression was almost completely abolished following differentiation (Fig. 5.7A). Incubation of undifferentiated cells with 30nM BoNT/C led to almost complete cleavage of SNAP25 and syntaxin 1, and a small amount of cleavage of syntaxin 2 (Fig. 5.7B). Syntaxins 3 and 4, and SNAP23 were all resistant to BoNT/C cleavage at this concentration. This cleavage resistance provides a possible mechanistic insight into why differentiation sensitises SiMa cells to BoNT/C-induced toxicity.

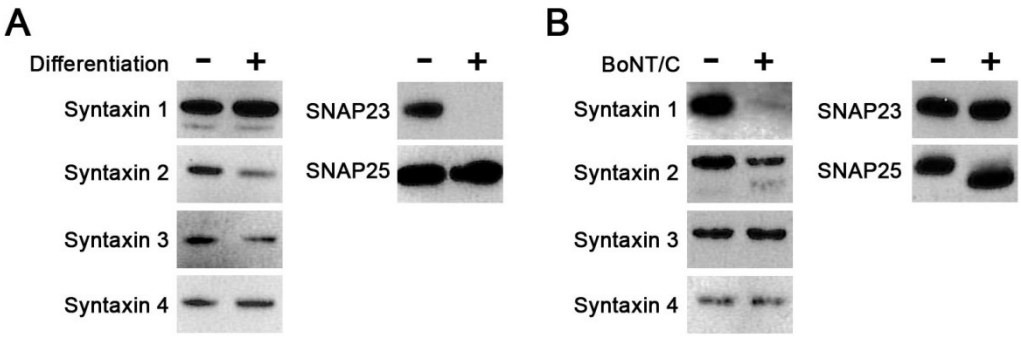

Figure 5.7: A, Immunoblot showing that differentiation of SiMa cells has little effect on syntaxin 1-4 protein levels but down-regulates SNAP23. **B,** Immunoblot showing near complete cleavage of Syntaxin 1 and SNAP25 following incubation of undifferentiated SiMa cells with 30nM BoNT/C. A small level of syntaxin 2 cleavage is seen, but syntaxins 3 and 4, and SNAP23 are resistant to BoNT/C activity at this concentration.

5.2.5 BoNT/C protease shows higher potency in differentiated SiMa cells than other cytotoxic toxins

In order to be a viable candidate for an immunotoxin, BoNT/C needs to show very high potency upon entry into the cell. To establish whether BoNT/C is potent enough, the enzymatic light chain was used without the Rbd or translocation domains. This enzymatic region is unable to bind and enter cells, so delivery into cells was carried out via LF3000 as described previously (Rust, Hassan et al. 2015). Enzymatic activity in SiMa cells was confirmed via immunoblotting which showed cleavage of both SNAP25 and syntaxin 1 by 10nM C protease only in the presence of LF3000 (Fig. 5.8A). Delivery of enzymes into cells using LF3000 allowed for the direct comparison of the efficacy of C protease with other cytotoxic enzymes. The enzymes used include the RIPs saporin and gelonin, and the enzymatic region of diphtheria toxin. Their relative sizes were visualised via Coomassie staining (Fig. 5.8B). All of these enzymes have previously been used as immunotoxins and are able to kill cells with high potency upon entry into the cytosol, meaning that only a small amount of toxin needs to be introduced into the cell to cause cell death (Kreitman 2006). Incubation of differentiated SiMa cells with the different enzymes for 72 hours revealed that the C protease has at least a 10-fold higher potency than the other toxins (Fig. 5.8C). This suggests that BoNT/C is highly toxic to differentiated neuroblastoma cells, making it a promising payload for targeted therapy.

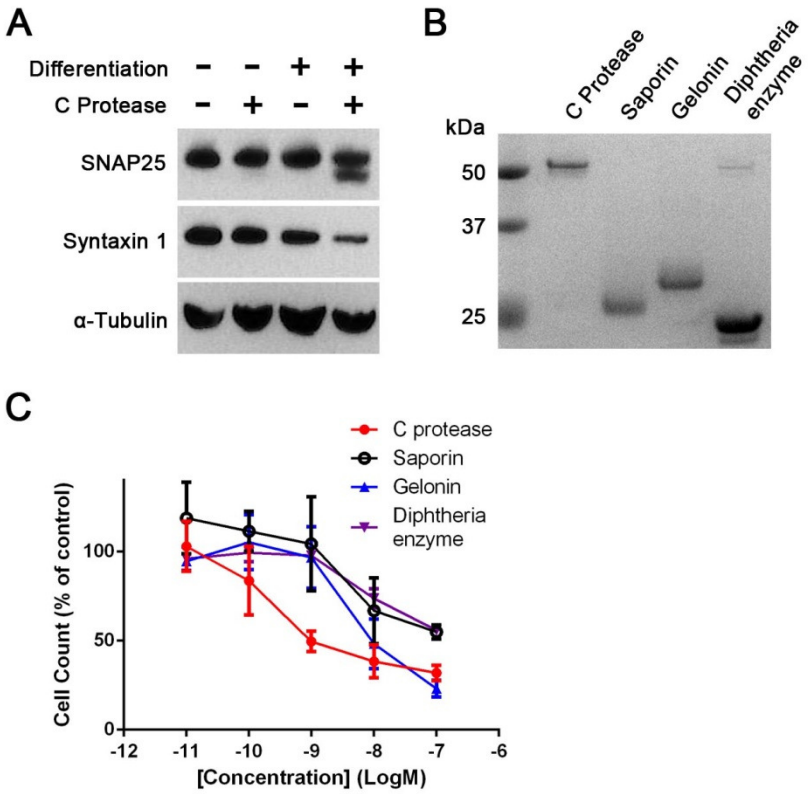

Figure 5.8: A, Immunoblot showing cleavage of SNAP25 and syntaxin 1 by 10nM C protease only in the presence of LF3000. **B,** Coomassie stain showing the different cytotoxic enzymes. **C,** CCK-8 assay following titration with enzymes shows the C protease is around 10 times more potent than the other enzymes tested (n=3, ± SD).

5.2.6 BoNT/C protease is cytotoxic in differentiated SH-SY5Y cells

To confirm that the cytotoxic effect of the BoNT/C protease is not specific to differentiated SiMa cells, SH-SY5Y cells were also tested. Differentiation of these cells using the same protocol developed for SiMa cells led to extensive neurite outgrowth and sensitised cells to BoNT/C protease-induced toxicity. This is demonstrated by microscopy which shows neurite loss and cell rounding following a 72 hour incubation with the protease, as well as a decrease in cell number as evidenced by CCK-8 assay (Fig. 5.9A and B). No effect was seen on the undifferentiated cells following incubation with BoNT/C protease. Activity of the protease was confirmed by immunoblotting which showed cleavage of SNAP25 in both differentiated and undifferentiated cells only in the presence of LF3000 (Fig. 5.9C). Differentiation of the SH-SY5Y cells also led to a substantial decrease in SNAP23 levels, as was

seen in the SiMa cell line (Fig. 5.9C). This data suggests that down-regulation of SNAP23 and BoNT/C-induced toxicity is a common feature of differentiation following treatment with retinoic acid.

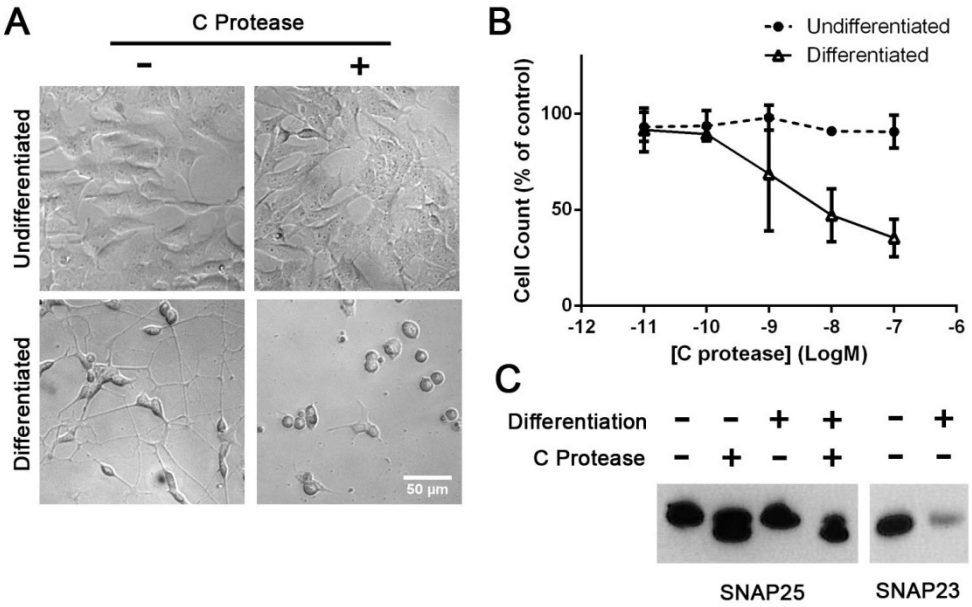

Figure 5.9: A, Light microscopy images show cell death of differentiated SH-SY5Y cells following incubation with 10 nM C protease in the presence of LF3000. No effect was observed on undifferentiated cells. **B,** CCK-8 assay following titration with C protease in the presence of LF3000 shows that C protease causes a reduction in number of differentiated cells (n=3, ± SD). **C,** Immunoblot shows cleavage of SNAP25 after incubation with C protease in the presence of LF3000. **D,** Immunoblot showing that differentiation causes a reduction in levels of SNAP23.

5.3 Discussion

This chapter has shown that BoNT/C induces apoptosis in differentiated neuroblastoma cells and that delivery of the BoNT/C protease into differentiated SiMa cells using LF3000 shows a 10-fold higher potency than currently used targeted toxins. This high level of potency makes the protease a promising candidate for targeted toxin therapy. Currently used toxins such as saporin and DT function by complete inhibition of protein synthesis and therefore have a non-specific cytotoxic mechanism (Alewine, Hassan et al. 2015). The BoNT/C protease may be a better form of treatment than these toxins as it has a more specific mechanism of action which will only affect neuroblastoma cells and mature neurons. This means that common, dose-limiting side effects such as VLS and hepatotoxicity should not occur. Additionally, the presence of a translocation domain in the full BoNT will help with delivery of the protease into the cytosol of target cells, as has been

demonstrated previously with the re-targeting of BoNT/A to non-neuronal cells (Somm, Bonnet et al. 2012, Arsenault, Ferrari et al. 2013).

The sensitisation of neuroblastoma cells to BoNT/C following differentiation is likely due to the down-regulation of SNAP23 combined with SNAP25 and syntaxin 1 cleavage. As has been shown previously, the exogenous expression of both SNAP23 and non-neuronal syntaxins in mature neurons rescues them from BoNT/C induced cytotoxicity (Peng, Liu et al. 2013). This is because these SNAREs play a functionally redundant role in membrane recycling processes that are necessary for cell survival. The SiMa cells express non-neuronal syntaxins which are not affected by differentiation. However, this is not enough to rescue the cells as SNAP23 is also necessary, likely due to the inability of cleaved SNAP25 to interact with these non-neuronal syntaxins. A recent study has shown that knockdown of SNAP23 leads to cell death in a number of cell models, suggesting a vital role for this SNARE in maintaining cell viability (Kaul, Mittal et al. 2015). This chapter further highlights the importance of SNARE proteins in cell survival.

An important limitation of using BoNT/C as a targeted toxin for neuroblastoma is that the cytotoxic action requires full differentiation of residual cells following retinoic acid therapy. The mechanism of resistance to retinoic acid is unclear and could be due intrinsic resistance where a small percentage of cells are resistant upon the onset of therapy and continue to divide until relapse (Joshi, Guleria et al. 2007). Another possible explanation is acquired resistance where cells develop resistance mechanisms during repeat exposure (Reynolds, Wang et al. 2000). It is likely that retinoic acid resistance is a combination of acquired and intrinsic resistance, meaning that targeted BoNT/C may only be effective for a small subset of patients. Further testing of SNAP23 levels and targeted-BoNT/C efficacy following retinoic acid treatment *in vivo* is therefore necessary to fully understand the potential benefits of the BoNT/C protease as a targeted toxin. Work is currently ongoing to increase the level of differentiation in neuroblastoma cells and the use of alternative differentiating reagents such as vasoactive intestinal peptide (VIP), brain derived neurotrophic factor (BDNF) and estradiol, with or without retinoic acid, is showing promising results in a number of studies (Teppola, Sarkanen et al. 2015, Boisvilliers, Perrin et al. 2016). Advances in this area may further increase the potential benefit of a BoNT/C based targeted therapy.

Chapter 6: General discussion

6.1 Summary of findings

All current immunotoxins have displayed dose-limiting side effects in clinical testing due to off-target toxicity in healthy cells. This thesis aimed to address this issue in the treatment of neuroblastoma by using protein delivery techniques to analyse the effects of two enzymes (BLF1 and BoNT/C protease) that have alternative cytotoxic mechanisms to conventional immunotoxin payloads. Findings are summarised in figure 6.1.

Enzymes are macromolecules that are unable to cross the cell membrane unaided. This makes the testing of intracellularly active enzymes more complex than small molecules as the large proteins need to be delivered into the cytosol of the cell for them to carry out their catalytic action. In chapter 3, two novel approaches were used to assess the action of these enzymes. Use of the DNA transfection reagent LF3000 or the J774.2 macrophage cell line allowed for efficient uptake of protein and an increase in potency by around 100-1000 fold. This allowed for analysis of enzyme action at low nano-molar to pico-molar concentrations which is similar to the effective concentrations of targeted-toxins seen *in vitro*. The LF3000 approach allows for the delivery of protein into a range of cell lines, whereas the J774.2 cell line approach allows for the direct comparison of different enzymes in a mammalian cell line without the need for any delivery techniques.

In chapter 4, both approaches reported in chapter 3 were used to assess the cytotoxic action of BLF1, an inhibitor of eIF4A. The J774.2 cell line was used to directly compare the effect of this enzyme with other translation inhibiting enzymes in a mammalian cell setting. Data showed that BLF1 inhibited growth with high potency similar to that seen with other toxins, but did not kill the cells at the concentrations tested. LF3000 was then used to deliver BLF1 into a range of different neuroblastoma cell lines. Again, BLF1 was shown to potently inhibit cell growth, but was cytotoxic only to MYCN amplified cell lines. No effect was seen on non-transformed primary fibroblasts. This suggests that BLF1 is cytostatic to rapidly dividing cells but shows selective cytotoxicity to cells that have a particular dependence on eIF4A.

In chapter 5, BoNT/C action was tested in neuroblastoma cells as it has previously been reported to be cytotoxic in neurons. Data showed that BoNT/C induces apoptosis in neuroblastoma cell lines, but only after neuronal differentiation with retinoic acid. LF3000 was again used to deliver the BoNT/C protease into differentiated cells and compare potency with translation inhibiting enzymes. This revealed that BoNT/C is highly potent and therefore a viable option for an immunotoxin payload.

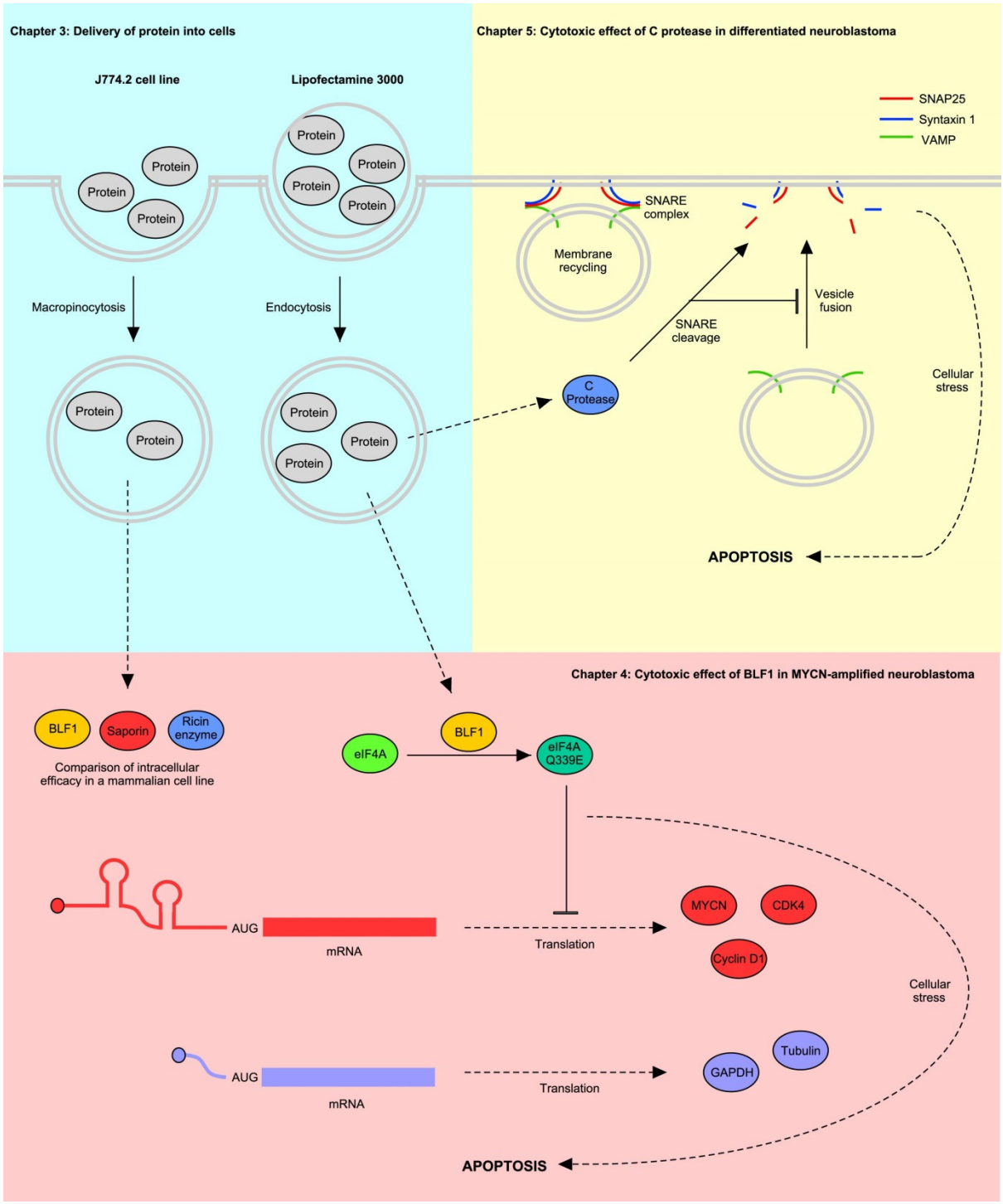

Figure 6.1: Schematic summarising work carried out in this thesis. Two protein delivery techniques allows for delivery of immunotoxin payloads into cells to assay intracellular function (Chapter 3). BLF1 inactivates eIF4A leading to downregulation of MYCN and other proteins necessary for cell survival of MYCN-amplified neuroblastoma (Chapter 4). Differentiation of neuroblastoma cells downregulates SNAP23 which sensitises cells to BoNT/C mediated toxicity due to inhibition of important membrane trafficking events (Chapter 5).

6.2 Significance

The findings from this thesis highlight two novel payloads, BLF1 and the BoNT/C protease, for targeted toxin design in the treatment of neuroblastoma. These toxins offer advantages over traditional, translation inhibiting enzymes as they show reduced off target toxicity whilst maintaining efficacy. The enzyme delivery techniques may also be of use in the future investigation of other intracellularly active enzymes or proteins.

Small molecule drugs have dominated drug development for decades. This is because they are cheap to produce, easy to modify and can be efficiently delivered into cells (Thompson and Ellman 1996). However, the simple structure of small molecules greatly reduces their ability to interact with cellular components. Indeed, estimates of the 'druggable' proteome - proteins that are predicted to be able to interact with small molecule inhibitors - reveals that only around 15 percent of proteins can be targeted by these drugs (Russ and Lampel 2005). Larger therapeutic molecules such as small peptides and proteins hold promise as they are able to overcome the inherent problems of small molecules and inhibit important targets normally considered undruggable. However, delivery of these large molecules into cells has remained a challenge both experimentally and clinically, which has limited therapeutic protein targets to mostly extracellular components.

Use of the DNA transfection reagent LF3000 to deliver proteins into cells holds a number of advantages over current protein delivery techniques. The method is almost identical to that used for DNA transfection and does not require any specialist equipment, unlike with the microinjection or electroporation techniques. This means that it should be easily adaptable for use in many different laboratories. Additionally, this approach is cost effective and simple, which allows for the easy comparison of different proteins in different cell lines. This is in contrast to microinjection, which requires delivery on a single cell basis; electroporation, which requires resuspension in suitable buffer; and the use of cell penetrating peptides, which requires recombinant modification of proteins tested. However, as with DNA transfection, delivery efficiency appears to vary between cell lines, making the use of positive controls (such as saporin in chapter 4) important when investigating potency and efficacy of proteins.

A surprising finding in chapter 3 was that the J774.2 macrophage cell line displays high sensitivity to cytotoxic enzymes even in the absence of delivery techniques. Further testing revealed that this was, in part, due to a high level of uptake via macropinocytosis. Indeed a protein delivery technique named iTOP (induced transduction by osmocytosis and propanebetaine) has recently been developed which functions by the induction of macropinocytosis (D'Astolfo, Pagliero et al. 2015). This demonstrates the efficiency of this cellular process for protein delivery. The iTOP technique

utilises high concentrations of NaCl in the cell media leading to hyperosmolality and increased uptake of protein via macropinocytosis. However, NaCl alone is not sufficient for cytoplasmic delivery as the internalised protein remains trapped in vesicles. To overcome this, a second compound, propanebetaine, was used to facilitate cytosolic entry. Propanebetaine is a zwitterionic compound at neutral pH and was shown to induce macropinosome leakage leading to protein escape into the cytosol. As with iTOP, data from chapter 3 suggests that macropinocytosis alone is not sufficient for cytoplasmic delivery of proteins. This is demonstrated by the lack of sensitivity of the RAW 264.7 cell line to BLF1, despite high macropinocytic activity. It is therefore likely, that a further characteristic of the J774.2 cell line allows entry of protein into the cytosol. However, this characteristic is currently unknown.

The biggest advantages of using the J774.2 cell line are its simplicity and the absence of any further reagents or compounds that may affect cell physiology. Although limited to one cell line, this approach nevertheless has a use for the testing of intracellularly active enzymes in a mammalian cell setting. Its simplicity makes it suitable for use in the initial screening of enzymes, and it may be extended for use with other macromolecules or drugs with low cell membrane permeability. As with HEK-293 cells, which are used for their ease of manipulation by nucleic acid transfection, the J774.2 cell line could be used as a cellular 'test tube', allowing for the easy delivery of proteins to directly disrupt or upregulate cellular processes. An example of this was the finding that cleavage of VAMP3 by BoNT/D light chain caused a dramatic change in cell morphology, implicating VAMP3 in macrophage cell adhesion and spreading. This has been observed in a previous study which utilised siRNA to knockdown VAMP3 (Veale, Offenhauser et al. 2011). However, siRNA mediated knockdown was less efficient than BoNT/D as low levels of VAMP3 were still present. The J774.2 cell line and enzyme-mediated inactivation may therefore be a more suitable model for investigating the role of VAMP3 in cell adhesion.

Both of these protein delivery approaches are particularly relevant for the delivery of immunotoxin payloads as they allow for analysis of action in the low nanomolar to picomolar range. This is comparable to the effect seen with many targeted toxins *in vitro*, suggesting that data is representative of the effect of a full targeted toxin (Polito, Bortolotti et al. 2013). Additionally, both approaches result in a large amount of protein remaining trapped in vesicles. This is again representative of what is seen following treatment with targeted toxins where endosome escape is seen as a rate limiting factor of toxicity (Pirie, Hackel et al. 2011). These approaches could therefore also be used for the investigation of endosome disruption and improving the efficiency of cytosolic delivery.

Since BLF1 was initially characterised, it has been postulated that it could be used as a novel payload for targeted toxin therapy (Hautbergue and Wilson 2012). Chapter 4, for the first time, demonstrated that BLF1 has high potency comparable to other translation inhibiting enzymes making it suitable for use in targeted toxins. Furthermore, the decreased potency of this enzyme in non-dividing cells and its selectivity towards cells with a dependence on high levels of translation initiation may make this a better choice as a targeted toxin payload for certain cancer types as it will have decreased side effects. Although validated in MYCN-driven neuroblastoma cell lines, BLF1 may also be of use in c-Myc driven tumours, as has been seen with small molecule inhibitors of eIF4A. This would greatly increase the applicability of this enzyme for cancer treatment as c-Myc is one of the most common driving mutations found in cancer. Indeed, it is thought that at least 50 percent of all cancers depend on c-Myc mutations, and Cancer Research UK has recently included the development of innovative approaches to target the cancer super-controller c-Myc as one of its Grand Challenges for cancer therapy development (Ledford 2015, Hartl 2016).

Chapter 4 also unveils eIF4A as a potential target for MYCN-amplified neuroblastoma. The prevalence of MYCN amplification in neuroblastoma and its major role in tumorigenesis makes the inhibition of MYCN a key therapeutic target for neuroblastoma treatment. Although considered undruggable by small molecules directly, a large amount of research is being carried out on identifying targets that indirectly interfere with MYCN activity. A number of these targets have been identified which can inhibit transcription and DNA binding, activate degradation, or cause cell death in a synthetically lethal manner (Huang and Weiss 2013). However, as well as contributing to cell growth and metastasis, MYCN also plays a role in the acquisition of resistance to drugs. The targeting of multiple pathways will help to reduce the likelihood of resistance, making the identification of novel targets of high importance. The identification of eIF4A as a target to translationally down-regulate MYCN is therefore significant as small molecule or enzymatic inhibition of this eIF may act synergistically with other drugs to help prevent resistance acquisition and relapse.

Previous work in the Davletov lab demonstrated that co-delivery of BoNT/C and /D light chains into neuroblastoma cells led to the induction of apoptosis (Arsenault, Cuijpers et al. 2013). This was the first time that BoNTs have been shown to be cytotoxic in non-neuronal cells and it was postulated that this was due to the formation of toxic SNARE fragments that interfere with correct SNARE complex formation and inhibit important membrane trafficking events. Chapter 5 demonstrated that BoNT/C alone is toxic to neuroblastoma cells, but only following differentiation with retinoic acid. This is likely because differentiation leads to the downregulation of SNAP23 meaning that it can no longer compensate for SNAP25 following cleavage. The specificity of BoNT/C for neuronal SNAREs

greatly reduces the types of cells that it can act on which, in turn, will reduce the level of possible side effects. Common dose limiting side effects of current immunotoxins such as VLS and hepatotoxicity would be abolished. Careful selection of a targeting ligand that does not bind to neurons could lead to a highly selective targeted toxin utilised in the maintenance phase of treatment for neuroblastoma which would help to prevent resistance to retinoic acid differentiation and relapse.

6.3 Limitations and future directions

The work carried out in this thesis validates both BLF1 and the BoNT/C protease as potential payloads for future targeted toxins. However, it is important to emphasise that both studies are in very early stages of research and a lot more work needs to be carried out to ascertain if these proteins will be of benefit to patients. Additionally, a greater understanding of the delivery techniques described in chapter 3 may help to improve delivery efficiency of proteins both experimentally and therapeutically.

The apparent sensitivity of the J774.2 cell line to intracellularly active enzymes cannot be explained by macropinocytosis alone. A second process which facilitates cytosolic entry must also be present and this will be an interesting avenue of research for the future. This increased cytosolic entry may be due to inherent 'leakiness' of the macropinosomes or an active mechanism where the protein is transported into the cytosol, as is seen during antigen presentation. Defects in protein degradation pathways such as the proteasome or lysosomal fusion may also play a part. One possible approach to test for macropinosome leakiness would be to use a fluorescent galectin-3 reporter system. This has been used previously to monitor vesicle leakage during pathogen infection as well as by D'Astolfo et al. to assess macropinosome membrane integrity (Paz, Sachse et al. 2010, D'Astolfo, Pagliero et al. 2015). Galectin-3 is a cytosolic protein that can bind to β-galactoside carbohydrates. These carbohydrates are normally localised to the extracellular side of the plasma membrane and the interior side of endocytic vesicles. Vesicle leakiness or membrane rupture allows entry of galectin-3 into the vesicle and binding to β-galactoside. This becomes apparent as intense fluorescent staining inside vesicles. Antigen cross presentation is the process by which exogenous peptides taken up by phagocytosis or endocytosis are presented on MHC class I molecules. For this to occur, exogenous proteins need to be exported into the cytosol where they are then processed for presentation by the proteasome. The mechanism by which this happens remains unclear, although it has been proposed that cytosolic entry could occur via the presence of ER protein channels on the endosome or be due to instability of the lysosomal membrane (Vyas, Van der Veen et al. 2008). A high level of cytosolic export followed by evasion of degradation may be a key

contributor to the sensitivity of the J774.2 cell line. Defects in protein degradation could be tested using a range of proteasome and lysosome inhibitors combined with comparison to the RAW 264.7 cell line which demonstrates high macropinocytic uptake but low sensitivity to enzymes. A better understanding of the mechanism that confers sensitivity to the J774.2 cell line may help with future research into antigen presentation and cytosolic delivery of macromolecules.

Despite protein reaching the cytosol, fluorescence microscopy revealed that the majority of uptaken protein remains trapped in vesicles in both the J774.2 cell line and following delivery with LF3000. This suggests that protein delivery efficiency could be increased even further by the addition of membrane disruptive agents. One obvious choice to increase cytosolic delivery would be the use of propanebetaine which has previously been used to induce macropinosome leakiness in the iTOP method (D'Astolfo, Pagliero et al. 2015). Alternatively, a range of endosome escape peptides are available which are able to disrupt membranes in a pH dependent manner. For instance, the GALA peptide is a short, 30-residue, synthetic peptide with a repeating Glu-Ala-Leu-Ala sequence (Nakase, Kobayashi et al. 2010). GALA mimics the function of viral fusion protein segments that mediate the escape of viral genes from endosomes into the cytosol. Endosomal acidification causes a rearrangement of the peptide structure from random to helical, giving it a high affinity for neutral or negatively charged membranes, leading to the formation of pores and destabilisation of the endosomal membrane (Nakase, Kobayashi et al. 2010). Following internalisation, the majority of protein is degraded in the endolysosomal pathway. Targeting this pathway by inhibiting lysosomal fusion or endosome acidification may also help to increase the delivery efficiency of these approaches. However, all of these methods involve the addition of compounds that may have further effects on cell physiology and function. This is important to take into account when testing intracellular action of enzymes.

The generation of targeted fusion proteins will be an important next step for further testing of BLF1 and BoNT/C protease both *in vitro* and *in vivo*. An obvious choice for targeting neuroblastoma is the use of an anti-GD2 antibody or Fv fragment, as this ganglioside has been shown to be highly expressed on neuroblastoma cells (Ahmed and Cheung 2014). However, generation of an immunotoxin is difficult as the antibody, or antibody fragment, cannot be generated recombinantly in a bacterial system (Yin, Li et al. 2007). Targeting using a ligand that binds a receptor highly expressed on neuroblastoma cells would be a useful proof of principle step for *in vitro* studies as well as for early *in vivo* testing. It has previously been shown in the Davletov lab that fusion of the BoNT/A light chain and translocation domain to ciliary neurotrophic factor (CNTF) allows for targeting of BoNT/A to the SH-SY5Y neuroblastoma cell line and cleavage of SNAP25 (Arsenault,

Ferrari et al. 2013). Efforts were made to generate a BLF1-CNTF fusion protein, but due to issues with cloning, this was not possible in the given timeframe. Successful generation of BLF1-CNTF would allow for *in vivo* testing, and use of the TH-MYCN mouse model would help to confirm the potent effects of BLF1 on MYCN-amplified cell lines observed *in vitro*. This mouse model utilises a tyrosine hydroxylase promoter to overexpress MYCN in cells derived from the neural crest (Weiss, Aldape et al. 1997). These animals develop neuroblastoma several months after birth. Xenografts of human neuroblastoma cells into nude mice would be of use to define the effect of BLF1-CNTF on human cells of different MYCN-amplification status *in vivo*. CNTF is a hormone that promotes survival, neurotransmitter synthesis and neurite outgrowth in certain neuronal populations (Ip and Yancopoulos 1992). Its specificity for neurons means that it could not be used for the targeting of BoNT/C protease, as this would lead to neuronal toxicity. A ligand specific for a receptor expressed on neuroblastoma that is not expressed on neurons would therefore need to be selected for *in vivo* testing.

In chapter 5, differentiation of neuroblastoma cells was shown to downregulate SNAP23, the non-neuronal homologue of SNAP25. It was postulated that this down-regulation of SNAP23 is what sensitises cells to BoNT/C protease mediated toxicity, as cells are no longer able to compensate for cleavage of SNAP25 and SNARE complex formation is disrupted. It appears that cleavage of both SNAP25 and syntaxin 1 are important for toxicity as cleavage of SNAP25 alone by BoNT/A does not affect cell viability. A possible explanation for this is that cleaved SNAP25 loses its ability to interact with non-neuronal syntaxins but is still able to interact with syntaxin 1 and form SNARE complexes, all be it with impaired efficiency. This disrupts rapid vesicle fusion necessary for neurotransmitter release but doesn't affect other vesicle fusion events, such as membrane recycling, which are necessary for cell survival. Co-cleavage of syntaxin 1 by BoNT/C inhibits this interaction leading to a complete block of SNARE-mediated vesicle fusion and cell death. The SNAREs affected by differentiation and BoNT treatment and their effect on cell viability are summarised in table 6.1. This reasoning is in support of a previous study which demonstrated that exogenous expression of both SNAP23 and a non-neuronal syntaxin can rescue primary hippocampal neurons from cell death following BoNT/C treatment (Peng, Liu et al. 2013). The same study also utilised a BoNT/C mutant that could only cleave syntaxin 1 and showed that this was still able to induce neuronal death. Cells could be rescued by the expression of non-neuronal syntaxins alone, without the need to express SNAP23. This suggests that non-cleaved SNAP25 retains the ability to interact with these syntaxins, but this ability is lost following cleavage. Exogenous expression of SNAP23 in neuroblastoma cells following differentiation would be a useful future experiment to confirm the link between down regulation of SNAP23 and BoNT/C mediated apoptosis. Expression of SNAP23 in differentiated

neuroblastoma cells was attempted using lipofection or retroviral transduction but was unsuccessful.

Condition	SNAP25/SNAP23	Syntaxins	Viable?
Undifferentiated, Untreated	SNAP25, SNAP23 -	Syntaxin 1, 2, 3, 4 -	Yes
Differentiated, Untreated	SNAP25 -	Syntaxin 1, 2, 3, 4 -	Yes
Undifferentiated, BoNT/A	SNAP23 Cleaved-SNAP25	Syntaxin 1, 2, 3, 4 -	Yes
Differentiated, BoNT/A	- Cleaved-SNAP25	Syntaxin 1, 2, 3, 4 -	Yes
Undifferentiated, BoNT/C	SNAP23 Cleaved-SNAP25	Syntaxin 2, 3, 4 Cleaved-Syntaxin 1	Yes
Differentiated, BoNT/C	- Cleaved-SNAP25	Syntaxin 2, 3, 4 Cleaved-Syntaxin 1	No

Table 6.1: The effects of differentiation and BoNTs on SNARE expression and cleavage. SNAREs able to form complexes are highlighted green, and those unable to form complexes are highlighted red. Differentiation and BoNT/C treatment are the only conditions that cause a complete block of complex formation and cell death.

The finding that differentiation of neuroblastoma cells downregulates SNAP23 also poses interesting questions about the role of SNAREs in neuron development. Previous studies have shown that SNAP23, along with other non-neuronal SNAREs such as VAMP3 and syntaxin 4, are expressed early in the differentiation of hippocampal neurons during axon outgrowth (Dabrowski, Aerts et al. 2003). It is thought that the regulated exocytosis carried out by these SNAREs allows cells to undergo processes of rapid surface dynamics necessary for axon growth (Cocucci, Racchetti et al. 2008). Additionally, SNAP23 is necessary for the exocytosis of IGF1 to the neuronal growth cone, which further contributes to axon growth (Grassi, Plonka et al. 2015). IGF1 has also been shown to be essential for the establishment of neuronal polarity in hippocampal neurons (Sosa, Dupraz et al. 2006). However, SNAP23 is almost undetectable in the adult brain which suggests that its down-regulation may be necessary for proper function of mature neurons (Wong, Daneman et al. 1997). It would be useful therefore, to establish a time course of SNAP23 expression following differentiation with retinoic acid in neuroblastoma cells and to observe the effect of exogenous SNAP23 expression on neurite outgrowth. Functional evaluation of the effect of SNAP23 on neurotransmitter release following differentiation may help to further elucidate the role of this SNARE in neuronal development.

The need to differentiate cells for BoNT/C sensitivity is a further limitation of this toxin. Although SNAP23 appears to be robustly down-regulated *in vitro* it would be important to observe the effect

of differentiation *in vivo*. Treatment with retinoic acid in mice models followed by detection of SNAP23 levels in neuroblastoma cells would be a useful future experiment to assess this and shed light on the feasibility of the use of BoNT/C protease as a targeted toxin. Pre-treatment with retinoic acid would also be necessary for testing the efficacy of any targeted toxin that is generated. Some neuroblastoma cell lines also show resistance to retinoic acid treatment which is an important consideration when using xenograft models.

Any targeted toxins generated using BLF1 or BoNT/C protease will potentially have decreased side effects when compared to conventional targeted toxins. However, the issues of efficient toxin delivery to target cells and immunogenicity would still persist and need to be addressed to give the most effective treatment. Immunogenicity of toxins can be greatly reduced by the removal of immunogenic epitopes. B-cell epitopes were successfully removed from PE by isolating antibodies from patients with immune resistance to PE and constructing a phage display library (Liu, Onda et al. 2012). Alanine scanning mutagenesis was then used to locate the epitopes and an alternative toxin was generated (LR-O10) which showed low reactivity with human antisera but maintained high cytotoxic and anti-tumour activity. Delivery efficiency can be greatly increased by the presence of a translocation domain. This is a benefit of BoNT/C as it already contains a translocation domain and only the targeting domain would need to be changed. BLF1 is a monomeric enzyme and so would need to be attached to the translocation domain of a different toxin or to other molecules that facilitate endosome escape. Fusion to endosome escape peptides such as GALA or co-administration with pore forming molecules such as saponin or listeriolysin could also increase cytosolic delivery. Promising results have been obtained using these approaches when using type I RIPs such as saporin (Fuchs, Bachran et al. 2009, Pirie, Liu et al. 2013).

Future work that combines knowledge obtained from this thesis with current research on immunotoxin design can be aimed at generating novel targeted toxins that show specificity by both targeting to neuroblastoma cells and by a selective cytotoxic mechanism of action. This dual specificity will offer advantages over small molecules due to uptake primarily into neuroblastoma cells and high potency. They will also offer advantages over current targeted toxins as the selective cytotoxicity will decrease side effects caused by uptake into healthy cells.

6.4 Concluding remarks

The development of cancer is an incredibly diverse and complex process which involves the acquirement of hundreds, if not thousands, of genetic mutations that contribute to cell growth, proliferation and survival (Loeb, Loeb et al. 2003). Neuroblastoma is a particularly diverse disease

with wide ranging outcomes and a high level of variation between patients (Brodeur 1995). This means it is unlikely that there will ever be a 'magic bullet', as was originally hoped for during the early development of targeted toxins (Vitetta, Thorpe et al. 1993). Instead, therapy requires a range of treatments that have different mechanisms of action and act synergistically to help eradicate cancerous cells and prevent relapse. The identification of novel targets for specific cancers, or subsets of cancers, is incredibly important to help combat tumour heterogeneity and drug resistance. The studies on BLF1 and BoNT/C highlight the importance of translation initiation and membrane recycling for cell survival in neuroblastoma cells, and the protein delivery techniques developed in this thesis may also help in the elucidation of future targets for cancer therapy.

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Appendix figures

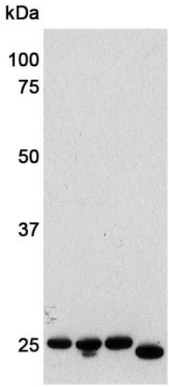


Fig. 3.2B
N2a, SNAP25

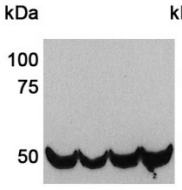


Fig. 3.2B
N2a, α-tubulin

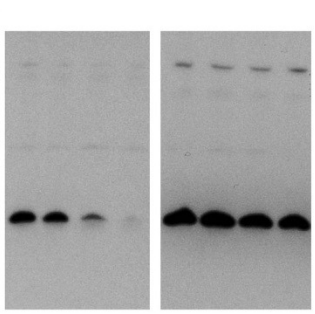


Fig. 3.4C
J774.2, VAMP3

Fig. 3.4C
N2a, VAMP3

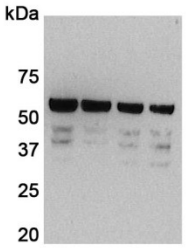


Fig. 4.1A
Amp, MYCN

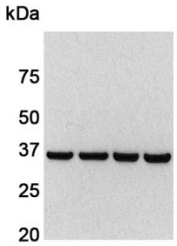


Fig. 4.1A
Amp, GAPDH

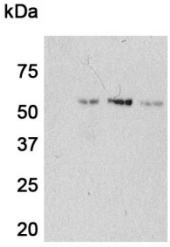


Fig. 4.1A
Non-amp, MYCN

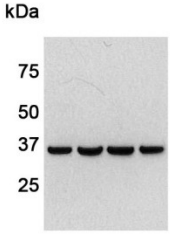


Fig. 4.1A
Non-amp, GAPDH

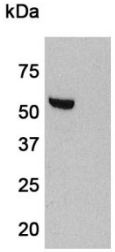


Fig.4.4A
SHEP,
MYCN

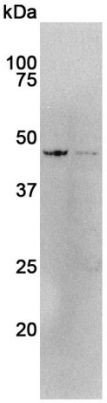


Fig. 4.4A
SHEP, eIF4A

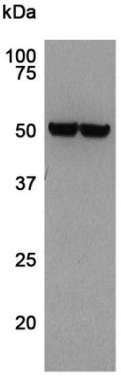


Fig. 4.4A
SHEP,
α-tubulin

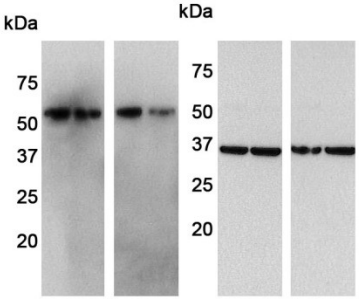


Fig. 4.5A
IMR-32, MYCN

Fig. 4.5A
IMR-32, GAPDH

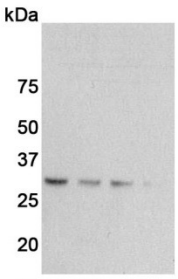
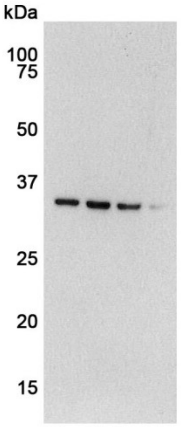


Fig. 4.5C
IMR-32, Cyclin D1



Fig, 4.5C
IMR-32, CDK4

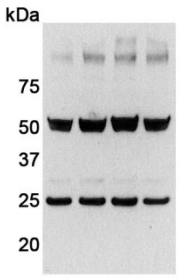


Fig. 4.5C
IMR-32, α-tubulin
SNAP25 (co-stain)

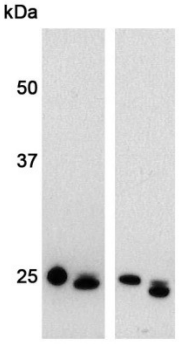


Fig. 5.1A
SiMa/SH-SY5Y,
SNAP25

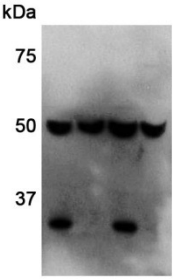


Fig. 5.1A
SiMa/SH-SY5Y,
α-tubulin
Syntaxin 1 (co-stain)

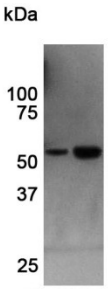


Fig. 5.3B
SiMa,
β-III Tubulin

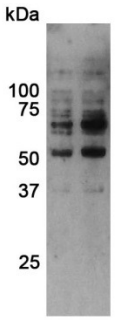


Fig. 5.3B
SiMa, Tau

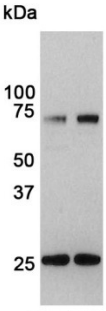


Fig. 5.3B
SiMa, SV2A
SNAP25
(co-stain)

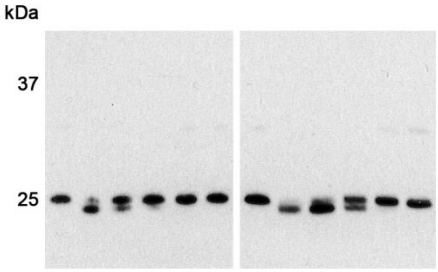
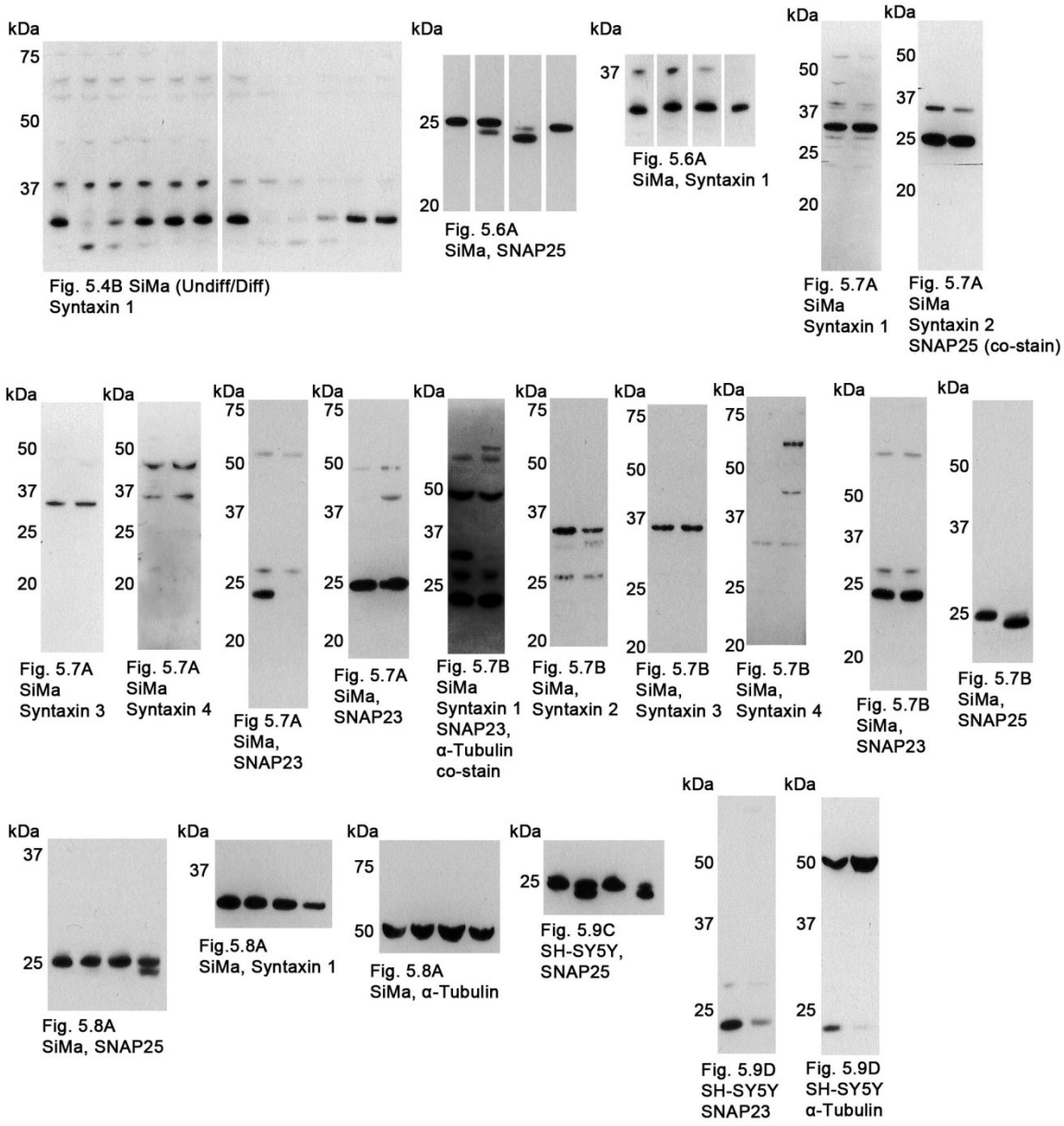


Fig. 5.4B SiMa (Undiff/Diff),
SNAP25



Appendix Figure 1: Full, unaltered blots for all immunoblot figures presented in this thesis including molecular size markers.

Fig. 3.6B

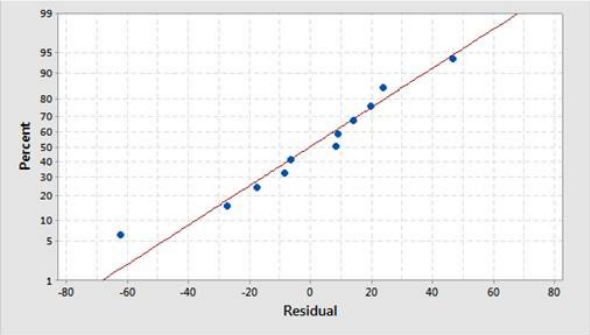


Fig. 4.3C

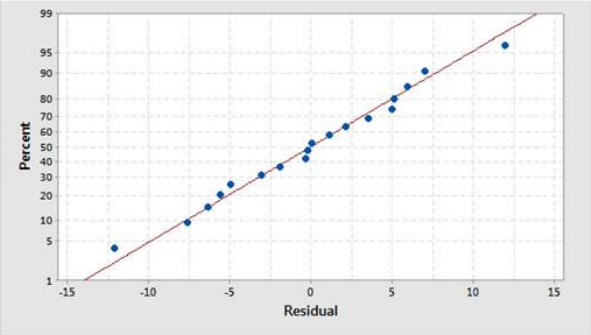


Fig. 4.1E

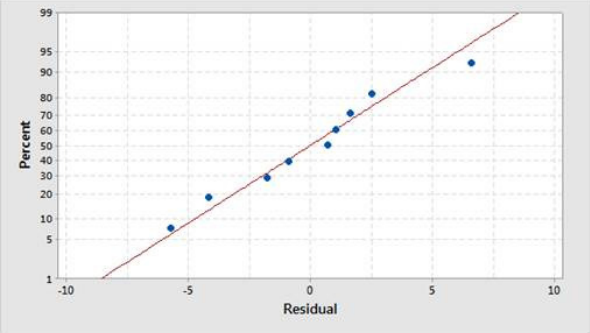


Fig. 4.6C

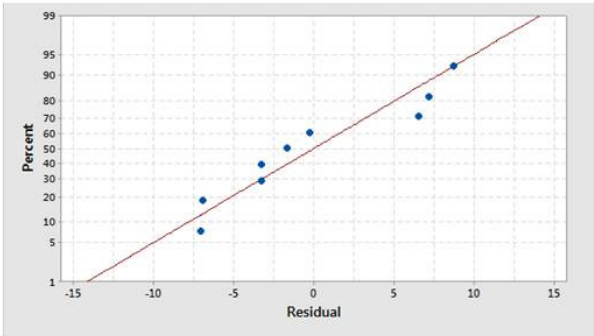


Fig. 4.3B

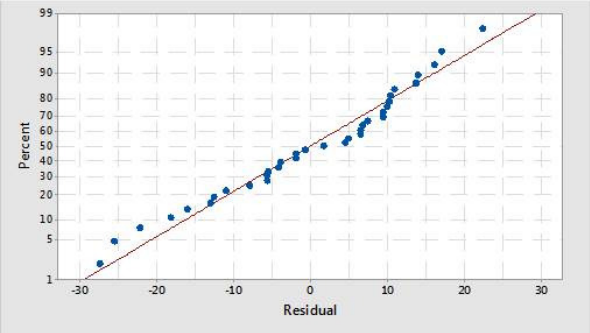
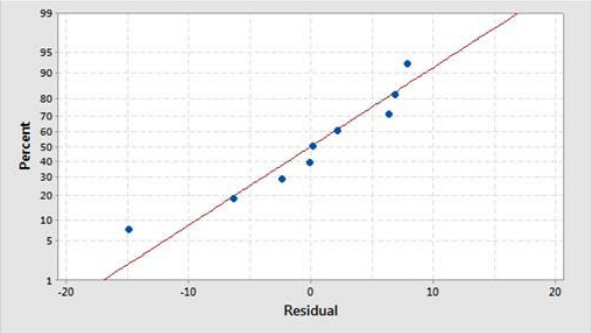


Fig. 5.2C



Appendix Figure 2: Normal probability plots from multiple-comparison analyses demonstrate the linear distribution of the data tested.